RESEARCH ARTICLE

TO MONITOR TREATMENT OF A LARGE COHORT OF PATIENTS WITH β-TALASSAEMIA MAJOR, INTERMEDIA & SICKLE CELL ANAEMIA IN ORDER TO ESTABLISH THE RESPONSE TO TRANS-RESVERATROL AND THE ASSOCIATED ELEMENTS.

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Abstract

Though several genetic, non-genetic and pharmacological factors reported to influence the Trans-Resveratrol response in different early studies, the response to Trans-Resveratrol is significantly different among good, moderate and non-responders irrespective of the IVS I-5 (G→C), the common beta mutation here and even among other β0 or β+thalassaemia mutations. Here we study whether HbF level has any relation to beta variants responding to Trans-Resveratrol therapy, it has been shown that even among good responders in some cases (8.39 %) patients are not showing high HbF values ( < 20% HbF values are taken).

In our study (among 220 patients), Trans-Resveratrol therapy completely replace blood transfusion in as expected in this Eastern part of India, the predominant β-thalassemia defect is the IVS1-5(G→C), the most frequent β0-thal mutation in the area. This mutation is found in 87.66% of the CR patients and in 86.66% of the PR / NR patients. The next represented defects are the β+-thalassemia mutations like Cod 8/9, Fr. 41/42, Cod 15, Cod 30 etc. The common IVS1-5 (G→C) mutation in beta thalassemia patients (either in homozygous or heterozygous form) is present in 31.5% among the good responders (CR) while it is present in 66.66% among PR / NR (the moderate responders / non-responders) and in HbE-beta patients present in 73.28% among good responders (CR) while in 33.33% among moderate / non responders (PR / NR).

Introduction:

β-thalassemias (β-thal) are common inherited red cell disorders characterized by absent or reduced synthesis of β-globin chains. Despite extensive knowledge of the molecular defects causing β-thalassemia, less is known about the mechanisms responsible for the associated ineffective erythropoiesis and reduced red cell survival (1-7). Increased levels of reactive oxygen species (ROS) have been reported to contribute to the anemia of β-thalassemia, although the effects of ROS have not been fully defined (1, 2-8). Exogenous anti-oxidant molecules might represent complementary therapeutic strategies to counteract the toxic effects of ROS in β-thalassemia. However, few of them
have been shown to beneficially affect \textit{in vivo} \(\beta\)-thalassaemic red cell features and/or thalassaemic ineffective erythropoiesis \textit{in vivo}. (3-8)

Recent studies have shown that Resveratrol is a ribo-nucleotide reductase inhibitor, much more effective than hydroxyurea (HU), a molecule that has been used clinically as therapeutic agent for the treatment of Sickle cell anaemia, a haemoglobinopathy characterized by the polymerization of a mutated form of adult haemoglobin (HbS) leading to erythrocyte sickling which plays an important part in the disease morbidity. Hydroxyurea has proven its clinical benefit as it can increase fetal haemoglobin (HbF) levels in the red cells of sickle cell patients inhibiting HbS polymer formation.

Hence, we would like to study the nucleotide variations in the \(\beta\)-globin gene cluster and their association with the Trans-Resveratrol response so that we could explore the genetic basis of the clinical diversity of the Trans-Resveratrol therapy in beta-thalassaemic and sickle cell anaemia patients. Following regions have been suggested to examine the portions of the \(\beta\)-globin gene cluster, locus control region

- HS 1, 2, 3, and 4
  - \((\mathrm{CA})x(\mathrm{TA})y\) repeat motif,
  - \((\mathrm{AT})x\mathrm{Ny}(\mathrm{AT})z\) repeat motif,
  - inverted repeat sequence \(\text{TGGGGACCCCA}\)
- the promoter region of the \(A\gamma\) and \(G\gamma\)-globin gene
- \((\mathrm{AT})x(\mathrm{TA})y\) repeat in \(G\gamma\)-globin gene
- 5’ of the silencer region, the \(\beta\)-globin gene and its 3’ flanking region

Again, we may assume that a large proportion of the thalassaemia patients in Eastern India have a \textit{molecular background favorable to Trans-Resveratrol response}, because, during the one year of treatment (followed by Thalassaemia Foundation) we did not observe any significant problems regarding drug compliance and no myelogenic or clinically adverse events occurred. This in agreement with other long-term clinical trials which reported no significant increases in secondary malignancy following Trans-Resveratrol therapy. The good response to Trans-Resveratrol treatment that a significant number of Eastern Indian patients with \(\beta\)-thalassemia had seems to correlate not with the particular haplotypes, but with some other molecular factors, which we have to screen further.

