RESULTS

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The present study was carried out in an attempt to determine the most common β-globin gene mutations of β-thalassemic children in Sharkia province. This study included 50 patients in addition to 25 healthy children as a control group. Both groups were matched as regards age, sex and residence. Most of patients were blood transfusion dependant. The patients were previously diagnosed clinically as β-thalassemia in Hematology Unit, Pediatric Department, Zagazig University and Biological Application Department of Nuclear Research Center, Atomic Energy Authority.

The age of both groups ranged from 3–15 years with a mean value 8.99±0.45 for patient and a mean value 9.96±0.61 for control groups. The patients included 33 males (66%) and 17 females (34%), whereas the control group formed of 17 males (68%) and 8 females (32%).Twenty seven patients (54%) gave positive consanguinity, while a positive consanguinity was found in 7 of the control group (28%). A positive family history of thalassemia was found in 17 patients (34%), whereas no one was found in control. All the demography of both groups was represented in table (1).

(1) Biochemical analysis:

(a) Hb concentration:

The patients Hb concentration ranged from 5.47 to 10.30 g/dl with a mean value of 7.45 g/dl ± 0.16, while its level in the control group ranged

from 11 to 13 g/dl with a mean value of 12.06 g/dl ± 0.13. The difference between both groups was statistically analyzied and represented in table (2) and illustrate in histogram No. (1). This difference showed that the patient Hb concentration was significantly lower than the control group ($p<0.001$).

(b) Serum iron:

The patients serum iron ranged from 205.22 to 279.85 µg/dl with a mean value of 244.02 µg/dl ± 2.20, whereas its level in the control ranged from 75 to 112 µg/dl with a mean value of 92.52 µg/dl ± 2.18. as shown in table (2) and the histogram (2) illustrates that the thalassemic patients showed significantly higher serum iron than the control group ($p< 0.001$).

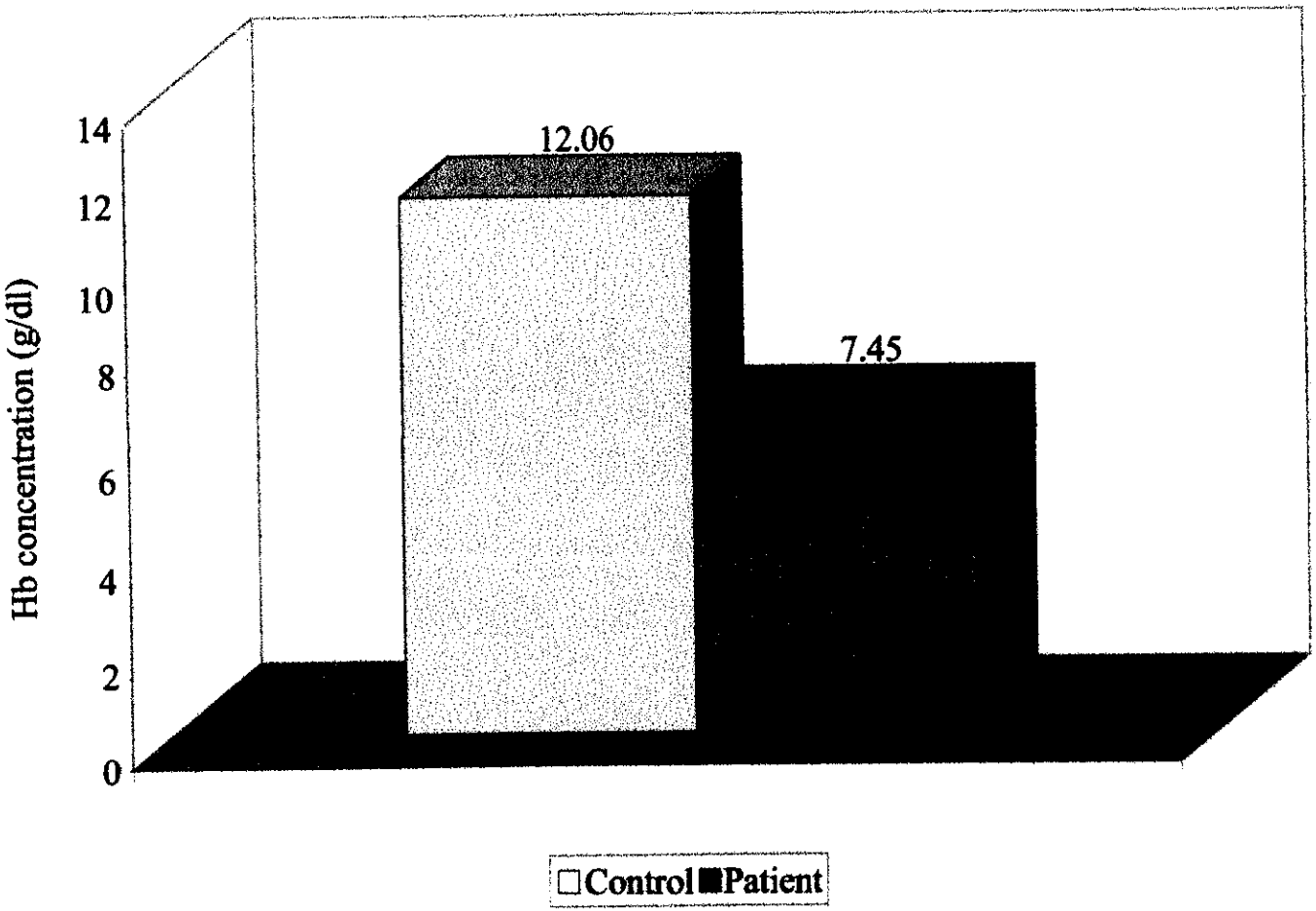
(c) Total iron binding capacity:

On the other hand, the TIBC level in patients ranged from 210.22 to 280.85 µg/dl with a mean value of 246.81 ± 2.14 µg/dl, whereas its level in the control group ranged from 264 to 385 µg/dl with a mean value of 320.40 µg/dl ± 8.36 as represented in table (2). The histogram (3) shows that the patients had significantly decreased level of TIBC than the control group ($p< 0.001$).

