Implication of therapeutic outcomes associated with molecular characterization of paediatric aplastic anaemia

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ABSTRACT

Objectives: Severe aplastic anemia is characterized by a hypocellular bone marrow and peripheral cytopenia. Mesenchymal stem cells (MSCs) play a crucial role in haematopoietic stem cells (HSCs) development and the development of microenvironment suitable for hematopoiesis. Molecular characterization of telomere maintenance pathway and gene expression profiling of MSCs can be important for the therapeutic interventions among paediatric aplastic anaemia patients.

Methods: The study involved paediatric aplastic anaemia patients (n = 10) and age matched paediatric healthy donors (n = 8). Peripheral blood samples were collected from the individuals. Average leucocyte telomere length and gene expression of the telomere maintenance genes were determined by quantitative real time PCR. Microarray based gene expression profiles (GSE33812) of MSCs for five paediatric aplastic anaemia patients were analyzed compared to five healthy controls and the data was downloaded from the GEO database.

Results: The telomere length was significantly shorter among paediatric AA patients compared to age matched healthy donors. Interestingly, one subgroup (n = 2) of paediatric AA patients has moderate telomere length comparable to age matched healthy donors. Based on the gene expression analysis of telomere maintenance pathway, TERF2 was significantly downregulated among paediatric patients with shorter telomere length but not among paediatric patients with moderate telomere length. Gene expression profiling of MSCs revealed three differentially expressed genes (GAS2L3, MK167 and TMSB15A) among the patients and was associated with therapeutic outcome.

Conclusion: Telomere length estimation and gene expression patterns of the MSCs and telomere length maintenance pathway may serve as a potential biomarker and could be associated with therapeutic choice of paediatric aplastic anaemia patients.

1. Introduction

Aplastic anemia (AA) is a rare, immune-mediated haematopoietic disorder associated with significant morbidity and mortality [1]. AA can be diagnosed in patients with pancytopenia and a hypocellular bone marrow. Typical symptoms include fatigue and easy bruising or bleeding. Infections may be present but generally there is no long-standing illness [2]. In patients with suspected AA, rapid and accurate diagnosis and concomitant supportive care are critical. Historically, immunosuppressive therapy (IST) and bone marrow transplantation (BMT) in eligible patients have been the mainstay of AA treatment [1]. In paediatric patients, new transplant strategies and improvements in supportive care have led to greatly improved outcomes and increasing application of BMT were reported in AA [1,3].

The incidence of AA varies with geography and it was found to be higher in Asia and lower in Europe, North America and Brazil according to the International Agranulocytosis and Aplastic Anemia Study (IAAAS) [4–7]. It was also identified that the incidence of that disease was 2-to 3-fold higher in Asia than in the West [8]. The great variation of the incidence of the disease is due to differential environmental exposure such as use of certain drugs and chemicals or by infectious agents such as viruses and bacteria. Besides the environmental agents the genetic background of different ethnic population may confer the risk of that disease [9–11]. It is really a great challenge to characterize the
paediatric patients with aplastic anaemia than an adult because of the incidence of numerous inherited bone marrow failures and they are also associated with aplastic anaemia. Therefore, a precise diagnostic technique is essential for the application of suitable therapeutics among children with aplastic anaemia [12]. Different demographic factors were already reported to be associated with aplastic anaemia among paediatric individuals and disease severity [13]. As the children are more sensitive to newer therapeutic agents in respect to their tolerability and suitability in contrast with chemotherapy or stem cell transplant, it’s necessary to establish optimal treatment strategies. A recent study reported that there is a substantial difference of the outcome of immunosuppressive therapy among children with aplastic anaemia compared to adult one [14].

Telomere repeats serve to protect the ends of chromosomes and can prevent end-to-end fusion, DNA damage and recombination [15]. Among aplastic anaemia patients, telomere and genes involved in telomere maintenance pathway were reported to be associated with therapeutic outcome. Measurement of the telomere length of lymphocytes was used as a promising diagnostic assay for the application of suitable therapeutic regimen in children with aplastic anaemia [16]. Transcriptome analysis can clearly differentiate healthy controls from AA patients. A study on transcriptome analysis among paediatric aplastic anaemia patients identified differentially expressed genes involved in cell metabolism and cell communication or adhesion [17]. MSCs usually resides within the stroma and they are derived from bone marrow. They play significant role in hematopoiesis and immunomodulation. Various studies have reported the differential gene expression in MSCs among patients with aplastic anaemia compared to healthy controls. A study by Li et al., 2012 identified over 300 differentially expressed genes among aplastic anaemia compared with healthy controls [18]. These differentially expressed gene were involved in apoptosis, adipogenesis, and the immune response. Another study also reported increased MSCs apoptosis in AA patients [19]. Consequent studies also revealed that MSCs among AA patients have lower proliferation potential [20,21].

