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Integrated analysis of global gene and microRNA expression profiling associated with aplastic anaemia

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ABSTRACT

Aims: Aplastic anaemia is a rare disorder characterized by peripheral pancytopenia and hypocellular bone marrow. Recent advancement of miRNA technologies, new promising therapy using small molecule inhibitors was suggested as efficient treatment option. Therefore, the study was undertaken to identify the significantly altered miRNA (miR-1202-upregulation) among aplastic anaemia patients compared to healthy controls by global miRNA expression profiling of bone marrow.

Materials and methods: miRNA and gene expression profiles for all the categories of aplastic anaemia patients and healthy controls were generated using Affymetrix probes.

Key findings: The study was based on freely available miRNA and host gene expression in NCBI GEO dataset. Microarray based gene expression profiling (GSE3807) revealed that RAPGEF5 and MANEA genes were significantly downregulated among aplastic anaemia patients compared to healthy controls and the expression of these genes were again upregulated after application of therapy among those patients compared to pre-therapy condition. RAPGEF5 was involved in Rap1 and Ras signaling pathways those were significantly enriched among aplastic anaemia patients and could be relevant for that phenotype. Microarray based miRNA expression profiling (GSE82095) also identified that miR-1202 was significantly upregulated among aplastic anaemia patients compared to controls and can potentially target RAPGEF5 and MANEA genes based on target prediction of miRNAs.

Significance: Thus synthetic miRNA inhibitors of miR-1202 can be used as a possible therapeutic agent to target miR-1202 and this inhibition can lead to its corresponding target gene upregulation for reversal of disease phenotype.

1. Introduction

Aplastic anaemia [AA] is an acquired rare life-threatening disease characterized by bone marrow failure which is characterized by lower blood count and hypocellularity of the bone marrow [1]. 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] [2–5]. It was also identified that the incidence of that disease was 2-to 3-fold higher in Asia than in the West [6]. 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 [7–9].

The mortality rate of the disease was seems to be high by many studies. Antithymocyte globulin [ATG] based therapy, immunosuppressive therapy and allogenic stem cell transplantation was reported to be efficient and improved outcome was observed with a 5-year overall survival [10–13]. The response to the therapy is depended upon the severity of the disease, age, and also by the choice of the initial treatment.

Microarray based gene expression profiling was implicated in the development of AA. The study by Li et al. [14] identified the aberrant expression of the genes in bone marrow mesenchymal stem cells related to increased adipogenesis mediated by genes [Tumor Necrosis Factor Receptor Type 1-Associated DEATH Domain Protein (TRADD), Protein Kinase AMP-Activated Non-Catalytic Subunit Beta 2 (PRKAB2), Leptin (LEP), Solute Carrier Family 2 Member 1 (SLC2A1) and Suppressor Of Cytokine Signaling 3 (SOCS3)] and decreased osteogenesis Another microarray based approach also showed the differential expression of cytokines and chemokines such as Interferon gamma and beta (IFN-γ and –β), C-X-C motif chemokine 2 (MIP2A) and C—C Motif Chemokine

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Table 1
Characteristics of the samples for miRNA expression profiling.

Sample id	Categories of samples	Pool of samples	Tissue/cell type	Age	Subject status
AA patient G	Aplastic anaemia	1	Bone marrow/CD3+ T cells	37 years	Acquired aplastic anaemia
AA patient B	Aplastic anaemia	1	Bone marrow/CD3+ T cells	40 years	Acquired aplastic anaemia
AA patient C	Aplastic anaemia	1	Bone marrow/CD3+ T cells	25 years	Acquired aplastic anaemia
Healthy control D	Healthy control	1	Bone marrow/CD3+ T cells	28 years	Healthy volunteers
Healthy control E	Healthy control	1	Bone marrow/CD3+ T cells	33 years	Healthy volunteers
Healthy control F	Healthy control	1	Bone marrow/CD3+ T cells	42 years	Healthy volunteers