(d) Ferritin:

As regards patients serum ferritin, its ranged from 1980 to 2094 ng/dl with a mean of 2030.04 ± 3.95 ng/dl, while its level in the control group ranged from 104 to143 ng/dl with a mean value of 130.68 ± 1.89. as shown

Fig. (1) The level of Hb concentration in control and patient groups.



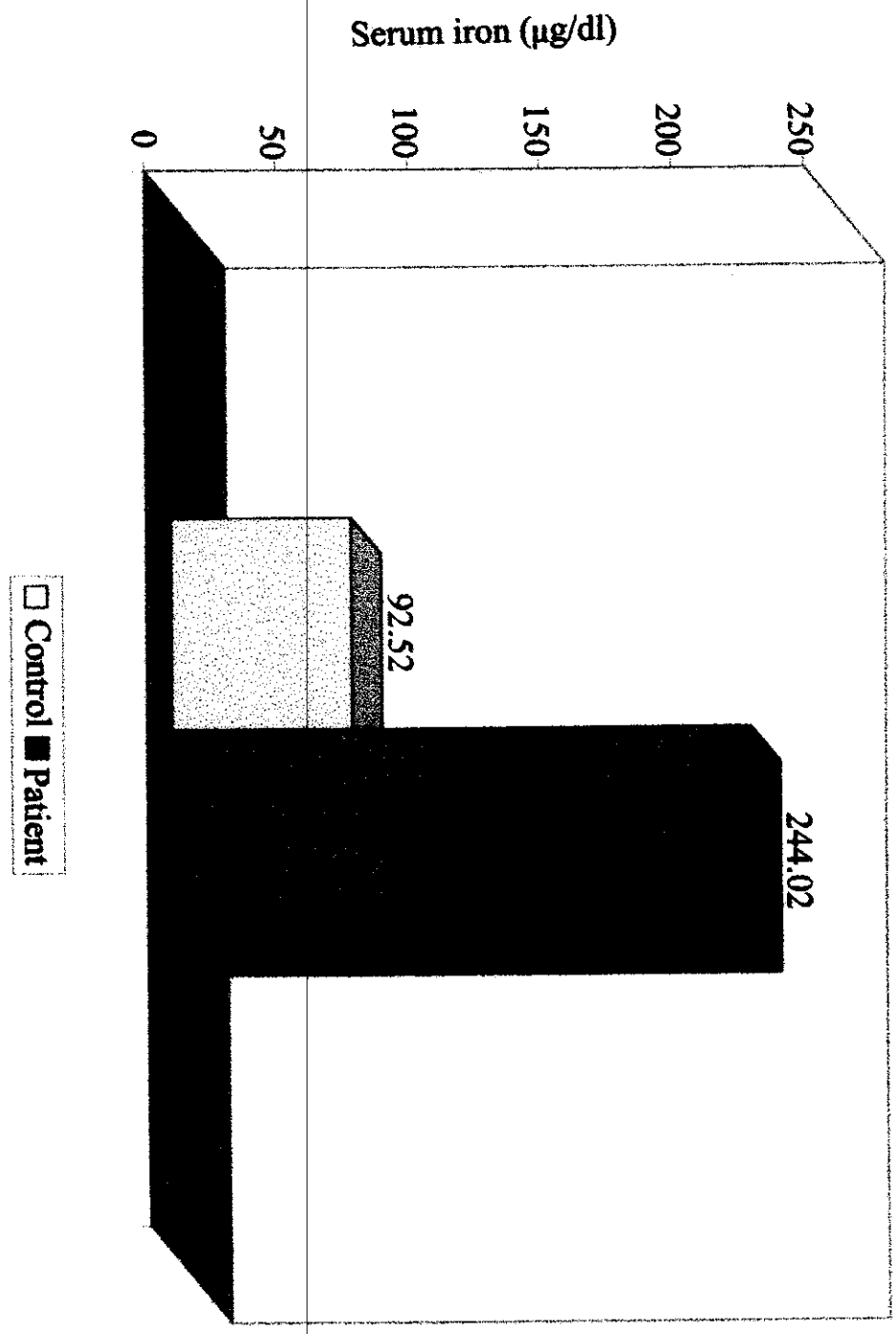


Fig. (2) The values of serum iron in control and patient groups.

