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BCL11B Upregulates the Expression of NF- κ B in T Cells Stimulated with SEA

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Abstract

Background: NF- κ B is one of the most important inflammatory mediators in the tumour microenvironment promotes inflammation-induced cancer. Many studies report that NF- κ B is activated in many kinds of leukaemia, so that some researchers by inhibiting NF- κ B to treat leukaemia. The overexpression of BCL11B has been primarily reported in T cell malignancies. Some studies have reported that BCL11B is a potential transcriptional repressor which has several splice isoforms. MiR-21-5p promotes cell proliferation by targeting BCL11B in Thp-1 human monocytes. Both NF- κ B and BCL11B transcription factors are related to inflammation and leukaemia. Whether there is correlation between NF- κ B and BCL11B transcription factors? If BCL11B is a potential important transcription factor in treatment of T lymphocyte leukaemia? In this study, we stimulated Jurkat cells and normal peripheral blood T cells with staphylococcal enterotoxin A(SEA), and we detected the expression of both the BCL11B and NF- κ B genes and their respective proteins at different stimulation times. Then, the BCL11B gene was suppressed by BCL11B-siRNA and detected at another stimulation time.

Results: We found that the mRNA expression of BCL11B and NF- κ B increased in two kinds of T cell lines over time which stimulated by SEA or PMA+IO. A similar result was confirmed for BCL11B and NF- κ B protein expression. While the expression of the NF- κ B protein did not increase on equal conditions in the BCL11B-knockdown group.

Conclusion: Our result suggested that the gene and protein expression levels of both BCL11B and NF- κ B were related, and BCL11B regulates NF- κ B expression in Jurkat cells and healthy human peripheral blood through the TCR signalling pathway. This study reveals that BCL11B can be used as a new therapeutic target for chronic inflammation and T cell leukaemia pathogenesis.

Key words: BCL11B; NF- κ B ; SEA; T lymphocyte leukaemia

1. Introduction

Staphylococcal enterotoxin A (SEA) is not only a superantigen that can powerfully activate T cells by combining the V region of the TCR with the MHC of presenting cells but is also an extracellular toxin that can cause inflammatory effects.

Numerous studies have shown a close correlation between inflammation and cancer. Inflammation plays a critical role in tumorigenesis, and these studies have elucidated some of the underlying molecular mechanisms. The formation and development of tumours can be prompted or accelerated by chronic inflammation[1]. The NF- κ B family of transcription factors plays essential roles in inflammation and innate immunity; furthermore, according to recent studies, NF- κ B plays a critical role in cancer initiation and progression [2-4]. NF- κ B dimerizes and is sequestered in the cytoplasm by I κ B (I κ Bs), which prevents the nuclear translocation of NF- κ B. And the nucleus is the place where NF- κ B stimulates or represses the transcription of many genes. NF- κ B heterodimer is activated and translocates to the nucleus to change genes transcriptions, after phosphorylation and degradation of I κ B kinase. Under homeostatic conditions, NF- κ B is deactivated after combining with inhibitor I κ B. Many studies report that the NF- κ B is activated in many kinds of leukaemia, such as acute myelocytic leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myeloid leukaemia (CML)[5, 6]. Clinically, suppression of NF- κ B or separation of I κ B kinase (IKK) complex units have achieved therapeutic effects. For example, L-antioxidant vitamin C (LAA), an NF- κ B inhibitor, has been used in AML, CML treatment. LAA inhibits NF- κ B by preventing RelA localization and restraining the combination of the NF- κ B and cox-2 to decrease cox-2 expression[7]. CHS-828 restrains the IKK complex separation to prevent I κ B α and I κ B β degradation. NF- κ B inhibitors, nucleosides, ribozymes, and siRNA are tumour treatment agents that inhibit NF- κ B and enhance tumour sensitivity to drugs.

