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Original Article

IFN-γ induces telomere attrition in aplastic anemia

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Abstract: Telomere attrition was reported in about 1/3 AA patients, but the precise pathogenesis is unclear. Our previously study found telomere length (TL) was closely related to patients' immune status and the severity of disease condition. In this study we detected TL and IFN-γ of AA patients, we found a closely inverse relationship between serum IFN-γ level and TL of PWBC from AA. We followed up 4 AA patients who received ATG-based treatment and found that the recovery of IFN-γ level and telomere length was important for a good therapeutic response, poor prognosis and high clone transformation rate always couple with continuous high level of serum IFN-γ and extremely short telomere length (lower than 5 kb) for whatever reason. Cell experiment showed that both serum form SAA and IFN-γ could reduce the expression of hTERT of MOLT-4, which meant IFN-γ could induce telomere attrition by inhibiting telomerase activity. Our study suggested high level of IFN-γ may one of the most important causes that contributed to telomere attrition in AA patients.

Keywords: Aplastic anemia, telomere attrition, IFN-γ, hTERT

Introduction

Acquired aplastic anemia is a life-threatening disease characterized by pancytopenia and hypocellular bone marrow. The incidence of aplastic anemia in the West is 2 per million and is about 2- to 3-fold higher in Asia. In China, the incidence is even higher with 7.4 cases per million per year. Although hematopoietic stem cell transplantation (HSCT) is curative in majority of AA patients, and immunosuppressive therapy (IST) often associate with a hematologic response rate ranging form 60% to 75% across many large studies, there also 30% patients die of the disease. In China, the percents might be higher than that.

The pathogenesis of AA is not clearly, immune-mediated processes are recognized by most researchers. Actived cytotoxic T cells secrete marrow-suppressive cytokines (e.g, IFN-γ, TNF-α) which reduce numbers of human hemotopoietic progenitor-derived colonies and induce apoptosis through the Fas-dependent and perforin pathways of cell death [1-5].

Telomeres are located at the end of liner eukaryotic chromosomes and consist of repetitive TTAGGG DNA sequences and specific interacting proteins that together form a capping structure that prevents chromosomal damage and degradation [7]. Telomerase play a very important role in telomere formation, and hTERT (telomerase reverse transcriptase) is the rate-limiting component and always be used to express the activity of telomerase. Short telomere in AA was first reported by St George's Hospital in 1998 [6]. About 1/3 AA patients was detected with short telomere, but mutations in telomerase gene (e.g. TERC or hTERT) was found only in about 10% of patients with AA [7-10].

Recently studies found telomere attrition was detected at the condition of inflammation, stress and (or) aging [11]. Cytokines such as TNF-α, IFN-γ show significantly correlation with telomere shortening of PWBC. AA is exactly the disease that triggered by cytotoxic T cells who release IFN-γ to serum of patients [1]. In this study we follow up parts of AA patients who responded to antithymocyte globulin (ATG) coupled with cyclosporine, glucocorticoid, hematopoietic stimulating factors (HSF, recombinant human erythropoietin, granulocyte colony-stimulating factor, recombinant human thrombopoietin, and/or IL-11 in combination), with or with-

Table 1. Patients' characteristics

Diagnosis	No.	Age (y)	N (*10^9/L)	Hb (g/L)	Plts (*10^9/L)	Ret (*10^9/L)	Therapy
Untreated SAA	7	27 (16-49)	0.68 ± 0.72	90.71 ± 27.11	24.28 ± 26.13	14.57 ± 8.94	Not previously treated except for transfusions
Recovering SAA	11	29 (18-50)	2.28 ± 1.65	103.37 ± 22.64	42.12 ± 50.29	29.11 ± 15.50	Treated with combination indicated

out androgen, try to find the relationship between short telomere and IFN-γ in AA.

Materials and methods

Patients and controls

Peripheral blood samples were obtained from 18 AA patients who were hospitalized in Department of Hematology General Hospital of Tianjin Medical University during Jan. 2011-Dec. 2015 and healthy controls after written the Declaration of Helsinki. AA diagnosis was established by bone marrow smear and peripheral blood cell count following criteria performed by the British committee for standards in haematology (BCSH). There were 18 AA patients included in the study, 13 males vs. 5 females, median age was 27 (16-49) years old. 7 of them were newly diagnosed patients and 11 of them were recovering patients. Recovering patients were patients with bone marrow hematopoietic recovery, which was defined as substantial improvement in 2-3 lineages. 9 age matched health controls were enrolled in this study, including 6 males and 3 females, median age was 29 (18-50) years old. There were no significant differences between patients and controls about their gender and age. This study was approved by our hospital ethics committee, all participants had signed the informed consent. Patients' information is performed in **Table 1**.