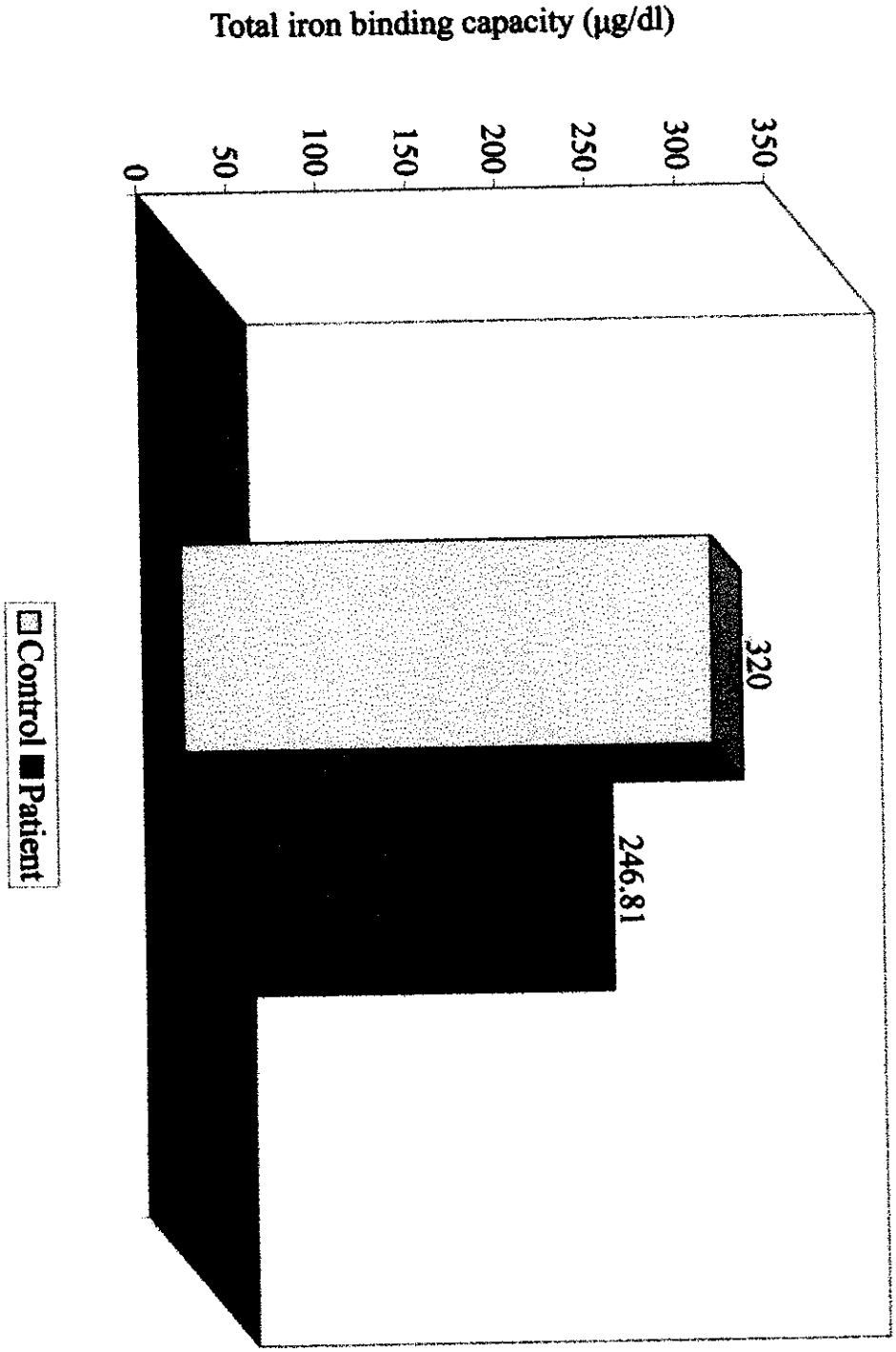


Fig. (3) The total iron binding capacity level in control and patient groups.

in table (2) and the histogram (4) shows that the patients had highly significant increased level of ferritin than the control group ($p< 0.001$).

(e) Hb electrophoresis:

The hemoglobin electrophoresis was carried out for the studied cases to confirm that these patients had β-thalassemia. The hemoglobin electrophoresis was only done for cases who had stopped blood transfusion for 2 months (41cases) or had no transfusion yet (early diagnosed cases). Nine patients were not included because they could not stop without transfusion for 2 months, and thus they were not included, and the results of Hb electrophoresis were represented in table (3). For example the histogram of the hemoglobin electrophoresis of one patient versus one control is represent in Fig. (5) and (6).

In this study, the mean level of HbA in thalassemic patients was 48.88 % ± 3.13 and ranged from 15 % to 82 %. This value was significantly lower ($p<0.001$) than that of control group 97.45 % ± 0.06 and its range from 96.5 % to 98 % as shown in fig.(7).

On the other hand, there was a pronounced increase in the value of HbF recording 47.74 % ± 3.10 and ranged from 16 % to 83 % in the patients group, this increase being more highly significantly ($p<0.001$) than the corresponding control group recording 0.79 % ± 0.05 and its ranged from 0.5 % to 1.5 % as represented in fig.(7).

Table (3) The hemoglobin electrophoresis patterns for control and patient groups.

Hb elecrophoresis	Control N =25	Patient N = 41	Test of significant	P - value
HbA%				
Mean ± SE	97.45%±0.06	48.88±3.13	6.78	<0.001
Median	97.5	55		
Range	96.5% - 98%	15% – 82%		
HbF%				
Mean ± SE	0.79%±0.05	47.74%±3.1	6.78	<0.001
Median	0.8%	41		
Range	0.5% - 1.5%	16% – 83%		
HbA2%				
Mean ± SE	1.74%±0.04	3.36%±0.24	6.46	<0.001
Median	1.8	3		
Range	1.4% – 2%	2% - 7.7%		

P <0.001 highly significant.

Note: This table represent 41 cases who had blood transfusion before 2 months.

