

Expression Profiling of FasL Gene in Human Blood Tissues and its Correlation with Severe Acute Pancreatitis (SAP)

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Received: 29 Apr 2018/ Revised: 14 Jun 2018/ Accepted: 25 Aug 2018

ABSTRACT

Blood leukocytes have a vital role in easing general inflammation through acute pancreatitis. Irrespective of topical growth in the considerate to the intricate pathogenesis of pancreatitis and the existence of the disease remnants sub-optimal are found. The present work attempted to detect the gene expression level of Fas Ligand (FasL) cDNA in four blood samples. The blood samples consisted of different age groups (7 years, 18 years, 25 years, and 50 years), whose expression for the gene FasL was studied by real time PCR. From the study, it was observed, in the sample no. 5 of 50 years age the expression was highest, followed by sample no. 1 of 25 years > sample no. 4 of 18 years > sample no. 3 of 7 years. The FasL gene was over expressed in the sample no. 5 and under expressed in the sample no. 1, 4, and 3.

Key-words: Apoptosis, cDNA, Cycle threshold, FasL, Immunosuppression, Lymphocytes, Severe acute pancreatitis

INTRODUCTION

The beginning of real-time PCR and present opposite transcription PCR (real-time RT-PCR) has intensely altered the ground of calculating gene expression. Definite PCR is the method of gathering information through cellus uniting strengthening and discovery into a solitary stage. This is attained by means of a diversity of dissimilar glowing chemistries that relate PCR artifact concentration to fluorescence strength. Reactions are branded by the fact in time (or PCR cycle) when the board intensification is primarily noticed ^[1-3]. This value is usually referred to as cycle threshold (Ct), the time at which fluorescence intensity is better than related fluorescence.

Accordingly, the better the amount of target DNA in the preliminary substantial, the earlier an important upsurge in the fluorescent sign will seem, yielding a lesser Ct ^[4].

There are numerous aids of consuming real-time PCR over additional approaches to enumerate gene expression. It can yield measurable information with a precise active variety of 7 to 8 log commands of the extent and does not need post-amplification operation ^[4]. The overall stages achieved throughout a real-time PCR research, after RNA separation to data investigation are momentarily conferred.

One-step versus Two-step Real-Time PCR- There is several pros and cons associated with each method. One-step real-time PCR is thought to minimize experimental variation because both enzymatic reactions occur in a single tube ^[4]. One-step protocols are also reportedly less sensitive than two-step protocols ^[4]. Two step real-time PCR separates the reverse transcription reaction from the real-time PCR assay, allowing several different real-time PCR assays on dilutions of a single cDNA.

How to cite this article

Biswas S, Singh MK. Expression Profiling of FasL Gene in Human Blood Tissues and its Correlation with Severe Acute Pancreatitis (SAP). Int. J. Life Sci. Scienti. Res., 2018; 4(5): 1986-1992.



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The 40 kDa type- The II transmembrane protein is the main Data of (CD95L or FasL) Fas ligand that convulsions to the Tumour necrosis factor (TNF) domestic that is necessary with its receptors persuades apoptosis. Fas ligand/receptors connections demonstration an important portion in the instruction of the immune system & the growth of cancer ^[5].

The human FasL gene is riven into four exons and contains about eight kb. The human FasL gene was charted on genetic material 1q23 by in situ hybridization in contradiction of human metaphase DNAs ^[5]. The Fas receptor FasR is the greatest strongly deliberate associate of the decease receptor family ^[5-8]. Apoptosis-inducing Fas receptor is termed isoform 1 and is a type 1 transmembrane protein which contains an intracellular death domain and a transmembrane domain ^[5,9].

Decoy receptor 3 (DcR3) is a newly exposed decoy receptor of the tumor necrosis factor superfamily that dilemmas to FasL, LIGHT, and TL1A ^[5,10]. DcR3 is a soluble receptor that has no signal transduction capabilities (hence a "decoy") and functions to prevent FasR-FasL

interactions by competitively binding to membrane bound Fas ligand and rendering them inactive ^[5,9]. Fas systems the death-inducing signaling complex (DISC) upon ligand compulsory ^[11,12]. This happening is also imitated by compulsory of an agonistic Fas antibody, however approximately indication proposes that the apoptotic signal persuaded by the antibody is variable in the learning of Fas signaling ^[13,14].