BCL11B is also called the CTIP2 gene. This gene is 7603 bp in length and is located on chromosome 14q32.2, in the T cell receptor sequence adjacent to the α/δ gene location. It is mainly expressed in T cells, thymus cells, and brain tissue. Some studies have shown that there is a strong correlation between the BCL11B gene and lymphocyte development, proliferation, differentiation and survival [6][7][8][9]. The expression of BCL11B varies at each developmental

phase. BCL11B overexpression has been observed primarily in T cell malignancies, and the expression of the BCL11B in normal T lymphocytes is not as high as that in T lymphoblastic leukaemia[9-11]. BCL11B may be associated with the occurrence of T lymphocyte leukaemia. Loss or low of BCL11B function contributes to lymphoblastic leukaemia[9]. Some researchers considered that it is a cancer suppressor gene that inhibits cancer cell growth[12]. However, other studies showed that suppressing the expression of BCL11B restrained Molt4 cell proliferation. Inhibition of BCL11B expression resulted in Jurkat cell death[13]. The BCL11B gene is relevant to T cell ALL, and BCL11B loses wild-type expression upon forming a new fusion gene[14]. Recent study reported that miR-21-5p promotes cell proliferation by targeting BCL11B in Thp-1 human monocytes, which is a kind of acute myeloid leukemia(AML)[15]. All the studies implied that BCL11B is important in the development of T lymphocyte, and in the occurrence of T lymphocyte leukaemia. If BCL11B is a potential important transcription factor in treatment of T lymphocyte leukaemia?

The presence of inflammatory mediators in the tumour microenvironment promotes inflammation-induced cancer. NF- κ B is one of the most important mediators. With reference to both NF- κ B and BCL11B transcription factors related to inflammation and leukaemia, especially under chronic antigen stimulation. Whether there is correlation between NF- κ B and BCL11B transcription factors? If BCL11B is a potential important transcription factor in treatment of T lymphocyte leukaemia? In this study, we used SEA to stimulate Jurkat cells and normal peripheral blood T cells and measured the expression of both the BCL11B and NF- κ B genes and their respective proteins at different times. We found that the mRNA expression of BCL11B and NF- κ B increased in the two kinds of T cells with time. A similar result was confirmed for BCL11B and NF- κ B protein expression. BCL11B-siRNA was used to inhibit BCL11B in Jurkat cells. The results indicated that the expression of the NF- κ B protein did not change in the BCL11B-siRNA group. BCL11B may be used as a new therapeutic target for chronic inflammation and T cell leukaemia pathogenesis.

2. Materials and Methods

2.1 Cell Preparation, Reagents, and Antibodies

Jurkat and Raji cells were obtained from American Type Culture Collection (A.T.C.C.). SEA and

phorbol-12-myristate-13-acetate (PMA) were purchased from Sigma (USA) and used at final concentrations of 1 µg/mL and 20 ng/mL, respectively. Ionomycin (IO) was obtained from Beijing Cowin Biotech Co., Ltd. (China) and used at a concentration of 1 µM. TNF-α was purchased from Beijing DingGuo Biotechnology Co., Ltd. (China) and used at a concentration of 20 ng/mL. The rabbit monoclonal anti-human NF-κB antibody RelA was purchased from ABclonal, and the anti-BCL11B antibody was purchased from Abcam. Mouse monoclonal anti-human GAPDH and secondary antibodies were purchased from Santa Cruz Biotechnology (USA).

2.2 Cell Cultures

Jurkat and Raji cells were maintained in RPMI 1640 medium (Gibco, USA) supplemented with 10% foetal bovine serum (Hangzhou Sijiqing Company, China), 100 U/mL penicillin, and 100 µg/mL streptomycin (Invitrogen) at 37°C in a humidified incubator with 5% CO₂. The culture medium was changed every 2-3 days.

The experimental cells were divided into the SEA group, PMA+IO group, and TNF-α group. In each group, Jurkat cells and Raji cells[10](Jurkat:Raji=10:1) were cultured in 12-well plates (1×10⁶cells/mL medium) in different wells; these cells were induced separately with SEA (1µg/mL), PMA (20 ng/mL), IO (1 µM), and TNF-α (20 ng/mL) for approximately 0 min, 15 min, 45 min, 180 min, 360 min.

2.3 RNA Extraction and cDNA Synthesis

RNA was extracted from the cells after stimulation. TRIzol was used, and the RNA was reverse transcribed into first-strand cDNA using random hexamer primers and a reverse transcriptase kit (TOKOYO, Japan) according to the manufacturer's instructions.