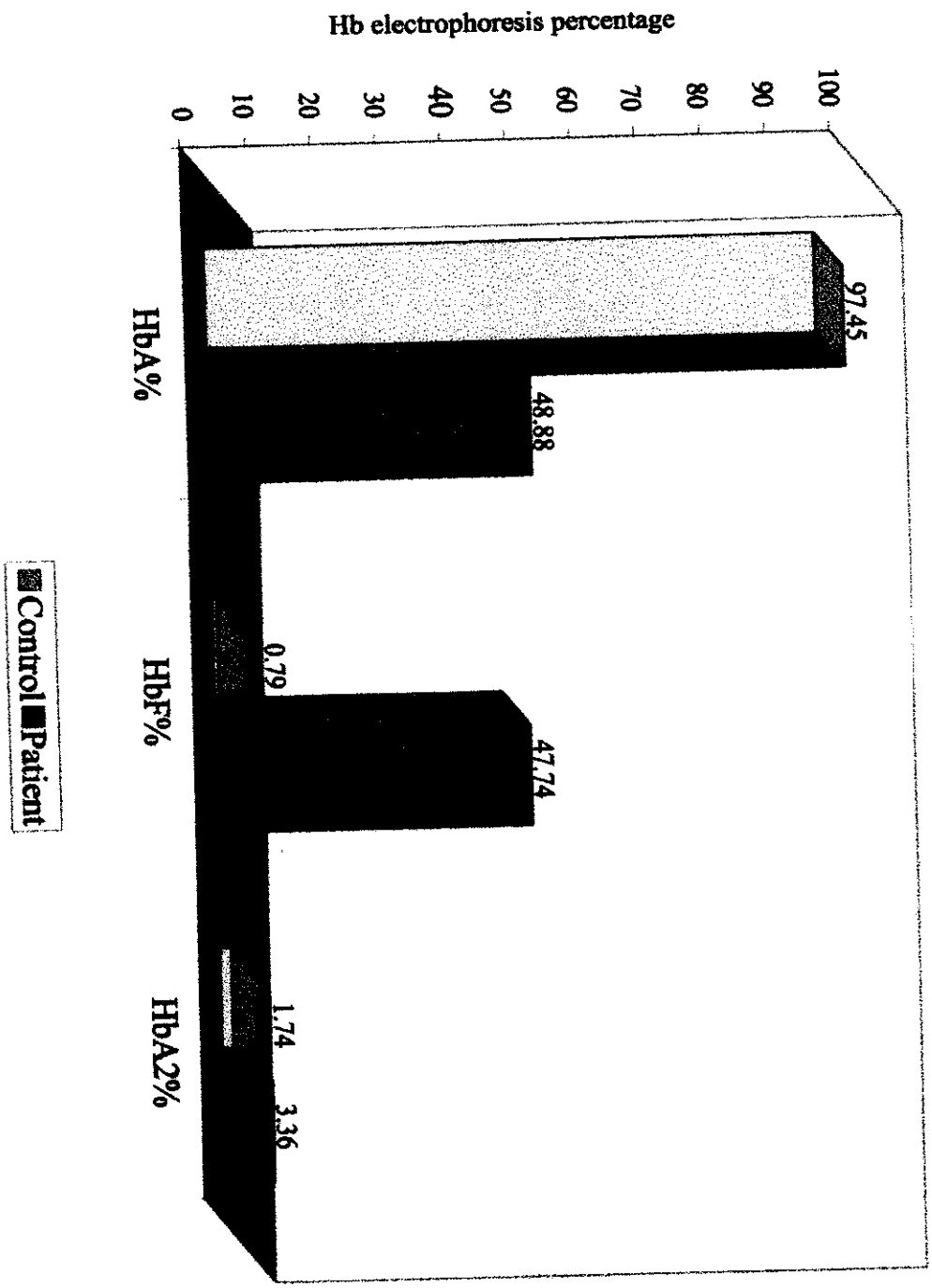


Fig. (7) The comparasion between Hb electrophoresis values in control and patient groups.

The patients level of HbA2 showed more highly significantly ($p<0.001$) increase recording 3.36 % ± 0.24 and its ranged from 2 % to 7.7 %, while in the control group this level ranged from 1.4 % to 2 % with a mean value of 1.74 % ± 0.04. The mean value of this fractions in both of controls and patients were illustrated in fig (7).

From the data of present study showed a negative correlation between age of the patients and its level of Hb conc. which was more highly significant ($P<0.001$) as shown in table (4) and illustrated in fig. (8).

There was a positive correlation between age and iron status (serum iron, TIBC and ferritin), which was more highly significant ($P<0.001$) as shown in table (4) and illustrated in figures (9,10 and 11) respectively.

On the other hand, the correlation between age of patients and HbA and HbF was non significant while it was significant between age and HbA2 were showed in Table (4) and illustrated in figures(12, 13 and 14)

The relation between sex in thalassemic patients and Hb conc. revealed that female had mean value 7.45±0.28 while, male had mean value 7.40±0.20, which was non significant as shown in Table (5).

The relation between sex in thalassemic patients and serum iron revealed that female had mean value 243.11±3.51 while male had mean value 244.48±2.84, which was non significant as shown in Table (5).

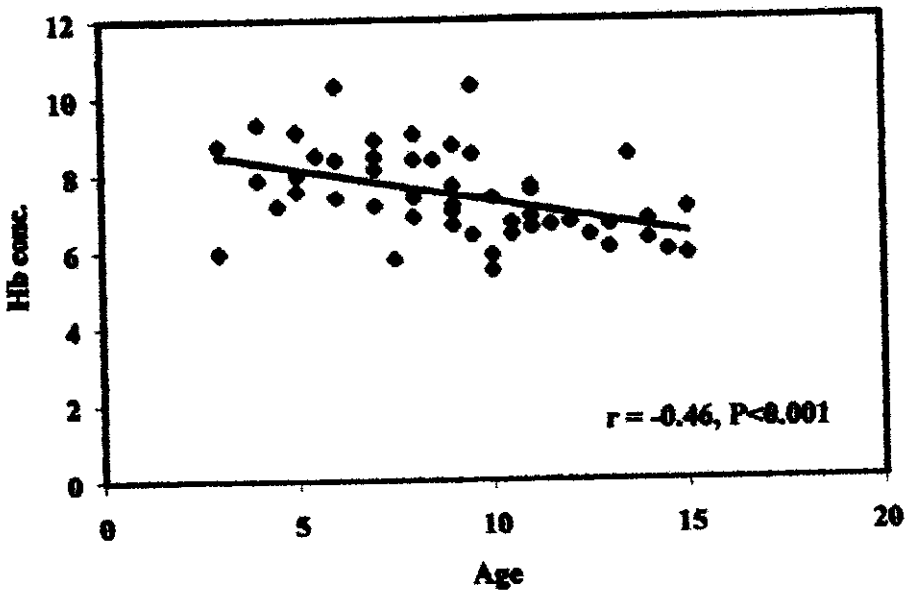


Fig. (8) Correlation between age and Hb concentration.