Various studies have already reported gene expression profiling of bone marrow MSCs from aplastic anaemia patients and they identified several genes those are involved in various biological processes such as cell cycle, cell division, proliferation, chemotaxis, adipogenesis-cytokine signalling and haematopoietic cell lineage differentiation which suggest that impaired cellular function is the hallmark of this disease [22–24]. Chao et al., 2015 also suggested the role of deregulation of cytokine genes in MSCs among children with aplastic anaemia [23].

Therefore, we undertook the current study to determine the impact of telomere maintenance pathway and gene expression status of MSCs associated with therapeutic outcome of paediatric patients with aplastic anaemia.

2. Material and methods

2.1. Subjects and samples

The study is a part of ongoing cohort study involving 10 paediatric aplastic anaemia cases and 8 healthy paediatric samples. The aplastic anaemia patients were selected on the basis of peripheral pancytopenia and hypocellular bone marrow [25]. Additionally, we excluded other cases of pancytopenia patients with HIV, hepatitis, parovirus B19, vitamin B12 and folate deficiencies, autoimmune disease, leukemia, lymphoma, solid malignancies, and fibrosis. Peripheral blood samples were collected from the subject attending a referral hospital (Burdwan Medical College & Hospital, Burdwan, West Bengal, India) and collected within a period of 2018–2020 with informed consent from the participants. The study was approved by the Institutional Clinical Ethical Committee of The University of Burdwan. The details of categories of samples selected for the study were depicted in Table 1.

2.2. DNA isolation from peripheral blood samples

Peripheral blood samples were collected from aplastic anaemia patients and healthy donor by aseptic method in Vacutainer spray-coated EDTA Tubes. DNA isolation from an aliquot of peripheral blood sample was done by QIAamp Blood DNA mini kit (Qiagen) following manufacturer’s protocol. Quality and concentration of stock DNA was therefore determined by estimation of optical density of the samples at wavelengths of 260 nm and 280 nm using a UV–Vis Spectrophotometer (Simadzu). The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA. The genomic DNA was subjected to 1% agarose gel electrophoresis to ensure the integrity of DNA samples. The samples which failed to reach the optimum concentration were subjected to whole genome amplification by REPLI-g mini kit (Qiagen).

2.3. Telomere length estimation

Leucocyte telomere length was estimated by quantitative real time PCR by Cawthon’s method [26] with the modifications by O’Callaghan et al., 2008 [27]. For this, 20 ng of genomic DNA was subjected to quantitative real time PCR by using two sets of oligomer and PCR primers. One set of oligomer and primers were used to quantify the telomeric sequence and another one was used to quantify the single copy gene standard (3684 nucleotides) as described previously [27]. By this method, two standard curves were obtained for telomeric sequence and single copy gene standard and the length per telomere per sample was estimated by the formula described previously [27]. The reactions were performed by Maxima SYBR Green master mix (Thermo Fisher Scientific) on LC480 real time PCR platform (Roche). All reactions were performed in triplicate with both aplastic anaemia patients and healthy donor’s DNA and both assays were performed in the same plate.