2.4 Real-time Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Quantitative real-time PCR was conducted with an MJ Research DNA EngineOpticon 2 instrument (Bio-Rad, USA) in a 20µl volume solution containing 10 µl of Real Master Mix; 0.5 mmol/L upstream and downstream primers; BCL11B (Accession No. NM 001282238) forward primer, CTTGGGTGCCTGCTATGAC, and reverse primer, TTCTCCTGCTTGGGACAGAT; NF-κB (Accession No.

NM 001165412.1) forward primer, CCACAAGACAGAAGCTGAAG, and reverse primer, AGATACTATCTGTAAGTGAACC; β_2m (Accession No. NM_004048.2) forward primer, TAACTGAATTCACCCCCAC, and reverse primer, CATCCAATCCAAATGCGGCA; 1 μ L of cDNA; and 8 μ L of distilled water. PCR cycling was conducted with initial denaturation at 95°C for 1 min, 40 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 15 s, and final cooling at 40°C for 1 min. A melting curve was obtained after cycling by an initial ramping from 40°C to 70°C at 2°C/s and a subsequent higher resolution melting programme was run from 65°C to 95°C at 0.1°C/s. Every 1 s, the fluorescence value was recorded.

2.5 Western Blot Analysis

Jurkat cells (3×10^6 cells/well) and normal T cells (3×10^6 cells/well) were cultured in 12-well plates with different chemicals for different times. The expression levels of BCL11B and NF- κ B were examined by Western blotting. The cells were destroyed in 60 μ L of lysis buffer containing 1% protease inhibitor. Protein concentrations were determined by Bradford assay. The proteins were separated by electrophoresis in a 10% SDS-polyacrylamide gel and transferred onto a PVDF membrane. The blots were blocked with 5% (w/v) non-fat milk constituted in 1x TBST for 1 h at room temperature. Membranes were incubated overnight at 4°C with a primary monoclonal antibody in 1x TBST and subsequently incubated with the respective horseradish peroxidase-conjugated secondary antibody as directed by the manufacturer for 1 h at room temperature. Immunoreactive bands were visualized using enhanced chemiluminescence light (ECL) detection reagent.

2.6 siRNA and Cell Transfection

BCL11B-siRNA, positive, negative and fluorescent transfection reagents were designed and synthesized by Ribobio (China). LiporNAiMAX and PTIOPTI-MEN were purchased from Life technology (USA)[16]. Jurkat cells (1×10^6 /well) were respectively cultured in 6-well plates in Opti-MEM after transfection with BCL11B-siRNA or negative transfection reagent according to the manufacturers' instructions and incubated for 48 h in a humidified incubator at 37°C with 5% CO₂. The cells were collected for PCR and Western blot analysis.

2.7 Human CD3+T Cell Isolation by MACS

CD3 microbeads were used for the positive selection of T cells according to the manufacturer's instructions (Miltenyi Biotec, Germany). The methods were based on those previously described [17]. PBMCs were resuspended in 800 μ L of buffer. CD3 microbeads (200 μ L) were added and incubated for 15 min at 4-8°C. To wash the cells, 10 mL of buffer was added, and then, the cells were centrifuged, resuspended in 500 μ L of wash buffer and magnetically separated on an AutoMACS magnet fitted with a MACS MS column. The cell suspension was applied to the column; sieved unlabelled cells and three 500- μ L washes were collected as the negative fractions. The magnetically labelled cells were immediately collected from the column in two 1-mL buffer solutions after the separating apparatus was removed. The purity of the cells was determined by flow cytometry (BD, USA). Most of the isolated cells (>95%) in this study were positive for CD3 T cell markers.

3. Statistical Analysis

The data were analysed by SPSS 16.0 statistical software. Data are reported as the means $\pm$ standard deviation ($\bar{x} \pm s$). Statistical analyses included analysis of variance for repeated data and paired samples T-tests.

Results

3.1 Expressions of BCL11B and NF- κ B Genes in Jurkat Cells

The gene expression value was calculated using the q-PCR relative quantitative formula $2^{-\Delta\Delta CT}$ with β_2M used as the reference gene[18]. Jurkat cells were stimulated by SEA (1 μ g/mL) for 15 min, 45 min, 180 min, 360 min, and the results showed that the mRNA expression of both BCL11B and NF- κ B gradually increased over time ($P<0.01$). After 180 min, the mRNA BCL11B expression level was much higher than that of NF- κ B mRNA ($P<0.05$) (Fig. 1A).