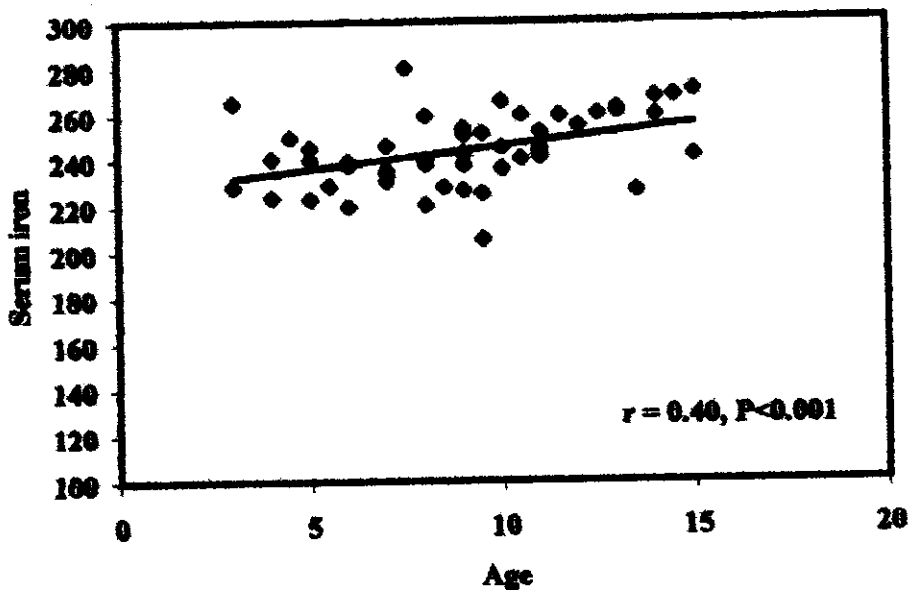


Fig. (9) Correlation between age and serum iron.

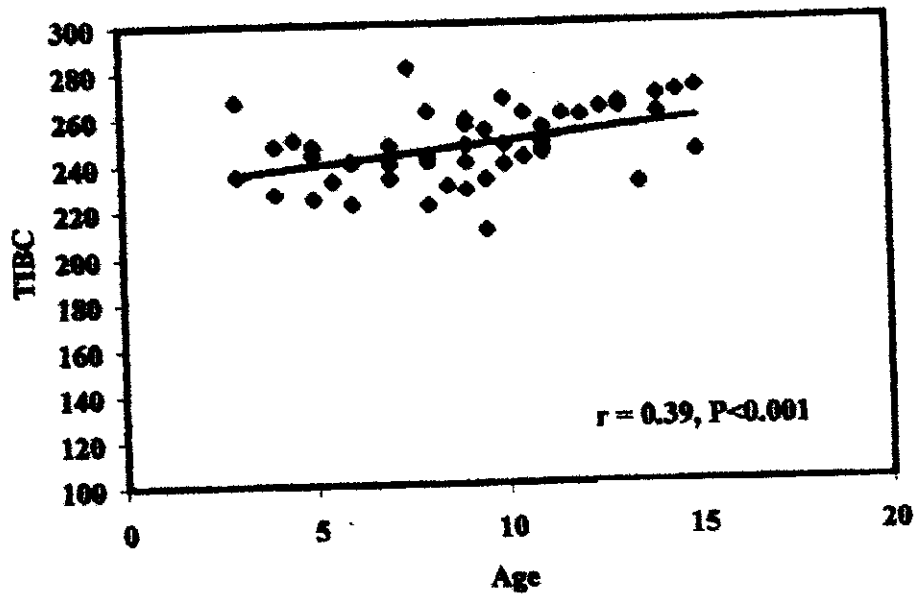


Fig. (10) Correlation between age and total iron binding capacity

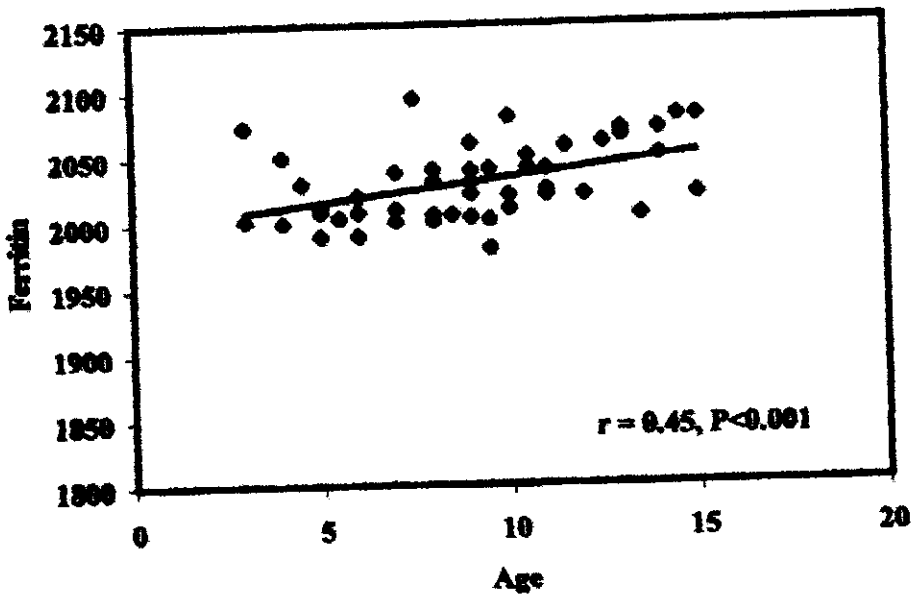


Fig. (11) Correlation between age and ferritin

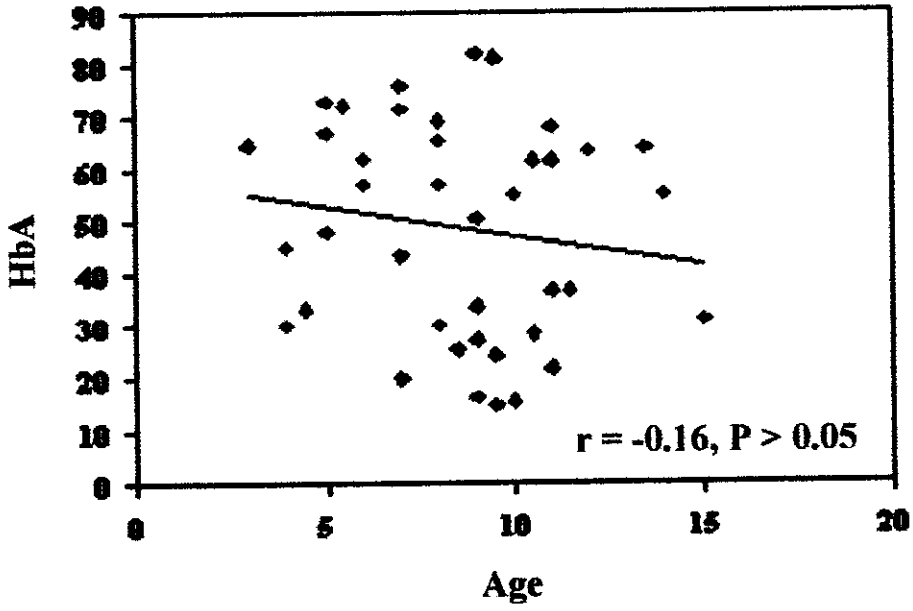


Fig. (12) Correlation between age and HbA.

