

## SISTER CHROMATID EXCHANGES IN HETEROZYGOTE $\beta$ THALASSAEMIC PATIENTS WITH NORMAL FOLATE LEVELS

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### ABSTRACT

Lymphocytes from  $\beta$  thalassaemia heterozygote patients, with normal levels of plasma folic acid cultured for 72 h in a folate rich medium, were not found to contain increased rates of sister chromatid exchanges (SCE). A wide intra and interindividual variability was found in both thalassaemic and control groups and methodological and biological factors, such as types of peripheral lymphocytes, sex, age, and smoking, alcohol and coffee drinking, as well as dietary habits, are possibly responsible for these variations.

### INTRODUCTION

The  $\beta$  thalassaemias comprise an heterogeneous group of inherited haematologic disorders characterized by reduced or absent synthesis of the  $\beta$  globin polypeptide chain of normal adult haemoglobin (Hb A). Since there is no change in the synthesis of  $\alpha$  globins, the patients exhibit an accumulation of unstable aggregates of unpaired  $\alpha$  chains which is responsible for the anemia (Weatherall and Clegg, 1981). Patients with heterozygous  $\beta$  thalassaemia usually present no clinical symptoms and show a mild hypochromic microcytic anemia with variations in red cell size and shape. The variable clinical features of heterozygous  $\beta$  thalassaemia have been assembled under the designation of  $\beta$  thalassaemia *minor*. The haemoglobin pattern in  $\beta$  thalassaemia *minor* is characterized by an increase of Hb A<sub>2</sub> into the 3.5% to 7% range and in about

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half of the cases a mild elevation of Hb F (2% to 5%) (Ohene-Frempong and Schwartz, 1980).

Increased erythropoiesis rates leading to increased requirements of folic acid, an essential cofactor in the incorporation of nucleotides into DNA, have been found in heterozygous  $\beta$  thalassaemic patients who consequently present some degree of folate deficiency (Castaldi *et al.*, 1983; Castagna *et al.*, 1984; Saraya *et al.*, 1984).

A high frequency of chromosomal anomalies in homozygous  $\beta$  thalassaemic patients was reported by Côté and Papadakou-Lagoyanni (1979). Since a folic acid deficiency is usually found in these patients, the authors hypothesized that the findings were due to a failure in the DNA repair mechanism related with such deficiency. A higher frequency of sister chromatid exchanges (SCE) in cultured lymphocytes was displayed by thalassaemic patients than by controls (Côté *et al.*, 1982).

The incidence of haemoglobinopathies in the region of São José do Rio Preto (State of São Paulo) is around 3.5%, 17% of these consisting of heterozygous  $\beta$  thalassaemia (Naoum *et al.*, 1985). This prompted us to investigate the rates of sister chromatid exchanges in a sample of heterozygous  $\beta$  thalassaemic patients and to relate these rates with plasma folate levels.

## MATERIAL AND METHODS

A sample of 21 unrelated heterozygous  $\beta$  thalassaemic patients (8 males and 13 females) from São José do Rio Preto was analyzed. The control subjects (11 males and 10 females) were either siblings of patients (10 cases), or matched by sex, age and socioeconomic status to one of the patients (11 cases); all were clinically healthy and none carried a thalassaemia gene. Subjects from both samples ranged in age from 13 to 49 years, mean age ( $\pm$  SE) was  $28.8 \pm 10.3$  years for the thalassaemic group and  $27.9 \pm 10.3$  years for the normal group.

None of the subjects had taken any folic acid supplement or medication or had a viral infection or X-ray exposure for the previous two months. However, three patients (subjects 5, 11 and 16) and three controls (subjects 6, 9 and 15) smoked less than twenty cigarettes per day. Thalassaemic and normal subjects had similar dietary habits, and all had normal chromosome complements.