To test whether changes in the mRNA expression of the BCL11B and NF- κ B genes in T cells were induced through the T cell receptor (TCR) signalling pathway, Jurkat cells were simultaneously stimulated with PMA (20 ng/mL) and IO (1 μ M). PMA+IO were recognized to stimulate T cell through TCR pathway. The mRNA expression of both BCL11B and NF- κ B was greatly increased over time ($P<0.01$), similar to the effect of SEA simulation (Fig. 1B). The data revealed that the

mRNA expression level of BCL11B and NF- κ B in Jurkat cells through the TCR signalling pathway. To determine whether a similar pattern of BCL11B and NF- κ B mRNA expression in T cells was induced through another pathway, not merely the TCR pathway, after stimulation, TNF- α was used to stimulate the cells. Although the NF- κ B mRNA expression level was gradually increased in the Jurkat cells ($P < 0.05$), the results showed that the mRNA expression of BCL11B was not significantly changed by TNF- α stimulation (20 ng/mL). (Fig. 1C). The results revealed that the stimulating pathway is not through TNF- α .

Figure 1. (A) Jurkat cells were stimulated with SEA (1 μ g/mL) for 15 min, 45 min, 180 min, and 360 min. (B) Jurkat cells were simultaneously stimulated with PMA (20 ng/mL) and IO (1 μ M) for the same durations. (C) TNF- α (20 ng/mL) was used to stimulate Jurkat cells for the same durations as the other treatments.

3.2 Expression of the BCL11B and NF- κ B Proteins in Jurkat Cells

In these experiments, the GAPDH group was used as a loading control. Additionally, grey value analysis was performed with Image J software to quantify the protein bands, and the data were recorded. The ratio of the proteins of interest to the loading control protein is a relative expression. The dynamic changes in BCL11B and NF- κ B protein expression were similar to those of the BCL11B ($P < 0.05$) and NF- κ B genes ($P < 0.05$) (Fig. 2A). The BCL11B protein expression was substantially higher than that of the NF- κ B protein at 360 min ($P < 0.05$). The variation trends of the proteins were as follows: The expression levels of BCL11B ($P < 0.01$) and NF- κ B ($P < 0.01$) were similar to those of the respective gene after the cells were simultaneously stimulated with PMA (20 ng/mL) and IO (1 μ M). However, the NF- κ B protein expression level was obviously higher than that of the BCL11B ($P < 0.05$) (Fig. 2B). The data revealed that the protein expression level of BCL11B and NF- κ B in Jurkat cells through the TCR signalling pathway. When Jurkat cells were stimulated by TNF- α (20 ng/mL), the BCL11B protein expression was not changed, but the NF- κ B protein expression level was increased, similar to the mRNA expression trends ($P < 0.05$) (Fig. 2C). The results confirmed the previous hypothesis. Variation of BCL11B and NF- κ B in Jurkat cells through the TCR signalling pathway. In addition, BCL11B is associated with NF- κ B.

Figure 2

A. SEA Group

B. PMA+IO group

C. TNF- α group

Figure 2. (A) Jurkat cells were stimulated with SEA (1 μ g/mL) for 15 min, 45 min, 180 min, and 360 min. (B) Jurkat cells were simultaneously stimulated with PMA (20 ng/mL) and IO (1 μ M) for the same durations. (C) TNF- α (20 ng/mL) was used to stimulate Jurkat cells for the same durations. All the samples derived from the same experiment and that blots were processed in parallel.

3.3 Expression of NF- κ B protein in Jurkat Cells after BCL11B Knockdown

To explore the possible relationship between the BCL11B transcription factor and NF- κ B transcription factor, BCL11B-siRNA was used to suppress the BCL11B gene in Jurkat cells. The

delivery of siRNAs into Jurkat cells visualized by BLOCK-iT™ Alexa Fluor red fluorescent labelled siRNAs. As shown in Fig. 3. There was no significant change in NF- κ B protein expression in Jurkat cells stimulated with SEA (1 μ g/mL) 15 min, 45 min, 180 min, and 360 min after 48 h of transfection with BCL11B-siRNA. The expression of the NF- κ B protein did not differ from SEA group or PMA+IO group on equal conditions. As shown in Fig. 4A.