The cellular endosomal apparatus will be adopted through the receptor complex once the subsequent demise domain (DD) was grouped. This permits the device molecule Fas-linked death domain (FADD) to quandary the death domain of Fas ample one's death domain. FADD also comprises a decease effector domain (DED) close its amino terminus, that eases obligatory to the DED of FADD-like ICE (FLICE), additional usually mentioned to as caspase-8 and lively caspase-8 is formerly free from the DISC obsessed by the cytosol, once it slices additional effector caspases, finally chiefting to DNA deprivation, membrane blebbing, and additional symbols of apoptosis ^[13,15].

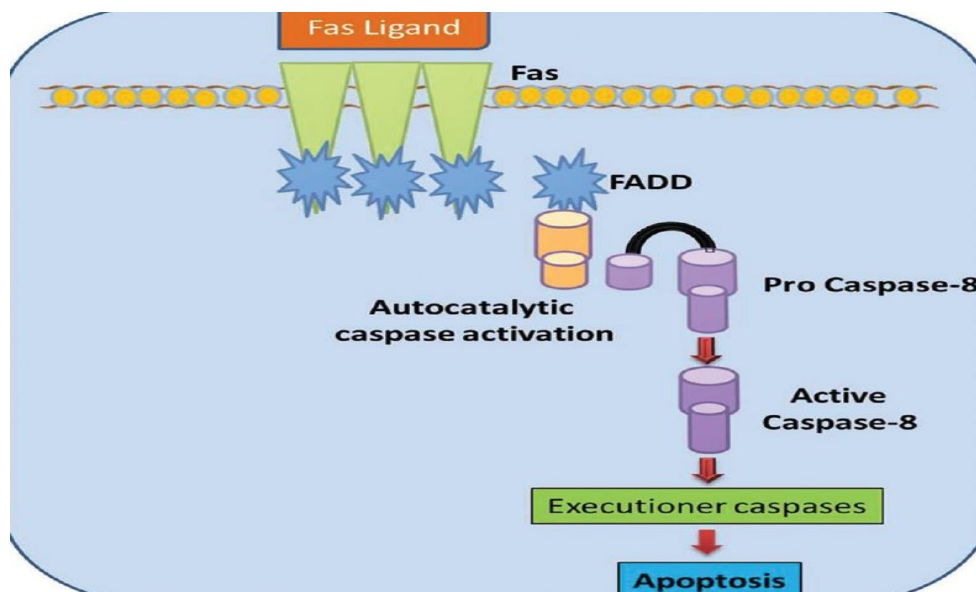


Fig. 1: Extrinsic pathway of apoptosis through FasL/Fas interaction

Source: http://www.jomfp.in/viewimage.asp?img=JOralMaxillofacPathol_2016_20_3_491_190953_f1.jpg

Roughly hearsays have recommended that the extrinsic Fas pathway is enough to induce complete apoptosis in certain cell types through DISC assembly and subsequent caspase-8 activation ^[15-18]. Characterized Type 1 cells comprise SKW6.4, H9, CH1, and SW480, all of that are lymphocyte lineages excluding the latter with colon adenocarcinoma lineage ^[16]. Cancerous cells for the

presences also distributes an approach for elusion for immune system ^[19,20].

MATERIALS AND METHODS

Initially, five fresh blood samples of different age group (7, 18, 25, 50, and 75 years) were collected from Evan Multispecialty Hospital and Research Centre, India. Following the collection of blood samples, reagent was

prepared with (a) 10X PBS with NaCl (0.8 g), KCl (0.2 g), Disodium hydrogen phosphate (1.44 g) and Potassium hydrogen phosphate (0.24 g), (b) Triazole Reagent with Phenol (3.8 ml), Guanidium Thiocyanate (0.8 M), Ammonium thiocyanate (0.4 M), Sodium Acetate (0.1 M) and Glycerol (500 μ L).