2.4. RNA isolation and cDNA preparation

Total RNA was isolated from another aliquot of peripheral blood samples from patients and healthy donors by the method described previously [28]. The integrity of each RNA sample was confirmed by running the RNA samples in 1% agarose gel electrophoresis. The concentration and purity of the RNA samples were determined by estimation of optical density of the samples at wavelengths of 260 nm and 280 nm.

| Table 1 | Categories of samples selected for the study. |
|---------|-----------------------------------------------|
| Categories of Samples | Subset of categories | No. of Samples | Median Age (Range) in yrs. | Diagnostic Features |
| Aplastic Anemia Patients | | 10 | 10.5 (2.5–15) | Peripheral Pancytopenia Hypocellular Bone Marrow |
| Aplastic Anemia Patients | Moderate Telomere Length | 2 | 10.25 (6.5–14) | Peripheral Pancytopenia Hypocellular Bone Marrow |
| Aplastic Anemia Patients | Shorter Telomere Length | 8 | 10.5 (2.5–15) | Peripheral Pancytopenia Hypocellular Bone Marrow |
| Healthy Donors | | 8 | 9 (3–15) | No indication of Peripheral Pancytopenia No history of aplastic anaemia or any chronic diseases |
nm using a UV-Vis Spectrophotometer (Simadzu). For cDNA preparation, 1 μg of total RNA from each sample was reverse transcribed using the High capacity cDNA Reverse Transcription kit (Applied Biosystems) following manufacturer’s protocol. Reverse transcription reaction, with mRNA and all reagents but no reverse transcriptase, was performed for the samples as negative controls. All cDNA samples were stored at −80 °C for long term usage.

2.5. Expression analysis of the candidate genes

The expression of some prioritized candidate genes involved in telomere length maintenance pathway were determined by SYBR Green based assay. The genes selected for the study are involved in telomere length maintenance pathway based on literature survey and the details of the genes were depicted in Table S1. For real-time PCR of SYBR Green based assays, 1 μl of cDNA was subjected to amplification by addition of 50 ng of forward primer, 50 ng of reverse primer, 5 μl Maxima SYBR Green PCR Master Mix (Thermo Fisher Scientific) and 3 μl nuclease-free water. The real time PCR program included initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 1 min. Melting curve analysis was done to rule out the non-specific amplification. The negative controls used for the amplification were RNase free water and cDNA prepared from mRNA without reverse transcriptase. Each assay was performed in triplicate per sample on MicroAmp optical 96-well plates using a LC480 PCR System (Roche). Relative expressions of the mRNAs were calculated using GAPDH as the endogenous control and calibrated to the healthy donor samples. The details of primers and PCR conditions for mRNA expression study were depicted in Table S2.

2.6. Gene expression profiling of MSCs

The expression analysis of the microarray datasets from the Gene Expression Omnibus (GEO) database with accession id GSE33812 [29] of the National Center for Biotechnology Information (NCBI) of the U.S. National Library of Medicine was used for the current study. There was single gene expression profiling dataset for aplastic anemia patients among children. This dataset included 5 severe paediatric aplastic anemia patients compared to 5 healthy donors. The details of patients and healthy donors and therapeutic interventions were depicted in Table 2. Agilent-014850 Whole Human Genome Microarray 4 × 44K G4112F GeneChips were used for gene expression profiling. The probe quality of the array was assessed before and after normalization and the background correction was done using bioconductor based limma package. To improve data quality, a filtering of the probes was applied. The probes containing repetitive sequences, binding to multiple sites of human transcriptome, were removed for further analysis. Earlier study on same sample set identified the role of cytokine genes in aplastic anaemia patients [23]. In this present communication, we took the approach to identify the differentially expressed genes in individual case sample compared to controls. Thus, we will able to identify the specific genes associated with therapeutic outcome. The differentially expressed genes for each aplastic anemia patient were selected on the basis of fold change of gene expression, compared to healthy controls (for up-regulation, fold change ≥2 and for down-regulation, fold change ≤−2).

2.7. Statistical analysis

Telomere length and mRNA expression was expressed as box plots to observe the difference in distributions. Kolmogorov-Smirnov test was performed to identify whether the test variables like expression of mRNA and telomere length, followed normal distribution. Non-parametric two sample Mann Whitney U test was used to identify association of disease categories and healthy donors with variables that did not follow normal distribution. A p value less than 0.05 was considered statistically significant. All statistical analyses were done using software packages SPSS (version 16.0 for windows), R ([www.r-project.org]) and Microsoft Excel 2007.