Diagnosis of heterozygous  $\beta$  thalassaemia was established on the basis of decreased osmotic red cell fragility (in 0.36% NaCl solution), abnormal red cell morphology and haematimetric values (mean cell haemoglobin, mean cell volume and Hb A<sub>2</sub> level) (Naoum, 1987). Plasma folate levels were determined on coded samples with a commercial radioassay kit (No Boil Dualcount-Solid Phase, Diagnostic Products Corporation, Los Angeles, CA), as previously described (Silva and Varella-Garcia, 1989).

Cytogenetic analysis was carried out on cultures of peripheral blood lymphocytes. Phytohemagglutinin stimulated cultures were set up by adding 0.8 ml of plasma from each subject to 9 ml of DIFCO RPMI 1640 medium supplemented with 20% of human AB serum, antibiotics and 5-bromodeoxyuridine in a final concentration of 10  $\mu\text{g/ml}$ , followed by dividing the volume into two equal portions. The cultures were incubated in the dark at 37°C for 72 h. Colchicine was added one hour before harvesting in a final concentration of  $4 \times 10^{-5}$  M. Hypotonic shock was performed with 0.075 M-KCl and chromosome fixation with 3:1 methanol-acetic acid. Differential staining of sister chromatids was obtained according to the technique of Korenberg and Freedlender (1974) with slight modifications. The slides were covered with  $10^{-4}$  M - Hoechst 33258 in Hanks solution and irradiated for 12 minutes with UV light (Philips UV, 15 W) before heating to 87°-89°C in 1 M- $\text{NaH}_2\text{PO}_4$  buffer. The slides were next washed and stained with Giemsa solution (2% in Sorensen buffer).

SCE were blind scored on coded slides by a single person in 50 second replicative cycle metaphases with 46 chromosomes per individual. To minimize laboratory variability the specimens were cultured and processed in groups with similar numbers of patients and controls.

Statistical analysis was performed on data with square root transformation by using General Linear Models (Statistical Analysis System - SAS Institute, Inc., Cary, NC). Association between SCE and plasma folate values was investigated by linear correlation analysis.

## RESULTS

Individual and pooled data of SCE per cell and folate level are presented in Table I for  $\beta$  thalassaemic patients and in Table II for controls. In the  $\beta$  thalassaemic group the mean  $\pm$  SE individual SCE/cell ranged from  $10.22 \pm 0.56$  to  $24.80 \pm 0.81$  with an average of  $16.90 \pm 0.78$ . The incidence of SCE varied from 4 to 39 per cell. The mean  $\pm$  SE SCE/cell for the control group was  $17.82 \pm 1.08$ , the individual mean  $\pm$  SE values ranging from  $7.82 \pm 0.50$  to  $29.38 \pm 0.72$ . The lowest value of SCE per cell was 1 and the highest was 40. No significant differences were found between samples (patients x controls:  $F(1 \text{ df})=1.38$ ;  $P > 0.05$ ), sex (males x females:  $F(1 \text{ df})=0.35$ ;  $P > 0.05$ ) or both variables considered together (sample x sex interaction:  $F(1 \text{ df})=2.79$ ;  $P > 0.05$ ).

Plasma folate levels in both samples were within the normal range (3-17 ng/ml). The mean  $\pm$  SE folic acid level for the  $\beta$  thalassaemic patients was  $10.5 \pm 1.2$  ng/ml and for the controls  $10.6 \pm 0.9$  ng/ml. No significant difference in mean folic acid levels was found between patient and control samples ( $t(20 \text{ df})=0.55$ ;  $P > 0.05$ ) or even between subsamples grouped according to sex (male patients x male controls:  $t(17 \text{ df})=0.30$ ;  $P > 0.05$ ; female patients x female controls:  $t(21 \text{ df})=0.79$ ;  $P > 0.05$ ) or consanguinity among

Table I - SCE frequencies in 50 cells and plasma folate levels in individual and pooled data of the  $\beta$  thalassaemic group.