The materials required for RNA Isolation from blood sample, Eppendorf tubes and micropipettes, PBS solution (phosphate-buffered saline), centrifugation machine, triazole solution, ice box, isopropanol, 70% ethanol, distilled water/TE buffer, chloroform, and gel electrophoresis kit. As the blood samples were collected and the materials required for the RNA isolation were collected. One ml of PBS 200 μ L of blood sample was added in all the respective Eppendorf tubes and the samples are centrifuged for 5 minutes at 3000 rpm (revolution per minute) with which the 500 μ L of triazole reagent was added along with 200 μ L chloroform and it was mixed thoroughly. Later, the mixture was incubated on ice for 15 minutes and centrifugation was performed at 10000 rpm for 10 minutes at 4°C. After centrifugation, the aqueous phase was transferred to fresh Eppendorf tubes. Once transferred, 0.5 ml isopropanol was added and incubated in ice for 10 minutes. Again, centrifugation was done at 10000 rpm for 10 minutes at 4°C. The supernatant formed was removed and carefully washed with 70% ethanol. After, the solution mixture was whirled at 7500 rpm for 5 minutes at 4°C. The supernatant formed was removed followed by air dry. The RNA pellet formed was dissolved in the appropriate volume of double distilled water or TE Buffer (100 μ L) and vortexed. Agarose gel electrophoresis was performed to see resultant bands present in the blood samples. The samples were positive for RNA as detected by agarose gel electrophoresis was stored for future use. The resultant bands for RNA of the different blood samples were observed under UV- transilluminator. The bands were seen orange in color due to the fluorescence action of Ethidium bromide (EB), which was an intercalating dye.

The blood samples for RNA were positive as the various orange color bands were observed under UV transilluminator after electrophoresis of the respective samples. The materials required for mRNA Isolation from RNA sample by D(T) column were spring column tube, Distilled water, RNA sample, Centrifugation machine, 100% alcohol, and Eppendorf tubes.

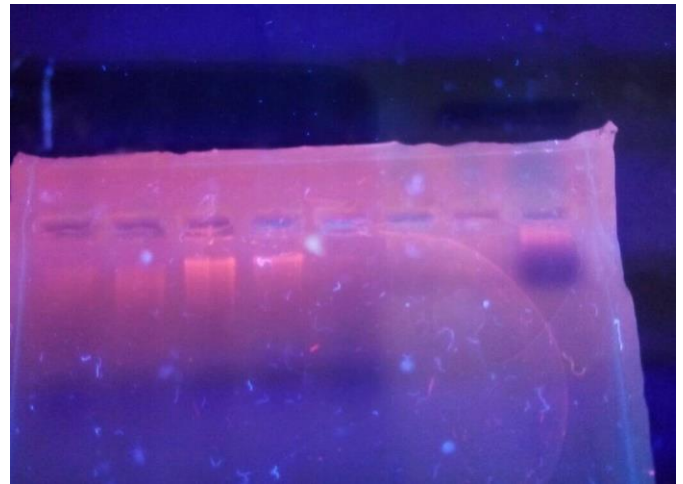


Fig. 2: Agarose gel electrophoresis of RNA samples

First and foremost, the column was cleaned by using the 500 μ L of distilled water which was centrifuged for 5 minutes at 5000 rpm (revolution per minute). After the column was cleaned the sample (RNA) of about 50 μ L and 200 μ L of distilled water was added, which was then centrifuged approximately for 10 minutes at 5000 rpm. The water which was beneath the column was discarded and 200 μ L of absolute alcohol (ethanol) was added and centrifuged for 10 minutes at 10000 rpm. After the centrifugation process over the supernatant (lower liquid after spin) was transferred into fresh Eppendorf tubes. After the supernatant collected the tubes were centrifuged for 10 minutes at 10000 rpm. The supernatant formed discarded and finally, the pellet formed was collected and followed by air dry for 5–10 minutes. Lastly, 50 μ L of double distilled water was added and the sample was stored for further use.

The materials required for cDNA synthesis are RNA (4 μ L), OLIGO d (T) (2 μ L), dNTP (2 μ L) and WATER (2 μ L). The mixture was incubated at 65°C for 5 minutes followed by incubation in ice for 1 minute. Both the mixture was mixed to form a total volume of 20 μ L. After mixing the total solution is incubated at 50°C for 50 minutes. After that, it was incubated at 85°C for 5 minutes followed by keeping in ice for 1 minute for stopping the reaction or else it was done by adding EDTA (5 μ L) to the total mixture then the total mixture was vortexed for 1 minute. After that 1 μ L of RNase H was added then it was further incubated at 37°C in BOD incubator for approximately 20 minutes. Finally, it was further stored at -20°C for further use.