Ligand 20 (MIP3A), C−C motif chemokine receptor 2 (CCR2), C−C motif chemokine ligand 2 (CCL2), Interleukin 8 (IL-8), Interleukin 1B (IL-1B) and Interleukin 1 receptor antagonist (IL-1RA), which are likely to play important roles in the recruitment and activation of lymphocytes into cytotoxic effectors for marrow hematopoietic target cells in AA [15]. There were also other studies which revealed the differential gene expression signature could be relevant for the development of the disease such as integrins, apoptosis and terminal cytolytic enzyme generation [16,17].

MicroRNA [miRNA]s are a family of small non-coding RNA that can primarily modulate gene expression at the post-transcriptional level by hybridization to complementary sequences in the 3′ untranslated region [3′UTR] of their corresponding mRNA [18]. miRNAs can regulate approximately 30% of the human genome, and a single miRNA have potential to regulate hundreds of proteins [19]. The emerging evidences showed that miRNAs play an important role in modulation of immune response. Deregulation of mi RNA expression was reported in many important human diseases like autoimmune diseases, such as rheumatoid arthritis and multiple sclerosis. Although a single miRNA can target many m RNAs, still the miRNA-m RNA interaction can produce dramatic changes in cell behaviour [20]. The study by Hosokawa et al., 2015 reported that downregulation of miR-126-3p and miR-145-5p promotes $CD4^+$ and $CD8^+$ T-cell activation by increasing *MYC* and *PIK3R2* expression levels in AA patients [21].

Recent studies have shown that miRNA can fine tune the gene expression in a broad spectrum of biological pathways. The potential property of the miRNAs indicates that miRNA modulation can be used as a viable therapeutic agent and promising tool. Due to the recent advancement on LNA anti-miR and Small molecule inhibitors of miRNAs [SMIRs]s, miRNAs have emerged to be promising novel therapeutic targets and intervention tools [22]. Locked nucleic acid (LNA) is inaccessible RNA in which the ribose moiety is modified with an extra bridge connecting the 2′ oxygen and 4′ carbon. The locked ribose conformation enhances base stacking and backbone pre-organization which significantly increases the hybridization properties.

We therefore undertook the study to determine the miRNA and gene expression profiling of CD3+ T cells derived from bone marrow of aplastic anaemia patients compared to control. We further explore the differential gene expression signature of bone marrow transplanted post therapy patients compared to pre therapy which calls for the identification of the particular miRNAs as novel therapeutic agents.

2. Materials and methods

2.1. Source data

The expression analysis of the microarray datasets from the Gene Expression Omnibus [GEO] database of the National Center for Biotechnology Information [NCBI] of the U.S. National Library of Medicine was used for the current study. There were single micro RNA expression dataset and two gene expression profiling datasets on aplastic anaemia patients. Of two gene expression profiling datasets, one dataset include only paediatric aplastic anaemia patients. Therefore, to get a clear view of molecular insights of aplastic anaemia irrespective of age, we used the other dataset of gene expression profiling for the study.

2.2. Definition of clinical datasets

For miRNA expression profiling, we have used GEO dataset with accession id GSE82095 which included bone marrow T cells of three acquired aplastic anaemia patients (Median age = 37 years, Range: 25–40 years) and three healthy individuals (Median age = 33 years, Range: 28–42 years). Affymetrix Gene Chip microRNA 2.0 Array was used for detection of this miRNA expression analysis [23]. The characteristics of the samples used for the study was depicted in Table 1. The raw data files were downloaded from the database and analyzed. For gene expression analysis, we have used GEO dataset with accession id GSE3807 which included CD3+ T cells derived from bone marrow or peripheral blood of pool of six aplastic anaemia patients (Median age = 60.5 years, Range:19–70 years), five healthy controls (Median age = 37 years, Range:25–58 years) and two post therapy aplastic anaemia patients (Median age = 67 years, Range:64–70 years). Affymetrix HG_U133A GeneChips were used for gene expression profiling [24]. The characteristics of the samples used for the study was depicted in Table 2. The raw data files were downloaded from the database and analyzed. The raw data files were downloaded from the database and analyzed further by us to identify the differentially expressed genes and significantly altered pathways relevant for aplastic anaemia.