Measurement of telomere length

DNA was extracted from 1-2 ×10^6 target cells by a DNA extraction kit (Tian'enze Biotech, Beijing, China). Mean length of terminal restriction fragments (TRF) was measured using the TeloTAGGG telomere length assay kit (Roche). Purified DNA (1-2 μg) was digested with HinfI/RsaI mixture. Following electrophoresisand transfer, the membrane containing DNA was hybridized to digoxigenin-labeled probe specific for telomeric DNA repeats. After incubation with a digoxigenin-specific antibody, TRF length was visualized by a highly sensitive chemiluminescent reagent in the kit by gel imaging analysis system (GenesnapG, GeneCompany, American). Overall mean TRF length was determined using Gene Tools software.

Isolation of serum and enzyme-linked immunosorbent assay (ELISA)

Blood was drawn from patients and controls by gel serum separating tubes. And the serum was isolated by brief centrifugation of the whole blood. The production of IFN-γ in serum was measured by ELISA MAXTM Standard Sets KIT II (Biolegend). Optical density was measured by a microplate reader at 450 nm (Elx 800, BioTek).

Cell culture and qPCR

Molt-4 cells which were used as a positive control in most telomere length detected study were cultured in different conditions and harvested at 6 hours. mRNA was isolated according to the manufacturer's protocol (Invitrogen).

Total amount of RNA were reverse transcribed using the High-Capacity Reverse Transcription kit (TianGen Biotech, China) according to manufacturer's protocol. Real-time PCR was performed according to the protocol of telomerase detected kit (JiKai Biotech, China), qPCR was carried out in a Bio-Rad PCR iQ5 (Bio-Rad, USA). Absorption values of the SYBR Green I in each tube were detected at the end of each cycle. A melting curve analysis of PCR products from 55 to 95°C was also performed after PCR amplification. The relative expression level of hTERT was calculated by the ΔΔCt method (User Bulletin number 2, ABI PRISM7700 Sequence Detection System).

Statistical analysis

All statistical calculations were performed using a software SPSS 22.0. Normal distribution of measurement datas were expressed by mean ± standard deviation (SD) at least three independent experiments. For analysis of three or more groups, data were analyzed by the analysis of variance (ANOVA). Spearman's rank correlation test was used to calculate the correlation between telomere length and

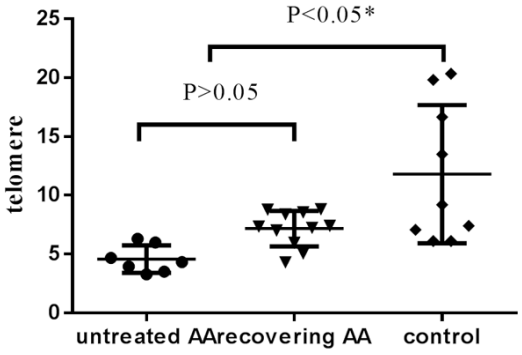

Figure 1. Telomere length of PWBC.

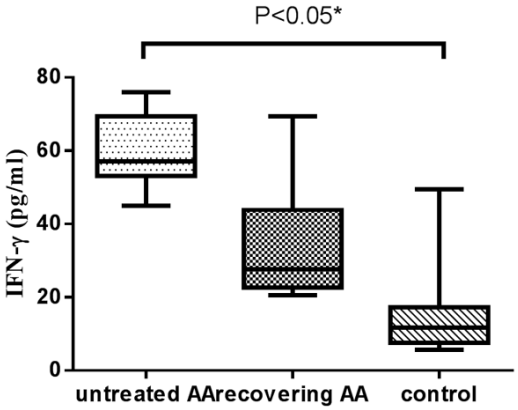

Figure 2. IFN-γ level of SAA serum.

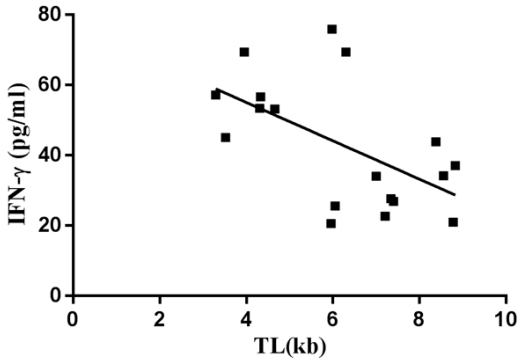

Figure 3. Relationship between TL and IFN-γ.

clinical data. $P < 0.05$ was considered to be signifcant.