3. Results

3.1. Telomere length estimation revealed characteristic differences among different categories of samples

Our quantitative real time PCR based estimation of telomere length identified the difference of telomere length between paediatric aplastic anaemia patients and age matched healthy donors. Telomere length estimation revealed that telomere length was significantly (p value < 0.001) reduced in aplastic anaemia patients compared to healthy donors. Interestingly, 2 out of 10 patients (2/10, 20%) showed moderate telomere length compared to age matched healthy donors and no indication of regression in telomere length was found. Therefore, we stratified our case samples into two subsets, one subset with shorter telomere length and another with moderate telomere length as depicted in Fig. 1.

3.2. Differential expression of telomere length maintenance genes among the subsets of aplastic anaemia cases

The 9 genes selected for the mRNA expression analysis were selected on the basis of their involvement in telomere length maintenance pathway. Among these 9 genes, only 1 gene (TERF2) revealed significant downregulation among case samples with shorter telomere length (fold change = −3.52-fold, p = 0.01) compared to healthy donors but not among cases with moderate telomere length as depicted in Fig. 2.

3.3. Gene expression profiling of MSCs revealed characteristic molecular differences among the subtypes of paediatric aplastic anaemia

We compared the gene expression status of every five paediatric aplastic anaemia samples compared to healthy donors. The earlier study on this gene expression profiling revealed the deregulation of cytokine genes which was associated aplastic anaemia cases [23]. Interestingly, 2 patients among them not responded to conventional immunosuppressive therapy and bone marrow transplantation was performed among them. Therefore, we critically looked at the gene expression profiling of individual patients compared to healthy donors. Interestingly, we found that three genes (GAS2L3, MK167, and TMSB15A) were significantly

Table 2

| Categories of Samples | Subset of categories | No. of Samples | Median Age (Range) in yrs. | Therapy applied |
|-----------------------|----------------------|----------------|---------------------------|-----------------|
| Aplastic Anemia Patients | Aplastic Anemia Patients with immunosuppressive therapy | 3 | 11.6 (10–12.2) | Recovery after immunosuppressive therapy |
| Aplastic Anemia Patients | Aplastic Anemia Patients with bone marrow transplantation | 2 | 13 (11.3–14.7) | Recovery after Haematopoietic stem cell transplantation |
| Healthy Donors | | 5 | 15 (2.8–17.8) | Nil |

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downregulated among four aplastic anaemia patients but not among one patient where bone marrow transplantation was recommended and the patient not responded to immunosuppressive therapy. On the contrary, angiotensinogen gene (AGT) was consistently downregulated among all the paediatric aplastic anaemia samples compared to healthy donors. The detailed gene expression profiling of these genes were depicted in Table 3.

3.4. Association of gene expression pattern with the therapeutic outcomes

Out of five paediatric aplastic anaemia patients, three patients were treated successfully with conventional immunosuppressive therapy. Other two patients were not responsive to immunosuppressive therapy and bone marrow transplantation was done for them. Based on therapeutic interventions, two subsets of paediatric aplastic anaemia patients were detected in the dataset. MSCs gene expression profiling revealed that three genes (GAS2L3, MK167, and TMSB15A) were downregulated among the patients with immunosuppressive therapy but not among one patient with bone marrow transplantation. However, we could not detect any differential gene expression in another patient with bone marrow transplantation. But, these three genes can be used as a biomarker for discriminating the paediatric aplastic anaemia subsets for the intervention of therapy.

4. Discussion

To know about the biology of aplastic anaemia, knowledge about the biology of mesenchymal stem cells and bone marrow microenvironment are essential. This enable to adopt the new strategy of personalized therapy for bone marrow failure. Another important aspect of therapeutic choice should be based on molecular characterization. Telomere length assessment and gene expression status may serve as an important diagnostic approach for the adoption of specific therapeutic interventions.

Table 3
Differentially downregulated genes among paediatric patients with aplastic anaemia compared to healthy donors.

