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Research Article

# IDENTIFICATION OF VARIATION IN CALRETICULIN GENE EXPRESSION LEVELS IN WHOLE BLOOD OF HEALTHY HUMAN SUBJECTS

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**ABSTRACT**

**Objective**: Calreticulin (CRT) is a multifunctional protein, ubiquitously present in endoplasmic reticulum and plays an important role in maintenance of cellular calcium homeostasis. In humans, age-related rate of CRT gene expression has not been reported.

**Methods**: In the present work we sought to investigate the level of CRT expression in healthy young and aged subjects. Blood samples from 24 healthy volunteers were examined for mRNA encoding CRT by Quantitative real-time-PCR.

**Results**: qRT-PCR analysis showed a slight reduction in CRT gene expression among aged samples as compared to young samples but these results were not statistically significant. This primary report demonstrates the expression of CRT in healthy volunteers.

**Conclusion**: Though preliminary, these findings merits further investigation to resolve the relationship between age and variation in expression of CRT.

**Keywords:** Calreticulin, age, gene expression, quantitative real time PCR.

## INTRODUCTION

Calreticulin (CRT) was first recognized as an endoplasmic reticulum protein with two major functions: $Ca^{2+}$ binding and molecular chaperoning [1]. Moreover, calreticulin implicated in variety of cellular roles, including modulation of $Ca^{2+}$ signals, $Ca^{2+}$ storage, regulation of steroid-sensitive gene expression, cell adhesion, chaperone in protein folding, autoimmune response and neuromodulations [2,3].

Calreticulin is a multifunctional protein that has been identified as a possible biomarker for varied diseases [4, 5]. For instance, calreticulin have been shown to rise in patients with lung cancer, breast cancer, gastric cancer, and rheumatoid arthritis [6, 7, 8, 9, 10]. Calreticulin has also been identified as a prognostic factor for neuroblastoma [11] and proved to be important for neural development [12]. Calreticulin has been found to influence cell vulnerability to apoptosis and to be overexpressed in apoptotic regions of the embryo [13]. Moreover, overexpression of calreticulin was shown to protect HeLa cells from apoptosis [14, 15] and alternatively, decreased expression of calreticulin before apoptosis in human leukemia cells [16]. Furthermore, a quite low CRT expression in canine kidney cells was reported [17].

Calreticulin is also found in extra cellular areas including the blood, and it has been related with regulation of immune responses [18]. Prior studies on calreticulin gene knock-out also indicate that the protein plays a role in the heart development [2, 19]. Earlier reports on calreticulin expression during embryonic cardiac development provide incite approaches into the functions of calreticulin. Although, calreticulin has numerous physiologic functions in the cell, its level in normal conditions has not been studied. At this point we sought to analyze the age related changes in calreticulin gene expression. This paper describes the level of calreticulin gene expression in blood of apparently healthy individuals. CRT expression in healthy blood was studied to analyze the analytical relevance of CRT expression in healthy group and the mRNA level of CRT in blood was quantified by real-time PCR.

## MATERIAL AND METHODS

### Samples

A total of 5 ml blood was withdrawn in EDTA tubes from healthy donors ($n$ = 24), The study was approved by the institutional ethics committee of the Karpagam University and has been carried out in compliance with the guidelines of the Helsinki Declaration of 1975. The study participants gave their written informed consent and samples and certain information were kept confidential for analysis.

### RNA isolation and cDNA synthesis

Total RNA from blood samples were extracted using a commercial kit (RNeasy Mini Kit; Qiagen, Inc.) according to the manufacturer's protocol. Then pellets were air-dried and dissolved in 20 µl DEPC-treated water. Concentration and ratio of isolated RNA were measured by Nanodrop-System-Nanophotometer (Implen, Munich, Germany), inhibition controls were carried out and RNA integrity was controlled by denaturing formaldehyde-gel electrophoresis. For reverse transcription an amount of 5 µg RNA in a maximum volume of 8 µl in DEPC treated water was used. A total of 10 µl 2× RT reaction mix (containing oligo(dT)20 (2.5 µm), random hexameres (2.5 ng/µl), 10mM $MgCl_2$ and dNTPs) and 2 µl RT enzyme mix (SuperScript III First Strand Synthesis Super Mix; Invitrogen) were added. The solution was incubated at 25°C for 10 min and at 42°C for 50 min. Following this, polymerase was heat-inactivated at 85°C for 5 min and subsequently chilled on ice. A total of 1 µl (2U) RNase H was added to the reaction and the whole solution was incubated at 37°C for 20 min. The obtained cDNA was stored at −20°C until use.