2.3. Microarray based miRNA and gene expression analysis

miRNA and gene expression profiles for all the categories of aplastic anaemia patients and healthy controls were generated using Affymetrix probes. 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. Downstream analysis was done to identify the differentially expressed genes, based on *t*-test among both the categories of aplastic anaemia patients compared to healthy controls and pre therapy aplastic anaemia patients compared to post therapy patients. The p-values were determined and multiple testing corrections [Benjamini Hochberg method] done to remove the false discovery rate. The differentially expressed genes were selected on the basis of p value < 0.05 and fold change of gene expression, compared to controls [for up-regulation, fold change ≥ 2 and for down-regulation, fold change ≤ -2].

2.4. Pathway analysis of significantly enriched genes

To get a global view of relevant biological pathways associated with disease progression, we used DAVID Bioinformatics Resources 6.8 of Laboratory of Human Retrovirology and Immunoinformatics [LHRI] and we considered KEGG based biological pathways for this study.

2.5. Target prediction of miRNAs

The target human genes corresponding to a set of significantly altered miRNA among aplastic anaemia patients compared to healthy

Table 2
Characteristics of the samples for host gene expression profiling.

Sample id	Categories of samples	Pool of samples	Gender	Age	Degree of illness
I	Aplastic anaemia (both pre-therapy and post-therapy)	Pool of four samples	Three female/one male	19/57/40/70 years	Severe aplastic anaemia
II	Aplastic anaemia (both pre-therapy and post-therapy)	Pool of two samples	One female/one male	70/64 years	Severe aplastic anaemia/in hematological remission
PB_III	Healthy controls	Pool of three samples	Two females, one male	25/25/37 years	Healthy volunteers
BM_III	Healthy controls	Pool of two samples	One female, one male	42/58 years	Healthy volunteers

controls in the study were predicted based on miRWalk 2.0 prediction software that included both predicted and validated miRNA binding sites.

2.6. Confirmation of miRNA target genes by microarray based gene expression profiling

Confirmation of significantly altered miRNA target genes was done, considering only those that appeared as targets of the significantly altered miRNA (Rap guanine nucleotide exchange factor 5 (RAPGEF5), α-endomannosidase (MANEA) and Lectin mannose binding 1L (MAN1L)) recorded among post-therapy patient compared to pre-therapy aplastic anaemia patients only.

3. Results

3.1. Differentially expressed genes in microarray based gene expression profiling

To identify the differentially expressed genes in aplastic anaemia patient we compared the gene expression profiling of pre-therapy aplastic anaemia patients compared to healthy controls. As we failed to identify any differentially expressed genes among pre-therapy aplastic anaemia patients compared to healthy controls based on adjusted p value < 0.05, we used unadjusted p value < 0.001 to identify the top most altered genes among aplastic anaemia patients. Microarray based global gene expression revealed that there were 34 differentially expressed genes among pre-therapy aplastic anaemia patients compared to healthy controls of which 20 genes were downregulated and 14 genes were upregulated (Table S1). When the analysis were done in post-therapy aplastic anaemia patients compared to healthy controls, it was found that only 12 genes were differentially expressed of which 5 genes were downregulated and 7 genes were upregulated (Table S2). The gene expression analysis also revealed that 38 genes were differentially expressed among post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients of which 16 genes were downregulated and 22 genes were upregulated (Table S3).