| Patient 1 log fold change | Patient 2 log fold change | Patient 3 log fold change | Patient 4 log fold change | Patient 5 log fold change | Gene       | Gene name                     | Remarks                             |
|--------------------------|--------------------------|--------------------------|--------------------------|--------------------------|------------|-------------------------------|-------------------------------------|
| -2.144                   | -2.524                   | -2.97                    | -2.841                   | -2.535                   | AGT        | angiotensinogen               | Universally downregulated among all patients |
| -1.048                   | -2.275                   | -2.343                   | -4.192                   | -2.003                   | GAS2L3     | growth arrest specific 2 like 3 | Differentially downregulated among patients with immunosuppressive therapy |
| -1.281                   | -2.809                   | -3.336                   | -3.586                   | -3.243                   | MKi67      | marker of proliferation Ki-67 | Differentially downregulated among patients with immunosuppressive therapy |
| -1.437                   | -4.012                   | -3.921                   | -3.828                   | -3.173                   | TMSB15A    | thymosin beta 15a             | Differentially downregulated among patients with immunosuppressive therapy |
It was identified in our dataset that there is a stratification of pediatric aplastic anaemia samples based on leucocyte telomere length; one subset with shorter telomere length and another subset with moderate telomere length compared to healthy donors. Earlier clinical trials suggested the effective use of Danazol drug (clinicaltrials.gov NCT01441037) in patients with a telomere disorder. Thus, this drug can be used to treat the aplastic anaemia patients if they have shorter leucocyte telomere length. Earlier study also reported that leucocyte telomere length was associated with therapeutic responses among aplastic anaemia patients [16]. Our study also revealed that TERF2 gene was differentially downregulated among patients with shorter telomere length but not among the patients with moderate telomere length. Earlier study reported that silencing of this gene was associated with bone marrow failure [38]. Thus TERF2 expression status can be used as a possible biomarker for discrimination of subsets of paediatric aplastic anaemia patients and may serve as therapeutic target for the subset of patients with aplastic anaemia.

Our study revealed that AGT gene was significantly downregulated among all the patients with aplastic anaemia. Angiotensinogen (AGT) is the precursor of the vaso-active peptide, angiotensin II. Variant of AGT gene was identified as a significant risk factor and hereditary marker for hypertension [31]. It was also reported to be associated with cytokine secretion which might be relevant for aplastic anaemia pathogenesis. Another study also demonstrated that AGT plays a role in MSCs differentiation into adipocytes [32]. Another study also reported that silencing of AGT gene was associated with impaired pro-inflammatory cytokine secretion and might be associated with immune disorders [33]. Taken together, these findings also suggested that downregulation of AGT gene could be relevant for development of aplastic anaemia in children.

The therapeutic intervention of five paediatric aplastic anaemia patients selected for mesenchymal stem cell gene expression profiling was characteristically different. Two out of five patients were not responded to conventional immunosuppressive therapy and three patients responded to such therapy. Among one non-responsive, there was no deregulation of GAS2L3, MK167, TMSB15A genes whereas the genes were downregulated among other four patients. GAS2L3 is a cytoskeleton-associated protein which was not found to be associated with aplastic anaemia. But germline deletion of this protein associated with decreased cardiomyocyte proliferation and cardiomyocyte hypertrophy in mice model which can be considered as a proliferation marker and its deregulation might be associated with aplastic anaemia [34]. Similarly MK167 protein was considered as a proliferation marker among aplastic anaemia patients and may be associated with treatment [35]. Deregulation of TMSB15A gene was not reported in aplastic anaemia but its RNA level was associated with chemotherapeutic drug response in breast cancer [36].

Once the genetic nature of aplastic anaemia is confirmed, novel drugs like small molecule inhibitors can be applied and offer a hope for the proper therapeutic interventions of the proper children with aplastic anaemia [37,38]. Recent study from our lab showed the potential of miRNA as therapeutic target for aplastic anaemia [39]. Thus proper molecular characterization may serve as a promising diagnostic approach for the choice of therapeutic strategy in upcoming days.

5. Conclusion

Children with aplastic anaemia have promising therapeutic outcomes if treated properly with immunosuppressive therapy and bone marrow transplantation. Leucocyte telomere length, TERF2, GAS2L3, MK167 and TMSB15A gene expression status was identified as a potential biomarker for the different subset of paediatric patients with aplastic anaemia. Based on these biomarker assay suitable therapeutic options can be adopted for this disorder.

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Ethical approval

The study was approved by the Institutional Clinical Ethical Committee of The University of Burdwan for the experiments on human samples.

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CRediT authorship contribution statement

Sarmistha Adhikari: Formal analysis, Writing - original draft. Kaustav Nayek: Formal analysis. Arghya Bandopadhyay: Formal analysis. Paramita Mandal: Formal analysis, Writing - original draft.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2020.100899.

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