### Real-time quantitative – PCR

Primers and probes for TaqMan® gene expression assay were designed for each gene using Primer Express Software (Perkin Elmer/Applied Biosystems) are given in Table 1. For each gene, real-time PCR was performed in 20µl target volume using 5µl of PolyA cDNA from each sample, 10µl master mix, and 0.5µl of gene-specific forward primer, 0.5µl of gene-specific reveres primer, 0.2µl of

TaqMan probe and 3.8µl of ddH2O. The PCR profile was 95°C for 1 min, 60°C for 50 s, and 72°C for 2 min for 40 cycles, followed by extension for 7 min at 72°C. All samples, as well as the human

genomic standards were measured in each gene. Negative controls were set each time. Samples were analyzed using sequence detection software (SeqScape, Applied Biosystems) as recommended by the manufacturer.

**Table 1: Sequences of primers used in RT-PCR analysis**

| Gene | Sequence (5′- 3′) |
|---|---|
| CRT | forward 5'-TCTCAGTTCCGGCAAGTTCT-3' |
| | reverse 5'-GTTGCTGAAAGGCTCGAAAC-3' |
| GAPDH | forward 5′-AAGGCTGTGGGCAAGG-3′ |
| | reverse 5′-TGGAGGAGTGGGTGTCG-3′ |

**Statistical analysis**

Statistical analysis of the expression levels of the genes were performed in each groups using non-parametric tests, $p<0.05$ for calculation of the statistical significance. All the tests were performed using the statistical package from the Social Science (SPSS) software.

**RESULTS AND DISCUSSION**

Calreticulin serves as multifunctional protein is a relating factor for many studies including disease diagnosis, transduction and signaling pathways, calcium and ER stress, molecular chaperoning and mitochondrial apoptosis [20]. The CRT serves as a key factor in the maintenance of calcium homeostasis and inability to retain homeostasis may lead to death or a disease. Identification of normal gene expression level is important in prognosis of diseases like cancer in addition several of the identified differentially expressed genes have been linked to diseases though either at a diverse level or diverse model. Hence analysis of CRT gene expression level is of critical importance to provide clues about the potentials of different disease states.

In the present study we investigated a possible association between the age and level of CRT expression in 24 healthy volunteers. The characteristics of the groups studied are presented in Table 2. To examine the expression pattern and quantification of the CRT gene, all samples were divided into two groups: young (age ≤30y, *n* = 12) and aged (age ≥60 y, *n* = 12). Within each age group, there were no significant differences between the ages of the male and female groups.

**Table 2: General characteristics of study population**

| Characteristics | N | |
|---|---|---|
| Number of volunteers enrolled | N | 24 |
| Gender | Male | 12 |
| | Female | 12 |
| Mean age (years) | Group I (young) | 12 |
| | Group II (aged) | 12 |
| Age range (years) | Group I (≤30) | 12 |
| | Group II (≥60) | 12 |

Values are shown as mean ± SD. **p* <0.05.

In this study, endogenous expression of CRT mRNA was provided specifically to measure up the gene expression between healthy individuals. Expression level of certain long-established reference genes were used for normalization in gene expression studies. Total RNA was isolated from blood samples of 24 healthy individuals. The RNA concentrations and A260/A280 ratios of each sample was obtained. In order to verify the integrity, all RNA samples for each of the extraction methods were analyzed using 2 % agarose gel electrophoresis. The RNA obtained from all samples showed more defined and visible bands, indicating a low level of degradation.

Following electrophoresis, RNA obtained from each subjects were assayed in reverse transcription polymerase chain reaction, as described. The produced cDNA from all of the samples were confirmed by gene expression in real-time PCR, specific PCR was performed for CRT genes. CRT was positive in all of the samples of both the groups and bands of suitable sizes were present in all of the samples (Figure 1).