Materials and Methods:-

Study groups:-

Patients with HPLC-screened documented Sickle cell anaemia, S-beta thalassemia, beta thalassaemia, HbE thalassaemia, HbE-beta thalassaemia, HPFH genotypes have been considered in this primary analysis.

Collection of Sample:-

Sample was collected from OPD of Thalassaemia Foundation, Kolkata. Total 220 patients were evaluated. Among which 140 patients with Hb-E-beta and 69 patients with Beta and HPFH and 11 patients with other hemoglobinopathies were observed.

Molecular Analysis:-

Samples for DNA isolation have been collected. DNA has been extracted and genotyping has been done by ARMS-PCR for the common beta mutations of this region, like IVSI-5(G\rightarrow C), Cod 8/9, Fr.41/42, Cod15, Cod26 and also for Cod6 (Sickle cell anaemia).

Fetal hemoglobin studies:-

Hb variants’ (HbA / HbA2 / HbF & others) levels was estimated by HPLC (High Performance Liquid Chromatography) (Bio-Rad, USA). Estimation of HbF was also done by using HPLC method.

Result:-

Clinical profile:-

We were able to classify \textit{three categories} of response: a \textit{Complete Response} (52.2%) in patients who can able to maintain at an average Hb level of 6-9 gm/dL without blood transfusion, in this group 12.3% patients are without any previous H/O blood transfusion, others shifted from monthly blood transfusion dependency to a stable transfusion-free condition; a \textit{Partial Response} (18.2%) in patients who remained transfusion dependent but at longer intervals (2-
3 months or more), and Non response (15.9%) in patients who, after more than one year of treatment, remained at the same level of transfusion dependency. [Table 1]

Table 1: Distribution of patients in different categories of response

| Groups of different categories | n (%) | HbE-beta (n=142) | Beta/HPFH (n=69) | Haemoglobinopathies (HbE, Sickle etc) (n=11) |
|-------------------------------|-------|------------------|------------------|--------------------------------------------|
| COMPLETE RESPONSE             |       |                  |                  |                                            |
| GROUP-I (withdrawal of BT)     | 88 (%)| Female=24 (%)    | Female = 5 (%)   | Female = 5 (%)                            |
|                               |       | Male = 46 (%)    | Male = 7 (%)     | Male = 1 (%)                              |
| GROUP-II (No H/O BT)          | 27 (%)| Female = 9 (%)   | Female = 2 (%)   | Female = 0 (%)                            |
|                               |       | Male = 12 (%)    | Male = 4 (%)     | Male = 0 (%)                              |
| NON RESPONSE                  | 35 (%)| Female = 2 (%)   | Female = 5 (%)   | Female = 0 (%)                            |
| GROUP-III                     |       | Male = 6 (%)     | Male = 22 (%)    | Male = 0 (%)                              |
| PARTIAL RESPONSE              | 40 (%)| Female = 9 (%)   | Female = 9 (%)   | Female = 0 (%)                            |
| GROUP-IV                      |       | Male = 11 (%)    | Male = 10 (%)    | Male = 1 (%)                              |
| CONTROL GROUP                 | 32 (%)| Female = 9 (%)   | Female = 2 (%)   | Female = 2 (%)                            |
| (without HU)                  |       | Male = 14 (%)    | Male = 3 (%)     | Male = 2 (%)                              |

Molecular Analysis:-
Samples for DNA isolation have been collected. DNA has been separated for mutation analysis by ARMS-PCR for common beta thal mutations of India. Patients were studied at the molecular level for their β-globin gene mutations.

As expected in this Eastern part of India, the predominant β-thalassemia defect is the IVSI-5(G→C), the most frequent β0-thal mutation in the area. This mutation is found in 87.66% of the CR patients and in 86.66% of the PR / NR patients. The next represented defects are the β0-thalassemia mutations like Cod 8/9, Fr. 41/42, Cod 15, Cod 30 etc. The distribution of the IVSI-5 (G→C) genotype among the CR, PR / NR categories is summarized in [Table 2].

Table 2: β-thalassaemia genotypes and Trans-Resveratrol response categories

| Genotype β/β | CR (%) | PR / NR (%) |
|--------------|--------|-------------|
| IVSI-5 (G→C) / IVSI-5 (G→C) | 14.38  | 53.33       |
| IVSI-5 (G→C) / cd 26 | 73.28  | 33.33       |
| IVSI-5 (G→C) / cd 6 | -----  | -----       |

The common IVSI-5 (G→C) mutation in beta thalassemia patients (either in homozygous or heterozygous form) is present in 31.5% among the good responders (CR) while it is present in 66.66% among PR / NR (the moderate responders / non-responders) and in HbE-beta patients present in 73.28% among good responders (CR) while in 33.33% among moderate / non responders (PR / NR). [Table 3].