Subject	Sex	SCE/Cell		Total folate (ng/ml)*
		Mean $\pm$ SE	Range	
1	M	13.24 $\pm$ 0.70	5-24	12.5
2	M	12.10 $\pm$ 0.55	5-24	5.8
3	M	13.74 $\pm$ 0.51	5-21	11.5
4	M	16.60 $\pm$ 0.52	9-25	14.0
5	M	19.32 $\pm$ 0.68	7-32	9.5
6	M	10.22 $\pm$ 0.56	4-20	5.2
7	M	16.94 $\pm$ 0.68	7-30	12.0
8	M	14.66 $\pm$ 0.54	5-22	20.0
9	F	17.06 $\pm$ 0.63	9-31	4.0
10	F	14.82 $\pm$ 0.65	4-24	8.5
11	F	15.34 $\pm$ 0.53	8-24	3.4
12	F	15.90 $\pm$ 0.52	10-27	14.5
13	F	21.08 $\pm$ 0.59	11-30	6.0
14	F	16.22 $\pm$ 0.71	8-37	12.5
15	F	18.82 $\pm$ 0.59	11-27	12.0
16	F	16.90 $\pm$ 0.69	8-29	4.5
17	F	15.24 $\pm$ 0.62	8-27	24.0
18	F	17.48 $\pm$ 0.66	6-32	16.0
19	F	24.80 $\pm$ 0.81	14-39	10.0
20	F	21.00 $\pm$ 0.66	12-36	8.5
21	F	23.34 $\pm$ 0.67	13-33	6.8
Pooled data:				
Male		14.60 $\pm$ 1.03	4-32	11.3 $\pm$ 1.7
Female		18.31 $\pm$ 0.91	4-39	10.0 $\pm$ 1.6
All patients		16.90 $\pm$ 0.78	4-39	10.5 $\pm$ 1.2

\*Normal values: 3-17 ng/ml.

Table II - SCE frequencies in 50 cells and plasma folate levels in individual and pooled data of the control group.

Subject	Sex	SCE/Cell		Total folate (ng/ml)*
		Mean $\pm$ SE	Range	
1	M	26.04 $\pm$ 0.74	15-38	9.0
2	M	20.96 $\pm$ 0.74	9-32	9.5
3	M	17.50 $\pm$ 0.76	5-30	16.0
4	M	17.68 $\pm$ 0.71	7-35	7.5
5	M	14.82 $\pm$ 0.55	7-23	6.5
6	M	29.38 $\pm$ 0.72	19-40	9.0
7	M	16.26 $\pm$ 0.51	10-24	9.5
8	M	20.76 $\pm$ 0.75	9-32	12.5
9	M	14.28 $\pm$ 0.57	6-23	7.5
10	M	14.92 $\pm$ 0.64	5-26	14.5
11	M	7.82 $\pm$ 0.50	1-20	11.5
12	F	17.22 $\pm$ 0.69	7-32	14.0
13	F	23.62 $\pm$ 0.78	14-35	6.4
14	F	16.52 $\pm$ 0.68	8-28	6.0
15	F	18.38 $\pm$ 0.61	10-29	9.5
16	F	21.60 $\pm$ 0.71	3-34	24.0
17	F	15.54 $\pm$ 0.59	6-27	14.0
18	F	12.66 $\pm$ 0.56	6-21	8.0
19	F	18.33 $\pm$ 0.80	9-35	9.0
20	F	10.48 $\pm$ 0.47	4-19	10.0
21	F	19.46 $\pm$ 0.64	11-31	9.5
Pooled data:				
Male		18.22 $\pm$ 1.78	1-40	10.3 $\pm$ 0.9
Female		17.38 $\pm$ 1.23	3-35	11.0 $\pm$ 1.7
All patients		17.82 $\pm$ 1.08	1-40	10.6 $\pm$ 0.9

\*Normal values: 3-17 ng/ml

individuals (sibling patients x controls:  $t(18 \text{ df})=0.05$ ;  $P > 0.05$ ; no sibling patients x controls:  $t(20 \text{ df})=0.75$ ;  $P > 0.05$ ).