3.2. Pathway analysis of differentially expressed genes in microarray based gene expression profiling

DAVID Bioinformatics Resources 6.8 based pathway analysis revealed that Rap1 signaling pathway and Ras signaling pathways were the top most significantly altered pathways among pre-therapy aplastic anaemia patients compared to healthy controls [Table 3]. Pathway analysis revealed that not a single pathway was significantly different between post-therapy aplastic anaemia patients versus healthy controls which strengthen the fact that therapy can reverse the disease progression. Pathway analysis also revealed that vascular smooth muscle contraction was the significantly altered pathway in post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients. The genes involved in significantly altered pathways and their expression status were depicted in Table 3.

3.3. Significantly altered genes among post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients

It is really important to identify the significantly altered genes among post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients those expression were reversed after the therapy. These altered genes can be used as the target for disease therapy. To, identify those genes, we compared the expression status of the altered genes among post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients. We were able to identify three genes such as RAPGEF5, MANEA and MAN1L. These three genes were significantly downregulated among pre-therapy aplastic

Table 3
Significantly enriched biological pathways associated with aplastic anaemia.

Comparisons	KEGG Pathways	p value	Upregulated genes	Downregulated genes
Control vs. Pre-therapy aplastic anaemia	Rap1 signaling pathway	0.001814	NGF, HGF, PARD6B	RAPGEF5, FGF22
	Ras signaling pathway	0.019592	NGF, HGF	RAPGEF5, FGF22
Control vs. Post-therapy aplastic anaemia	No significantly enriched pathways			
Pre-therapy vs. Post-therapy aplastic anaemia	Vascular smooth muscle contraction	0.023379	ACTA2, CACNA1D	EDNRA

NGF = nerve growth factor, HGF = hepatocyte growth factor, PARD6B = par-6 family cell polarity regulator beta, RAPGEF5 = Rap guanine nucleotide exchange factor 5, FGF22 = fibroblast growth factor 22, ACTA2 = actin, alpha 2, smooth muscle, aorta, CACNA1D = calcium voltage-gated channel subunit alpha1 D, EDNRA = endothelin receptor type A; p value denote hypergeometric p value.

anaemia patients compared to healthy controls. After therapy, these three genes expression were upregulated among post-therapy aplastic anaemia patients compared to pre-therapy aplastic anaemia patients. The detailed expression status of these three genes was depicted in Table 4.

3.4. Differentially expressed miRNAs based on microarray based miRNA expression profiling

Based on microarray based miRNA expression profiling, only two miRNAs revealed significant altered expression among aplastic anaemia patients compared to healthy controls [*t*-test; adjusted p-value < 0.05] such as hsa-miR-1202 and hsa-miR-3162 as depicted in Table 5. Of these two miRNAs, only hsa-miR-1202 showed significantly altered log fold change expression > 2. As the fold change expression of hsa-miR-3162 is < 2, this miRNA is not selected for the study.

3.5. miR-1202 expression level modulate the target gene expression after therapy of aplastic anaemia patients

miRNAs are known to target multiple transcripts and considering this property we identified the expression profiles of the predicted targets from the microarray based gene expression analysis. We selected only those target genes those exression level were reversed after the therapy of aplastic anaemia patients. Most of such target genes of the corresponding miRNAs seemed to be predicted and experimentally non-validated, as per the databases considered for target identification as detailed under the "Materials and methods" section. Two target genes of miR-1202 were identified such as RAPGEF5 and MANEA those expressions were reversed after the treatment of aplastic anaemia patients. Of these two target genes, RAPGEF5 was significantly down-regulated among aplastic anaemia patients compared to healthy controls and this gene also involved in significantly enriched pathways such as Rap1 signaling pathway and Ras signaling pathway.