RESULTS

The gene expression level of FasL cDNA was detected in four blood samples. Before qPCR normal PCR was done to check their amplification. The qPCR data was compared to cDNA expression level of healthy human blood, normalized to β -actin [21]. A standard curve with cDNA dilution of 1x, 3x, 9x, 27x and 81x. Ct values of dilution were used to prepare standard curve. A 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 FasL gene showed a slope of -3.404 and efficiency of 96.67% (Fig. 3 & Fig. 4 respectively).

Data Analysis of qPCR of FasL Gene- qPCR data was compared to cDNA expression level of healthy human blood, normalized to β -actin [21]. Standard curve with cDNA dilution of 1x, 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 FasL gene showed a slope of -3.404 and efficiency of 96.67 % (Fig. 3 & Fig. 4 respectively).

Quantification of FasL cDNA- The gene expression level of FasL cDNA was detected in four blood samples. Before qPCR normal PCR was done to check their amplification.

Standard curve of β -actin- Standard curve of β -actin housekeeping genes are shown in Fig. 3. The cDNA was diluted to 10 fold dilution of an initial amount of cDNA.

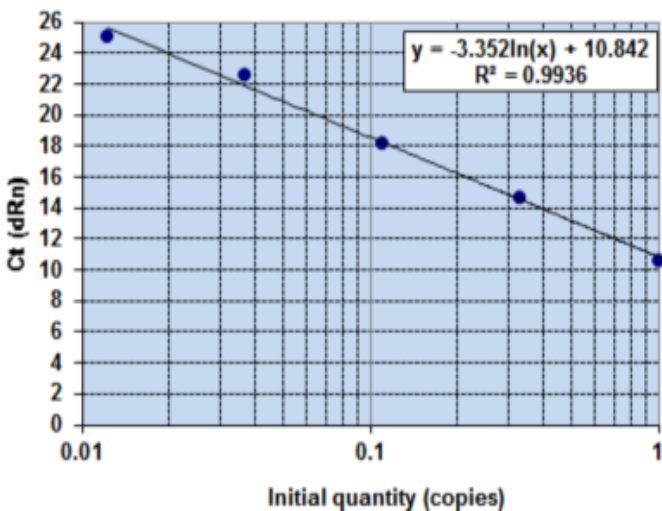


Fig. 3: Standard curve of β -actin housekeeping gene

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

Standard curve of FasL gene- Standard curve of FasL genes (randomly selected normal sample) are shown in Fig. 4. The cDNA was diluted to 10 fold dilution of an initial amount of cDNA.

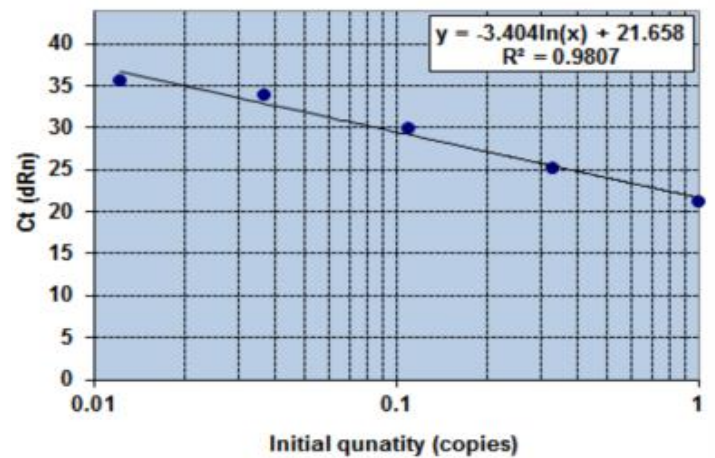


Fig. 4: Standard curve of FasL gene (randomly selected normal sample)

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

Threshold values obtained for FasL was 0.024. A threshold value for the FasL gene was calculated by setting automatic mode of PCR machine, while running the qPCR.

Quantification of gene- The quantification of the gene was obtained by qPCR, which was performed on healthy blood tissue and four blood samples for FasL gene. The data (Ct values) was normalized by calculating Δ ct followed by $\Delta\Delta$ ct. The $\Delta\Delta$ ct values were normalized with the formula $2^{-\Delta\Delta$ ct.

Ct values for β -actin, FasL gene- The data (Ct values) was normalized by calculating Δ Ct followed by $\Delta\Delta$ Ct. The $\Delta\Delta$ Ct values were normalized with the formula $2^{\Delta\Delta$ Ct [21].

Ct values for β -actin, FasL from normal blood samples- The values are obtained from qPCR for the samples, by using 3x cDNA dilutions.