No correlation was found between SCE values and folic acid levels, either in  $\beta$  thalassaemic patients ( $r = -0.10$ ;  $t = 1.97$ ;  $P > 0.05$ ) or in controls ( $r = -0.02$ ;  $t = 0.11$ ;  $P > 0.05$ ).

Wide interindividual variability in SCE/cell values was detected by Tukey's test. In male patients, 17 of 28 (61%) possible pairwise contrast comparisons showed significant differences in SCE/cell rates; in female patients 39 of 78 (50%) comparisons were significantly different. In male and female controls, respectively, 39 of 55 (71%), and 27 of 45 (60%) possible comparisons between individuals showed a significant difference.

## DISCUSSION

The heterozygous  $\beta$  thalassaemic patients analysed did not show an increase in the average number of SCEs. In contrast, Côté *et al.* (1982) found a higher rate of SCE in an heterogeneous assemblage of  $\beta$  thalassaemic patients than in controls. SCE rates found by these authors were slightly increased in the absence of blood transfusion but essentially independent of serum folate concentration, splenectomy, age, sex, thalassaemia genotype, haemoglobin level, lymphocyte count, and clinical status.

The discordant findings might be related to clinical and methodological conditions. Our study group was comprised of homogeneously asymptomatic heterozygous  $\beta$  thalassaemic patients, while Côté *et al.* (1982) analyzed a clinically diversified group of  $\beta$  thalassaemic patients. Furthermore, culture time also seems to play an important role in these results. A decreasing availability of nutrients and an increasing quantity of toxic metabolites have been found in long-term cultures, as in the 94 h cultures analyzed by Côté *et al.* (1982). Thus the increased rate of SCE observed by these authors could be due to such factors.

The lack of increase in SCE frequencies of the patients could be associated with their normal plasmatic folate levels. Moreover, no significant correlation was detected between folic acid levels and SCE rates in either  $\beta$  thalassaemia heterozygous and normal samples. Thus, our results do not show increased rates of SCE in heterozygous  $\beta$  thalassaemic patients in normal conditions of folic acid, either *in vivo*, since the patients had normal plasma level, or *in vitro*, since a folate rich culture medium was utilized and short term cultures (72 h) were carried out.

A large intra and interindividual variability was found in the frequency of SCE/cell within  $\beta$  thalassaemic and control groups, and several methodological and biological factors may account for this variability. Since T lymphocytes show higher SCE frequencies and slower proliferation rates than B lymphocytes (Lindblad and Lambert,

1981; Miller, 1988), variations in the frequencies of these cells could be one of the factors responsible for the intraindividual diversity. The interindividual variability found in this study is corroborated by the literature findings: a variation in the baseline SCE frequency in standardized lymphocyte cultures from different donors is usually mentioned (Carrano *et al.*, 1986; Wulf *et al.*, 1987; Bender *et al.*, 1988), probably due to the influence of factors like sex, age, smoking, alcohol and coffee consumption, as well as with some dietary habits, as discussed by Das (1988). These factors were taken into account in the matching of patients with controls, but nevertheless may have contributed to the large individual variability observed.

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### RESUMO

Linfócitos de pacientes  $\beta$  talassêmicos heterozigotos com níveis normais de ácido fólico plasmático, cultivados por 72 horas em meio rico em folato, não apresentaram frequências aumentadas de trocas entre cromátides-irmãs (TCI). Uma ampla variabilidade intra e interindividual foi encontrada nos grupos de talassêmicos e controles e fatores metodológicos e biológicos, tais como tipos de linfócitos periféricos, sexo, idade, cigarro, café e bebida alcoólica, como também hábito alimentar, são possivelmente responsáveis por essas variações.

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