Results

Telomeres length of patients with AA

The mean telomere length in peripheral white blood cells (PWBCs) was found to be significantly shorter in untreated SAA patients (4.58 ± 1.17) kb than in recovering SAA patients (7.26 ± 1.40) and in controls (11.79 ± 5.87) kb (**Figure 1**). No significant difference of telomere length was detected between recovering SAA patients and controls. We also found telomere length in AA was age independent (r=0.38, $P > 0.05$). Moreover, we divided AA patients into 15-30 years old group, 31-45 years old group and 45-60 years old group, no significant differences were found about telomere length among each groups.

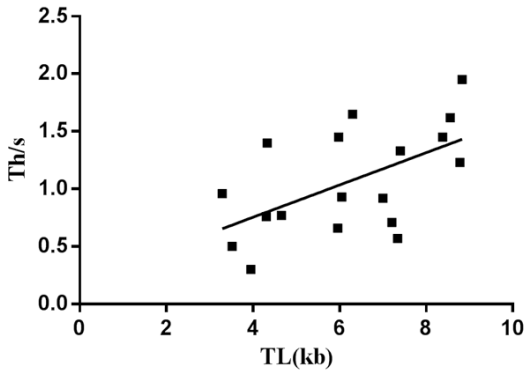

Figure 4. Relationship between TL and Th/s.

Serum IFN-γ level of the patients with AA

The serum IFN-γ level was much higher in untreated group (56.33 ± 13.32) pg/ml than in recovering SAA patients (34.46 ± 15.37) pg/ml and in controls (15.28 ± 13.58) pg/ml, significant differences were found between each other (**Figure 2**).

The relationship between telomere length and clinical parameters

We collected PWBC and serum of patients with AA simultaneously, and we found shortened telomere length correlated with high level of serum IFN-γ (r=-0.538, P=0.021) and low level of Th/s (r=0.533, P=0.023) (**Figures 3** and **4**).

There was no relationship between the telomere length of PWBCs in AA patients and the absolute value of neutrophil count (r=0.397, P=0.143), reticulocytes in peripheral blood (r=0.374, P=0.170), bone marrow erythroid proportion (r=0.324, P=0.478), myeloid cells proportion (r=0.036, P=0.939) and lymphoid cells proportion (r=-0.524, P=0.183), the recovery time of at least one lineage of blood cells (r=-0.429, P=0.188).

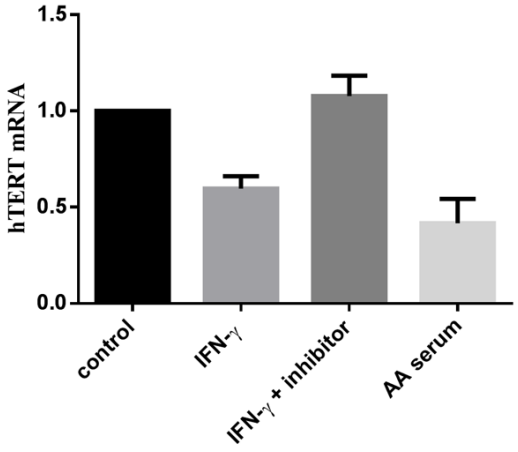

Figure 5. hTERT mRNA expression of MOLT-4 cells. Molt-4 cells were divided into 4 groups and cultured with 4 different culture mediums, Group 1: RPMI1640 supplemented with 10% fetal bovine serum; Group 2: 1640 medium supplemented with 10% FBS and 50 pg/mL IFN-γ; Group 3: 1640 medium supplemented with 10% FBS and 50 pg/mL IFN-γ plus adequate antagonist of IFN-γ; Group 4: 1640 medium supplemented with 10% SAA serum. The expression of hTERT was extremely low in AA and IFN-γ group, and got recovery in IFN-γ plus antagonist group.