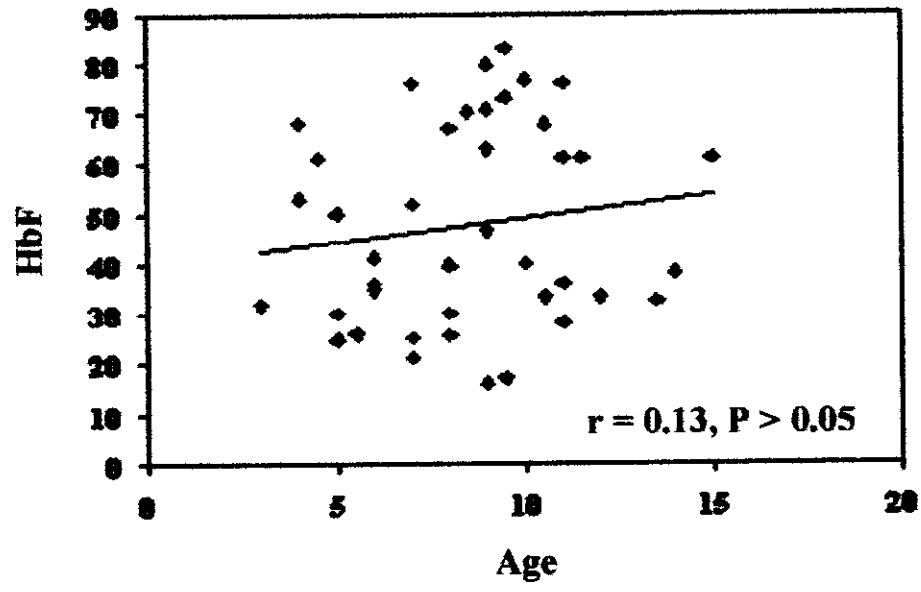


Fig. (13) Correlation between age and HbF.

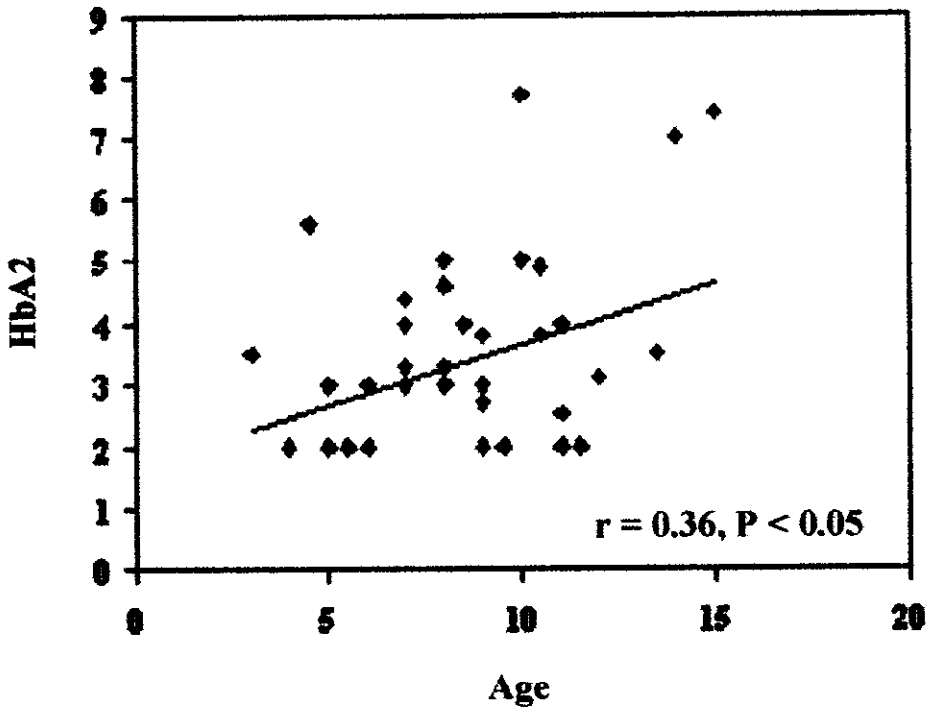


Fig. (14) Correlation between age and HbA2.

Table (5) Relation between sex and Hb conc. serum iron,TIBC and ferritin in patients

Parameters	Females N=17	Males N=33	T-Test	P-value
Hb conc g/dl Mean ± SE	7.54±0.28	7.40±0.20	0.40	>0.05
Serum iron µg/dl Mean ± SE	243.11±3.51	244.48±2.84	0.29	>0.05
TIBC µg/dl Mean ± SE	245.92±3.56	247.27±2.70	0.29	>0.05
Ferritin ng/dl Mean ± SE	2028.11±6.48	2031.03±5.04	0.34	>0.05

P >0.05 non significant.

The relation between sex in thalassemic patients and TIBC revealed that female had mean value 245.92±3.56 while male had mean value 247.27±2.70, which was non significant as shown in Table (5).

The relation between sex in thalassemic patients and ferritin revealed that female had mean value 2028.11±6.48 while male had mean value 2031.03±5.04, which was non significant as shown in Table (5).

The relation between sex of patients and Hb fractions showed that HbA in female recording 50.16±5.27 while in male recording 48.29±3.96 which was non significant as shown in Table (6).