Table 1: Ct values for β -actin and FasL from normal blood samples

Ct	β -actin	FasL
Control (Sample-2)	10	14
Sample-1	12	16.56
Sample-3	9	16
Sample-4	12	17.432
Sample-5	13	16.146

Table 2: Δ Ct values for β -actin, FasL

Δ Ct	FasL
Control (Sample-2)	4
Sample-1	4.56
Sample-3	7
Sample-4	5.432
Sample-5	3.146

Table 3: $\Delta\Delta$ Ct values for β -actin and FasL

$\Delta\Delta$ Ct	FasL
Control (Sample-2)	0
Sample-1	0.56
Sample-3	3
Sample-4	1.432
Sample-5	-0.854

Normalization of β -actin and FasL- The data are normalized of β -actin, FasL was done according to Livak and Schmittgen [21].

Table 4: Normalization of β -actin and FasL [21]

Normalization ($2^{\Delta\Delta$ Ct)	FasL
Control (Sample-2)	1
Sample-1	0.678302
Sample-3	0.125
Sample-4	0.370617
Sample 5	1.807505

Expression of FasL gene is highest in sample-5 followed by sample-1> sample-4> sample-3. FasL gene was over expressed in sample-5 while under expressed in sample-1, sample-3, and sample-4.

DISCUSSION

Out of the four blood samples of different age groups (7 years, 18 years, 25 years, and 50 years) whose expression for the gene FasL was studied by real time PCR, it was observed that in the sample no (5) of 50 years age the expression was highest followed by sample no (1) of 25 years> sample no (4) of 18 years> sample no (3) of 7 years. The FasL gene was over expressed in the sample no (5) and under-expressed in the sample no (1), (4) & (3).

Severe acute pancreatitis (SAP) is a disease that develops from local pancreatic inflammation to overwhelming systemic inflammation. It was associated with severe infectious complications and multiple organ failure [22]. Necrotic pancreatic tissue in SAP was one of the main reasons that lead to systemic inflammation and mortality. It was widely accepted that uncontrolled inflammatory response plays a key role in the occurrence of infection and sepsis and that the immune response such as immunosuppression was also involved. With the development of systemic inflammation, pro- and anti-inflammatory cytokines are released into the circulation, causing compensatory anti-inflammatory response syndrome (CARS) and subsequent immune deficiency or immunosuppression, which renders the host susceptible to secondary infections and to systemic sepsis [23]. However, the molecular mechanisms involved in the pathogenesis of this disease remain poorly understood. Extensive apoptosis of lymphocytes and intestinal epithelial cells in patients with sepsis, shock, and multiple organ dysfunctions were found, and these

results suggested that the changes of immune status contribute to the immunosuppression [24,25].

Fas and FasL play critical roles in delivering death signals to the immune system, and interactions of Fas–FasL can initiate the death signal pathway leading to lymphocyte apoptosis [26]. Mutation or downregulation of the expression of Fas and FasL genes resulted in lymphocyte proliferation and autoimmune disease. In contrast, upregulation of the expression of Fas and FasL may cause excessive apoptosis of lymphocytes leading to immunological impairment and immunosuppression [24,26].

CONCLUSIONS

From the result obtained after the data analysis of FasL gene expression by real time PCR we can conclude that the gene FasL was over expressed in 25% population and under expressed in 75% of the total population studied. Over expression was observed in the subject with the highest age of 50 years out of the total population of no. 4, so as per the data analysis report we can say that FasL induced apoptosis of lymphocytes & down-regulation of immune system, and its association with immuno suppression and related diseases such as severe acute pancreatitis (SAP) in individuals were more prone to advanced aged individuals & it directly proportional to ageing.

In this project, four normal blood samples of different age groups (7, 18, 25, & 50 years) for the expression of FasL gene was studied by real time PCR and correlated with severe acute pancreatitis. In future the same study can be done by using more number of samples of various age groups specifically for males and females related to up-regulation of apoptosis of lymphocytes and down-regulation of immune system and its relation with SAP and other immunosuppressive diseases for more appropriate and precise analysis.

ACKNOWLEDGMENTS

I express my heartily thanks to Mr. Krishnan Bahadur Singh, Director, Allele Life Sciences Pvt. Ltd., Noida, India. He was extremely supportive throughout this project.

CONTRIBUTION OF AUTHORS

All authors equally contributed in this article.

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