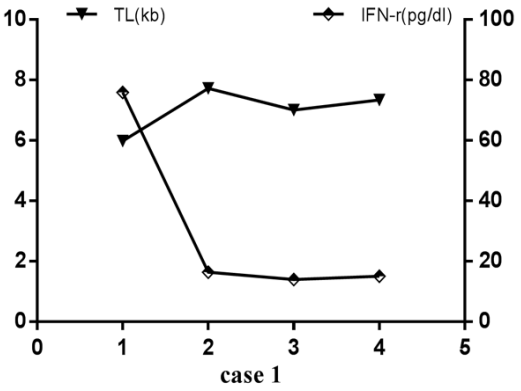

Figure 6. Case 1. The patient was first diagnosed severe aplastic anemia when he was 25 years old. We detected serum IFN-γ level (75.88 pg/ml) and the telomere length (5.89 kb) of PWBC before any other treatment except transfusion. After combined immunotherapy (including ATG, CsA, HSF) for six months, serum IFN-γ level decrease to 16.41 pg/ml, telomere length of PWBC increase to 7.21 kb, and the blood cells become totally normal after therapy for 11 months.

Molt-4 cells culture

Molt-4 cells were divided into 4 groups and cultured with 4 different culture mediums, Group 1: RPMI1640 (Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS, HYCLONE, USA); Group 2: 1640 medium supplemented with 10% FBS and 50 pg/mL IFN-γ (a similar IFN-γ level with untreated AA patients); Group 3: 1640 medium supplemented with 10% FBS and 50 pg/mL IFN-γ plus adequate antagonist of IFN-γ; Group 4: 1640 medium supplemented with 10% AA serum; The cells were incubated at 37°C in humidified 95% air with 5% CO_2 for 6 hours. Each experiment was repeated three times. We found the expression of hTERT was extremely low in AA (0.42 ± 0.13) and IFN-γ group (0.60 ± 0.06), and got recovery in IFN-γ plus antagonist group (1.077 ± 0.11) (**Figure 5**).

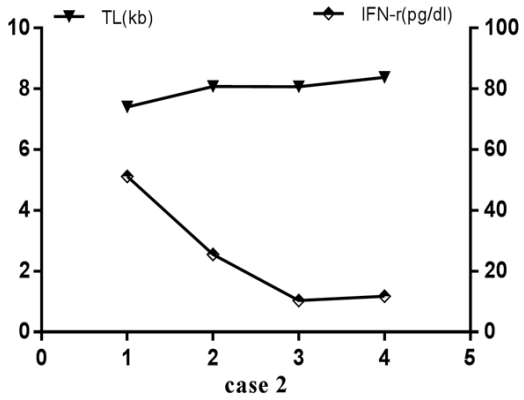

Figure 7. Case 2. The male patient was diagnosed hepatitis associated aplastic anemia when he was 19 years old. We detected the serum IFN-γ level (51.24 pg/ml) and the telomere length (7.4 kb) before any other treatment except transfusion. After combined immunotherapy (including ATG, CsA, HSF) for six months, the serum IFN-γ level decrease to 25.53 pg/ml, telomere length of PWBC turned to 8.08 kb, and the blood cells become totally normal after therapy for 15 months.

Four patients clinical parameters

Here we show some clinical data from 4 patients, who were followed up for at least 48 months by us. Blood routine was tested every week, bone marrow aspiration, PNH clone and chromosome were detected every 3 months. Telomere length and the serum IFN-γ were monitored every 12 months (**Figures 6**-**9**).

Discussion

AA is a critical hemotopoiesis failure disease characterized by bone marrow exhaustion and high mortality. Cytotoxic T cells are polarized to

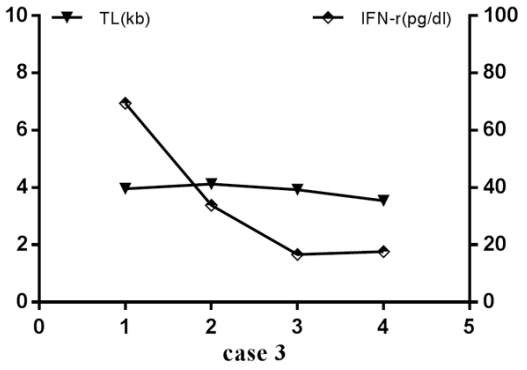

Figure 8. Case 3. The patient was first diagnosed severe aplastic anemia when he was 32 years old, we detected serum IFN-γ level (69.38 pg/ml) and the telomere length (3.95 kb) after he use CsA 3 mg/kg.d for one month but before ATG. After combined immunotherapy (including ATG, CsA, HSF, androgen) for six months, the serum IFN-γ level decrease to 33.82 pg/ml, but telomere length sustain a relative very level below 5 kb. This patient relapsed soon after a transient normal blood count period. PNH clone was detected at the sixth month after ATG therapy, and at last the patient developed acute myeloid leukemia (AML), without chromosome abnormality.