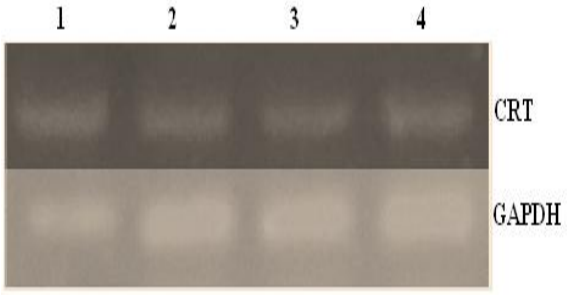


**Fig. 1: Amplification of the CRT and GAPDH genes using RT-PCR.**

Agarose gel electrophoresis of RT-PCR amplified products of total RNA extracted from healthy human blood: lane 1-2 (representative samples for group I) and lane 3-4 (representative samples for group II)

Negative template controls and standards were set all the time for qRT-PCR. Standard curves were plotted using Ct values of the five-fold serial dilutions of the human genomic DNA for the optimization and efficiency of qRT-PCR reactions. The efficiency of the GAPDH and CRT PCR reactions was calculated as 87.33%, and 85.71%, respectively. The qRT-PCR amplification plot of the CRT gene shows the amplification efficiencies of five-fold serial dilutions of the human genomic DNA. A representative qRT-PCR amplification plot of the CRT gene is shown in Figure 2.

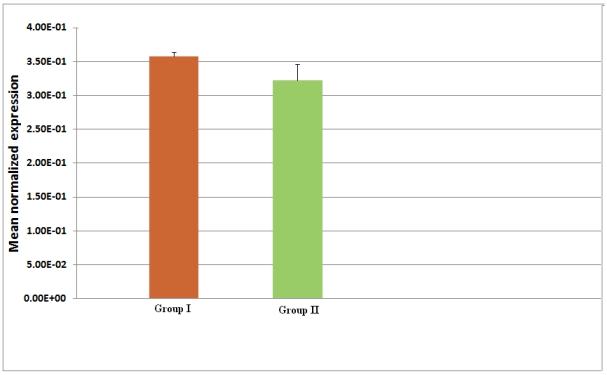


**Fig. 2: Real-time quantification of CRT by qRT-PCR.**

Data are presented as fold-changes of CRT levels in the young healthy subjects/ aged healthy subjects, quantitative analysis results expressed as a GAPDH ratio and recalculated as a percent of control value. Significance was set as (means ± SD) $P<0.05$.

Real-time PCR results showed slight elevation in the expression of CRT in young samples compared with aged samples. However, these results are not statistically significant ($p<0.05$). Conflictingly, several studies found the differential association of CRT gene with the diseases at different level or in different tissues [21]. CRT has been used as a target of gene therapy for cancers [22] and its inhibition was reported to induce cancer cell differentiation [23]. Inconsistently, induction of CRT was reported to inhibit breast cancer cell proliferation in our previous study [24]. Some studies verified CRT as a favorable free prognostic biomarker in cancer [10]. Moreover an ingenious study proved that CRT expression is highly associated with an increased risk of breast cancer [25].

In agreement with previous studies [26] GAPDH was used as stable gene for normalization of genes in lymphocytes, which had the stability expression levels between both the groups studied were almost equal. Comparison of young and aged individual's blood samples showed a significant difference between the two groups in the rate of CRT expression.

Finally, the cDNA of isolated sample confirmed the presence of CRT gene through gene sequencing. The rate of CRT expression was not

reported earlier with respect to age in healthy population. However, our study concludes that there is no substantial alteration in the expression level between the young and aged subjects.

## CONCLUSION

This is the first report to demonstrate the expression of CRT in normal healthy individual and indicates that this may serve as an appropriate path for further studies on age-related alteration in CRT expression and gene function. This groundwork, paves for future investigation to determine the relationship between age-related expression studies of CRT in healthy and diseased humans. The evaluation and identification of CRT gene with any altered expression could also be potential targets for novel and effective therapies.

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