Table 3: β-thalassaemia genotypes and Trans-Resveratrol response categories Genotype β/β

| Beta Mutations | Good Responder(%) | Non Responder(%) |
|---------------|------------------|-----------------|
| Beta Homozygous |                  |                 |
| IVSI-5 (G→C) / IVSI-5 (G→C) | 14.38  | 53.33       |
| Beta Heterozygous |                  |                 |
| IVSI(G→C) / cd8/9 | -----  | -----       |
| IVSI-5 (G→C) / Fr.41/42 | 17.12  | 13.33       |
| IVSI-5 (G→C) / cd15 | -----  | -----       |
| HbE-beta |                  |                 |
| IVSI-5 (G→C) / cd 26 | 73.28  | 33.33       |

The response to Trans-Resveratrol is equal in males and females and the age distribution is not significantly different in the three response categories. Similarly no significant difference in response was found between splenectomized and non-splenectomized patients.
Discussion:
It has been shown that low-dose resveratrol induces early maturation of normal erythroid precursors by activation of the FoxO3 transcriptional factor, inhibition of Akt and upregulation of antioxidant response genes such as catalase. The effects of resveratrol on cell maturation are highly dependent on resveratrol concentration and on cell types (1.5-8). The findings from previous studies are not directly applicable to the studies due to either the use of erythroid leukemia cells or study of primary erythroid cells without detailed characterization of their stage of cell differentiation (5-7). CFU-E cells are the most susceptible erythroid cell population to the effects of low dose resveratrol. Since resveratrol has no effect on the expression of erythropoietin receptors during erythroid differentiation (8), hence it can be proposed that resveratrol might hamper cell proliferation and may induce cell maturation as supported by the early expression of GPA and band 3 in resveratrol-treated cells and by similar observations in other cell models (7,17). We recently showed that resveratrol targets FoxO3 a key transcriptional factor in erythropoiesis involved in up-regulation of scavenging enzymes (4,12). We explored the possibility of resveratrol playing a pivotal role as an exogenous anti-oxidant agent and as a modulator of endogenous anti-oxidant systems.

In our present study we observed that whether HbF level has any relation to beta variants responding to Trans-Resveratrol therapy, it has been shown that even among good responders in some cases (8.39%) patients are not showing high HbF values (< 20% HbF values are taken).

In our study (among 220 patients), Trans-Resveratrol therapy completely replace blood transfusion in 88% cases (79.5% HbE-beta intermedia, 13.6% in beta thalassaemia major and 6.8% in other haemoglobinopathies like HbE disease & Sickle Cell anaemia).

According to Bianchi et al, when erythroid precursor cells from normal subjects were treated with increasing concentrations of resveratrol and analysis of accumulation of globin mRNA sequences was performed by quantitative RT-PCR, a clear increase in accumulation of γ-globin mRNA content was found. Increase in accumulation of α-globin and β-globin mRNA was much lower. Taken together these data strongly indicate resveratrol as a strong inducer of HbF and a selective stimulator of the expression in γ-globin genes.(17)

Conclusion:
1. Though several genetic, non-genetic and pharmacological factors reported to influence the Trans-Resveratrol response in different early studies, the response to Trans-Resveratrol is significantly different among good, moderate and non-responders irrespective of the IVS I-5 (G→C), the common beta mutation here and even among other β⁰ or β⁺ thalassaemia mutations.
2. To study whether HbF level has any relation to beta variants responding to Trans-Resveratrol therapy, it has been shown that even among good responders in some cases (8.39%) patients are not showing high HbF values (< 20% HbF values are taken).
3. In our study (among 220 patients), Trans-Resveratrol therapy completely replace blood transfusion in 88% cases (79.5% HbE-beta intermedia, 13.6% in beta thalassaemia major and 6.8% in other haemoglobinopathies like HbE disease & Sickle Cell anaemia).

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The authors declare that they have no competing interests.

Data Sharing Statement:—
We cannot share any unpublished data with other laboratory or person.

Patients Consent Statement:—
The signed consent from all the patients were taken before test was performed and kept them as official documents. In case of any unusual condition it will be presented in front of the concerned person.
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