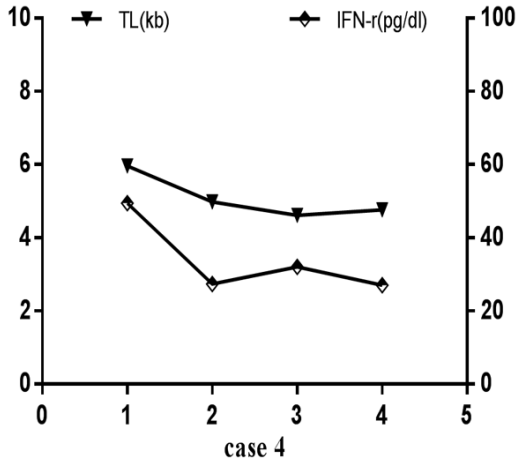

Figure 9. Case 4. The patient was first diagnosed severe aplastic anemia when he was 48 years old, we detected the serum IFN-γ level (49.47 pg/ml) and the telomere length (5.96 kb) after he received combined immunotherapy (including ATG, CsA, HSF, and rogen) for 31 months and hospitalized because of repeated fever. We followed up him once a year, he was transfusion dependence, got fever without clear foci of infection. The telomere length turned to about 5 kb after he received ATG 5 years. Finally this patient developed myelodysplastic syndrome (MDS) with normal chromosome.

Th1 profile and release suppressive cytokines such as TNF-α and IFN-γ, which attack bone marrow and induce apoptosis of hemopoietic stem cells and precursors [1-3]. This study proved the result once more, increased IFN-γ was detected in untreated AA patients than in recovery patients and controls. In our previous study, shortened TL was found correlated with low ratio of CD4+ T-helper lymphocytes to CD8+ T-suppressor (Th/s), low level of hemoglobin, platelet count, absolute neutrophil count and proportion of reticulocytes in peripheral blood. That means TL is closely related with the immune status and severity of AA [12].

In this study a strong inverse correlation was observed between TL and peripheral IFN-γ of AA, but not between TL and age. When we divided AA patients into three groups (15-30 years group, 31-45 years group and 45-60 years group), we still found no apparent intergroup difference of TL in AA. It seems telomere which was recognized life-clock of mammal is regulated by other mechanism besides time, but the pathogenesis is unknown.

Early report suggested that telomere attrition occurs under the condition of inflammation, stress or senility, this occurrence is closely related with cytokines, such as TNF-α and IFN-γ which induces TL decreasing by reducing the expression of hTERT and the activity of telomerase [13-16]. In this study, both serum from AA patients and IFN-γ can inhibit the expression of hTERT, and the expression of hTERT recover when IFN-γ plus antagonist. It seems that the inhibit effect of serum from AA patients is more severe than IFN-γ, so there may some other elements exist in serum from AA synergetic with IFN-γ can inhibit telomerase. Further studies are needed.

Treatment with androgen leading to telomere elongation was reported recently [17, 18], and so did we found in our research. Three out of four cases in my study had treated with androgen coupled with ATG-based immunosupression protocols. All these 4 patients response to initial immunosupressive treatment (IST). Case 3 maintained a critical short TL (< 5 kb) and had clonal evolution at the end. Case 4 had a relative longer telomere than Case 3, but the TL was detected after receiving ATG-based treatment not the baseline length. Maybe he experience a period of telomere elongation we hadn't caught, we only noted that telomere shorten gradually and malignant clonal conver-

sion. Case 1 and Case 2 have favorable response to IST, they both got completely remission with TL increasing and the level of IFN-γ decreasing. Interestingly, Case 2 got oral administration of androgen continuously, but Case 1 never used it. As we all know, androgen has many side effects especially to female and children. Therefore, it's necessary to distinguish the AA patients who get telomere recovery depend on androgen from who don't.

We showed all these 4 patients data about serum IFN-γ and TL of PWBC, and found IFN-γ decrease to a normal level and telomere elongation were the sufficient condition of AA recovery. No matter what reasons caused a persistently high level of IFN-γ (the high level of IFN-γ of case 4 may cause by infection) or a critical short of TL could lead to a extremely high cloning transformation rate and poor prognosis. Whether TL could be used as a marker to predict relapse and clonal evolution, our follow-up time was not enough. Further follow-up visit is necessary.

In brief, behind the veil of short telomere of AA are the intricacies of the mechanisms, immune abnormality may be one of the most important one of them, which deserves more in-depth study.

Acknowledgements

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Disclosure of conflict of interest

None.

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