4. Discussion

A number of previous microarray based global gene expression profiling studies were employed to identify the significantly enriched pathways relevant for development of aplastic anaemia [14–17,25,26] but the studies are limited to aplastic anaemia patients compared to healthy controls. No such microarray based gene expression profiling studies were reported among the post-therapy aplastic anaemia patients compared to pre-therapy condition. In the current study we analyzed the gene expression profiling of pre-therapy compared to post-therapy disease condition and able to identify the target genes relevant for therapeutic use. Beside this we were also able to identify the pathways in CD3+ T cells derived from bone marrow relevant for aplastic anaemia disease compared to healthy controls such as Rap1 signaling pathway and Ras signaling pathway. Oxidative stress induces premature senescence of hematopoietic stem cells by alteration of several major signaling pathways were induced by oxidative stress [27]. In-vitro study reported that oncogeneic Ras was also reported to arrest the cell growth after oxidative injury by activating the Mitogen-activated protein kinase (MAPK) pathway [28] which could be relevant for development of aplastic anaemia. This is the first step in the approach we took in this study of the significantly altered genes in CD3+ T cells derived from bone marrow those expression statuses were reversed after the therapy among aplastic anaemia patients. We also able to identify the significantly enriched vascular smooth muscle contraction pathway in CD3+ T cells derived from bone marrow among post-therapy patients compared to pre-therapy patients. Vascular smooth muscle contraction pathway is important for blood pressure maintenance as observed in animal model [29,30]. Recent evidence suggests that the recruitment of abundant stem/progenitor cells present in the vessel wall are largely responsible for smooth muscle cells accumulation during vascular remodelling such as neointimal hyperplasia and arteriosclerosis [31]. Thus this pathway could be relevant for aplastic anaemia.

In this study we were also able to identify the significantly altered genes those expression status were reversed after application of therapies such as RAPGEF5, MANEA and LMAN1L. Rap guanine nucleotide exchange factor 5 [RAPGEF5] is involved in Rap1 and Ras signaling pathways and these pathways are involved in premature senescence of cells subject to oxidative stress [27]. The knowledge of α-endomannosidase [*MANEA] gene are still not well characterized*, but studies reported that they are involved in alpha and beta mannosidosis and rare diseases are characterized by varying degrees of developmental impairment [32,33]. These findings point towards the biological plausibility of MANEA gene in aplastic anaemia. The role of lectin mannose binding 1 [LMAN1] is not reported till now but it was postulated by previous studies that moderate mannose binding lectin serum concentration may be favorable to healthy aging [34] and therefore its aberrant expression is relevant for development of aplastic anaemia. Many studies already identified that aplastic anaemia patients undergo massive premature aging such as progressive telomere shortening and other epigenetic signatures in human genome [35]. Therefore alteration of expression level of the genes involved in healthy aging could be associated with aplastic anaemia.

We also focused on miRNA expression profiling among aplastic anaemia patients compared to healthy controls to identify the significant miRNA which can modulate gene expression. Some studies previously reported that some miRNAs were associated with aplastic anaemia in terms of modulation of gene expression [36,37] but those studies are limited to aplastic anaemia disorder and no reports are available regarding the therapy in terms of miRNA expression. In this communication we are able to identify the significant miRNA which could be relevant for aplastic anaemia. We also identified the relevant target genes of this miRNA which were involved in reversal of disease phenotype after application of therapy. In this study we have identified has-miR-1202 which was significantly upregulated among aplastic anaemia patients compared to healthy controls. miR-1202 were reported to characterize the Treg phenotype of autoimmune diseases [38] and it was also known that such kind of autoimmune diseases can be associated with aplastic anaemia. Thus upregulation of miR-1202 expression can be used as a biomarker for defining aplastic anaemia. It was also identified by in-silico analysis that miR-1202 can target RAPGEF5 and MANEA genes out of three genes those are significantly

Table 4
Expression status of differentially expressed genes associated with therapy among aplastic anaemia.