There was also no significant change in NF- κ B protein expression in Jurkat cells stimulated with PMA (20 ng/mL) combined with IO (1 μ M) at 15 min, 45 min, 180 min, and 360 min after transfection for 48 h with BCL11B-siRNA. This observation was similar to the observation of the SEA group. As shown in Fig. 4B.

According to above, mRNA expression of BCL11B and NF- κ B increased in Jurkat cell line over time which stimulated by SEA or PMA+IO, a similar result was confirmed for BCL11B and NF- κ B protein expression. As shown in Fig.1, Fig.2. However, these similar results did not appear in the BCL11B Knockdown group. There was no significant change in NF- κ B protein expression in Jurkat cells stimulated with SEA or PMA+IO. Therefore, the results confirmed that BCL11B regulates NF- κ B expression in Jurkat cells. Otherwise, the similar of SEA group and PMA+IO group revealed again that variation of BCL11B and NF- κ B in Jurkat cells through the TCR signalling pathway.

Figure 3

Figure 3.(A) Jurkat cells transfected with BCL11B-siRNA according to the manufacturers' instructions visualized by fluorescent microscope. Figure 3.(B) Transfected Jurkat cells observed under light microscope.

Figure 4.

A

B

Figure 4. (A) Jurkat cells transfected with BCL11B-siRNA for 48 h were stimulated with SEA (1 $\mu\text{g/mL}$) for 15 min, 45 min, 180 min, and 360 min. (B) Transfected Jurkat cells stimulated with PMA (20 ng/mL) combined with IO (1 μM) for 15 min, 45 min, 180 min, and 360 min. All the samples derived from the same experiment and that blots were processed in parallel.

3.4 The Effects of SEA on the Protein Expression of BCL11B and NF- κ B in Normal Human T Lymphocytes

To determine the possibility of similar effects on BCL11B and NF- κ B protein expression can be detected in normal human T lymphocytes, CD3⁺ T lymphocytes obtained from normal human peripheral blood were stimulated with the same concentration of SEA as used to treat Jurkat cells. The results showed a variable trend of protein expression of BCL11B ($P < 0.05$) and NF- κ B ($P < 0.05$) in normal peripheral blood T cells, and this trend was similar to that of the Jurkat cells (Fig. 5).

Figure 5. CD3⁺ T lymphocytes obtained from normal human peripheral blood were stimulated with the same concentration of SEA for the same durations as used to treat Jurkat cells. All the samples derived from the same experiment and that blots were processed in parallel.

5. Discussion

This study shows that the gene and protein expression levels of both BCL11B and NF-κB were related, and the protein expression of NF-κB was regulated by BCL11B expression through the TCR signalling pathway in T cells stimulated with SEA. BCL11B is a novel transcription regulation factor that plays an important role in the development or differentiation of T lymphocytes, and it is also

associated with lymphocytic leukaemia occurrence and progression. BCL11B consists of 3 introns and 4 exons, but the fourth exon does not participate in transcription. After shearing 3 exons, mRNA precursors produce 2 isomers depending on whether they encode 823 or 894 amino acids. The NF- κ B protein is also a transcription factor that participates in the inflammatory response. There may be a relationship between BCL11B protein and NF- κ B protein during the inflammatory response. SEA, as a superantigen, stimulates 20% of lymphocytes through the TCR pathway and releases various kinds of bioactive cytokines, which induce biological and/or pathological physiological effects.