On the other hand, The HbF in female recording 46.68±5.28 while in male recording 48.29±3.91. While, the HbA2 in female recording 3.15±0.21 while in male recording 3.48±0.34 which was non significant as shown in Table (6).

There were 17 patients had positive family history in which the mean of Hb conc. recording 7.18±0.26 while, the mean of Hb conc. recording 7.59±0.21 in 33 patients had negative family history as shown in table (7).

There were 17 patients had positive family history in which the mean of serum iron recording 247.8±4.3 while, the mean of serum iron recording 242±2.46 in 33 patients had negative family history as shown in table (7).

There were 17 patients had positive family history in which the mean of TIBC recording 250.3 ±4.2 while, the mean of TIBC recording 245±2.4 in 33 patients had negative family history as shown in table (7).

There were 17 patient had positive family history in which the mean of ferritin recording 2037.5±7.8 while, the mean of ferritin recording 2026.1±4.3 in 33 patients had negative family history as shown in table (7).

There were 12 patients had positive family history in which the mean of HbA recording 51.2±5.82. While, the mean recording 47.9.1±3.77 in 29 patients had negative family history as shown in table (8).

On the other hand, The mean of HbF in positive family history recording 45.7±5.81 while, the mean recording 48.5±3.73 in patients had negative family history as shown in table (8). While, the mean HbA2 in positive family history recording 3.03±0.47 while the mean recording 3.5±0.27 which was non significant as shown in Table (8).

A highly significant relation between consanguinity and Hb conc. recording 6.69±0.11 in 27 patients had positive consanguinity and recording 8.34±0.21 in 23 patients had negative consanguinity as represented in table (9).

Table (7) Relation between Family history and Hb conc., serum iron TIBC and ferritin in patients.

Parameters	+Ve F. H. N=17	-Ve F. H. N=33	T-Test	P-value
Hb conc g/dl Mean ± SE	7.18±0.26	7.59±0.21	1.18	>0.05
Serum iron µg/dl Mean ± SE	247.8±4.3	242±2.46	1.25	>0.05
TIBC µg/dl Mean ± SE	250.3±4.2	245±2.4	1.18	>0.05
Ferritin ng/dl Mean ± SE	2037.5±7.8	2026.1±4.3	1.38	>0.05

P >0.05 non significant

Table (8) Relation between Family history and Hb electrophoresis in patients

Parameters	+Ve F. H. N=12	-Ve F. H. N=29	T-Test	P-value
Hb electrophoresis Hb A% Mean ± SE	51.2±5.82	47.9±3.77	0.48	>0.05
HbF% Mean ± SE	45.7±5.81	48.5±3.73	0.41	>0.05
Hb A2% Mean ± SE	3.03±0.47	3.5±0.27	0.89	>0.05

P >0.05 non significant

Table (9) Relation between consanguinity and and Hb conc.serum iron,TIBC and ferritin in patients

Parameters	+Ve Consagunity N=27	-Ve Consagunity N=23	T-Test	P-value
Hb conc g/dl Mean ± SE	6.69±0.11	8.34±0.21	6.99	<0.001
Serum iron µg/dl Mean ± SE	252.46±2.16	234.10±2.96	5.09	<0.001
TIBC µg/dl Mean ± SE	254.84±2.12	237.38±2.90	4.94	<0.001
Ferritin ng/dl Mean ± SE	2043.92±4.48	2013.73±5.05	4.48	<0.001

P <0.001 highly significant.

A highly significant relation between consanguinity and serum iron recording 252.46±2.16 in 27 patients had positive consanguinity and recording 234.10±2.96 in 23 patients had negative consanguinity as represented in table (9).

A highly significant relation between consanguinity and TIBC recording 254.84±2.12 in 27 patients had positive consanguinity and recording 237.38±2.90 in 23 patients had negative consanguinity as represented in table (9).

A highly significant relation between consanguinity and feritin recording 2043.92±4.48 in 27 patients had positive consanguinity and recording 2013.73±5.05 in 23 patients had negative consanguinity as represented in table (9).

A non significant relation between consanguinity and Hb electrophoresis where HbA% recording 47.03±4.49 in 21 patient had positive consanguinity and recording 50.83±4.43 in 20 patient had negative consanguinity as represented in table (10).

On the other hand, HbF% recording 49.20±4.38 in 21 patient had positive consanguinity and recording 49.21±4.49 in 20 patients had negative consanguinity. Where, HbA2% recording 3.76±0.40 in 21 patient had positive consanguinity and recording 2.95±0.22 in 20 patients had negative consanguinity.

Table (10) Relation between consanguinity and Hb electrophoresis in patients

Parameters	+Ve Consagunity N=21	-Ve Consagunity N=20	T-Test	P-value
Hb electrophoresis Hb A% Mean ± SE	47.03±4.49	50.83±4.43	0.60	>0.05
HbF% Mean ± SE	49.20±4.38	49.21±4.49	0.47	>0.05
Hb A2% Mean ± SE	3.76±0.40	2.95±0.22	1.71	>0.05

P >0.05 non significant.

(2) Molecular investigation:

***(a) DNA extraction*:**

The genomic DNA of the β-thalassemic children and control was extracted, run on 1% agarose and visualized under U. V. transilluminator as shown in photo (1).The extracted DNA of each patient or control was amplified using specific primers (A & B) to amplify 143 bp including β-thalassemic Intorn-1 and segments of the flanking sequences of exon-1 & exon-2.

(b) Amplification of DNA:

The amplified products were run on 2 % agarose gel electrophoresis, which revealed that all control (25) gave amplified DNA product of normal size as shown in Table (11). In addition, the electrophoresis revealed that 47 patients gave amplified DNA products of normal size, one patient produced no band (non detectable), whereas another one revealed small size DNA band and one gave extra size DNA band and these results are tabulated in Table (12). For instance, the amplified products of some patients and controls were run on 2 % agarose gel electrophoresis as shown in photo (2).