Genes	Log fold change expression among aplastic anaemia vs. healthy controls	p-Value	Log fold change expression among post-therapy aplastic anaemia vs. pre-therapy aplastic anaemia	p-Value	Gene description
RAPGEF5	-2.63	0.0005	2.99	0.00003	Rap guanine nucleotide exchange factor 5
MANEA	-3.64	0.0006	2.69	0.0003	Mannosidase endo-alpha
LMAN1L	-2.46	0.0007	1.76	0.0009	lectin, mannose binding 1 like

Table 5
Expression status of differentially expressed miRNAs associated with aplastic anaemia.

miRNAs	adj. p value	p. value	Log fold change expression compared to healthy controls
hsa-miR-1202	0.01831	2.66E−06	2.357164
hsa-miR-3162	0.03941	7.64E−06	1.570436

altered after therapy among aplastic anaemia. RAPGEF5 and MANEA genes were significantly downregulated among aplastic anaemia and after therapy they are again significantly upregulated compared to pre-therapy condition. These findings suggest that miR-1202 inhibitor can be used to repress those gene expressions as a therapeutic regimen. Numerous studies have reported that antisense oligonucleotides and antagomiRs are the synthetic miRNA inhibitors and these were extensively used in many hematological diseases [39].

Although the study point towards the promising and better targeted therapy for aplastic anaemia using antisense oligonucleotides of miR-1202, but the major limitation is first that the analyses were based on a very limited sample size and on Affimetryx chips only. No QPCR was performed to validate those findings in larger sample set. But still the findings on miR-1202 seems promising and targeting its expression could lead to a therapy and many studies and steps need to done in future before using this effective therapy.

In summary, microarray based miRNA expression analysis revealed that overexpression of miR-1202 were associated with development of aplastic anaemia and this can downregulate RAPGEF5 and MANEA genes in human. The expression of RAPGEF5 and MANEA genes were again upregulated subject to application of therapy among those aplastic anaemia patients as illustrated in Fig. 1. Thus synthetic inhibitors of miR-1202 can be used as a therapy for ameliorating aplastic anaemia phenotype.

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SA and PM: Conceived the objectives and performed the analysis, SA and PM: wrote the paper.

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Conflict of interest

Author SA declares that she has no conflict of interest. Author PM declares that she has no conflict of interest.

Ethical approval

This article does not contain any studies with human participants performed by any of the authors.

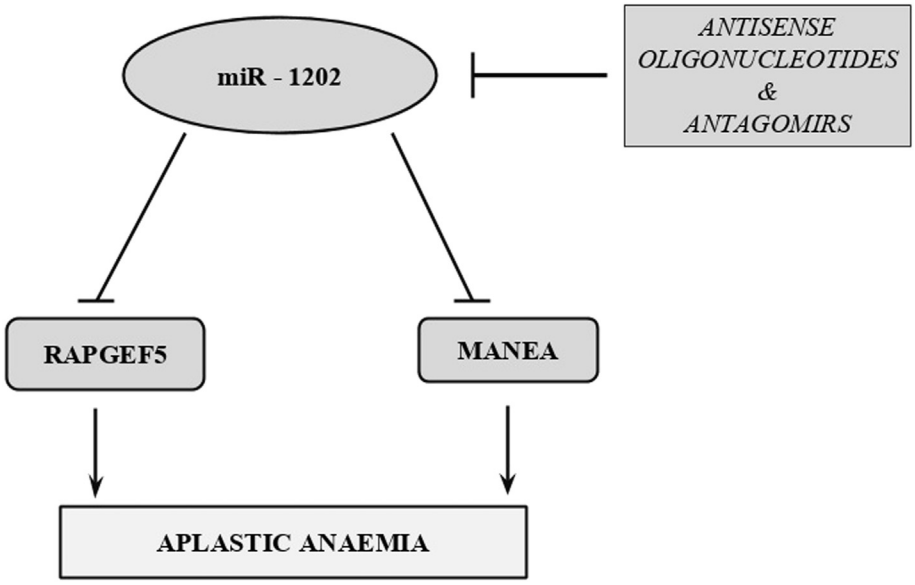

Fig. 1. Schematic overview of miR-1202 mediated gene regulation in aplastic anaemia.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2019.04.045.

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