Lck mediates canonical TCR signalling via activation of ZAP-70, phosphorylation of LAT, and conversion of phosphatidylinositol 4,5-bisphosphate to inositol 3,4,5-triphosphate and diacylglycerol by phospholipase C (PLC)- γ . The generation of inositol 3,4,5-triphosphate activates Ca^{2+} signalling and leads to the nuclear translocation of NFAT; diacylglycerol activates PKC and RAS-MAPK signalling[19]. PMA and IO have been widely used in the study of T cell activation because they activate protein kinase C (PKC) and calcineurin[20]. In this study, PMA combined with IO was used to stimulate Jurkat cells for approximately 15 min, 45 min, 180 min, and 360 min. The expression of BCL11B and NF- κ B was induced over time, and similar changes were found with SEA stimulation. The protein expression of NF- κ B and BCL11B in normal human peripheral blood T cells stimulated with SEA was similar to that in Jurkat cells. This result reveals that SEA, as a superantigen, induces the expression of BCL11B and NF- κ B through the TCR signalling pathway.

The NF- κ B signalling pathway can be activated by distinct substances through the TNF receptor, which is not the same pathway as that induced by TCR signalling. Tumour necrosis factor (TNF) can induce apoptosis and activate NF- κ B through signalling cascades emanating from TNF receptor 1 (TNFR1). Trimeric TNF induces TNFR1 aggregation, and TRADD associates via its own death domain with the aggregated death domain of TNFR1 to initiate signalling cascades leading to both apoptosis and NF- κ B activation. In this study, Jurkat T cells were stimulated with TNF- α under the same conditions (15 min, 45 min, 180 min, 360 min) as those of SEA stimulation. Increased expression was found only for NF- κ B mRNA and protein; increased expression of BCL11B was not detected. These results indicated that NF- κ B can be upregulated by the BCL11B transcription factor but not through the TCR signalling pathway.

BCL11B genes are expressed in all subsets of T cells and during the growth phase to the DN2 phase. The gene expression of BCL11B in original cells makes it possible to maintain TCR signalling, but this function is restrained in mature T cells[21]. BCL11B genes are important in T lymphocyte development and frequency, and their abnormal expression may affect T lymphocyte development, weakening the immune system by causing inflammation and/or tumours. Some studies have shown that the BCL11B gene is associated with inflammation. The BCL11B gene combines with the Gata-3 promoter to restrain Gata-3 protein expression, inhibit the Th2 gene,

and reduce Th17 cell production, ultimately leading to experimental autoimmune encephalomyelitis (EAE)[21]. The BCL11B gene is also highly expressed in ankylosing spondylitis (AS) and maternal placental dysfunction [22, 23]. Interleukin-2 (IL-2) is a vital inflammatory factor. Some surveys show that IL-2 is highly secreted by T cells because the US1 locus of the IL-2 promoter is combined with the BCL11B gene [24]. The NF- κ B protein is a major inflammation molecule. It is highly expressed in various inflammatory conditions, such as atherosclerosis, acute traumatic lung damage, rheumatoid arthritis, ulcerative colitis, and diabetes. It is widely believed that higher expression of NF- κ B protein can lead to inflammation.

The protein that combines with the BCL11B transcription factor remains unclear. Some studies have shown that BCL11B combines with a P300-binding protein to regulate IL-2 secretion. BCL11B inhibits IL-2 secretion by combining with MTA1 and MTA2 proteins, which are members of the NuRD[25, 26]. Some documents report that BCL11B enhances the Cot kinase reaction and then activates NF- κ B, and NF- κ B binds the promoter of IL-2 to enhance IL-2 cytokine expression[24]. In stimulated T lymphocytes, BCL11B can enhance Cot kinase expression [27] and increase NF- κ B gene expression. Therefore, BCL11B can play an important role in NF- κ B gene activation during inflammation.

SEA is not only an exotoxin; it is also a superantigen. Our results show that SEA, as a superantigen, can induce the expression of NF- κ B mRNA and protein in T lymphocytes through the TCR signalling pathway. SEA causes inflammation, which is related to the NF- κ B protein, and the NF- κ B protein is regulated by BCL11B. The exact mechanism of this abnormal T lymphocyte activation needs further investigation. This study provides new clues for the prevention and treatment of autoimmune diseases and abnormal T cell proliferation.

Declarations

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Authors' contributions

Yuhui Y and Senlin W participated in the experiments, Yuhui Y prepared figures 1-3. Senlin W prepared figure 4. Chen L made experimental plan. All authors reviewed the manuscript.

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Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The data used to support the findings of this study are included within the article and more detailed data of the current study are available from the corresponding author on reasonable request.

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