***(c) Purification of DNA*:**

Regarding the one who gave non detectable (no band). A question was raised, whether such non detectable product was due to DNA impurities or exclude of DNA during extraction. To solve such a question, extra purification of the extracted DNA was performed using phenol-chloroform method, reamplified by PCR using the same primers under the same previous condition and then visualized on 2% agarose gel. The electrophoresis pattern of this amplified product was shown in photo (3).

Table (11) The control groups' amplified DNA bands.

Total number	Products	No. of DNA samples	Size of DNA
25	Normal product (one band)	25 (100%)	143 bp

Table (12) The patients' amplified DNA bands

Total number	Products	No. of DNA samples	Size of DNA
50 cases	Normal product (one band)	47 94%	143 bp
	One normal & one small (two band)	1 2%	1st band = 143 bp 2 nd band = 126 bp
	One normal & one extra (two band)	1 2%	1st band = 143 bp 2 nd band = 162 bp
	Non detectable (no band)	1 2%	–

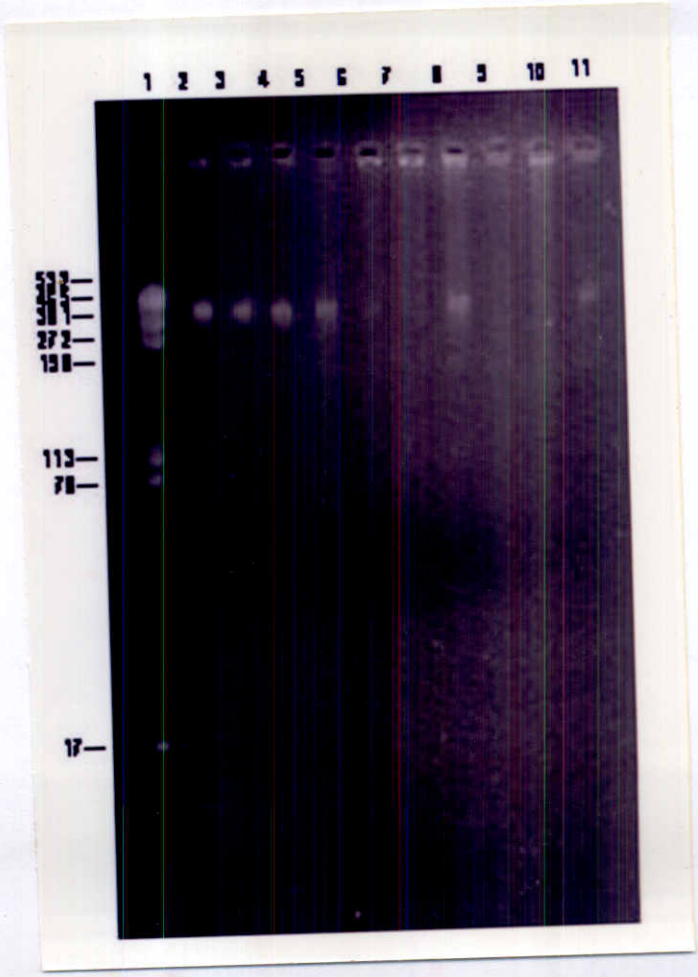


Photo. (1) Extracted DNA products of control and patients.

Each lane contained 10 µl of extracted DNA of control and patient. Lane2, even lane 5 contained DNA from control while, lane 6 even lane 10 contained DNA from patients while, A molecular weight DNA marker (17bp -532 bp) run in parallel, lane 1and lane11.

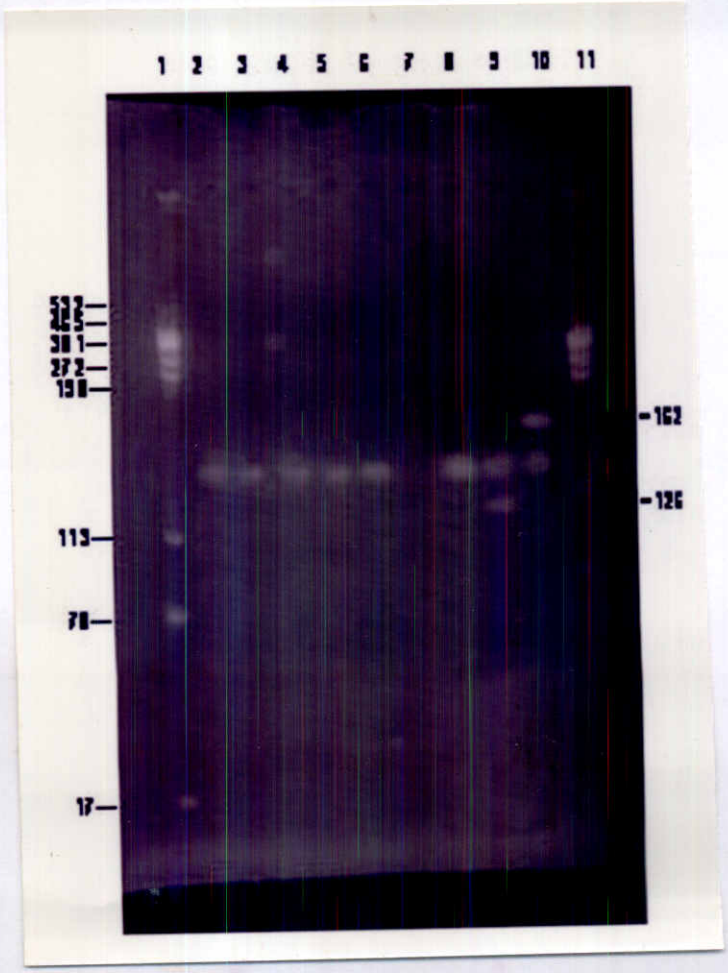


Photo (2) Amplified DNA products of control and patients.

Each lane contained 10 µl of amplified DNA of control and patients. Lane2 even lane 4 contained DNA from control while, lane 5, to lane 8 contained DNA from patients. On the other hand lane 7 contained DNA from patient N0.7 Lane 9 contained DNA from patient No. 12 and lane 10 contained DNA from patient No. 21. A molecular weight DNA marker (17bp -532 bp) run in parallel, lane 1and lane11.

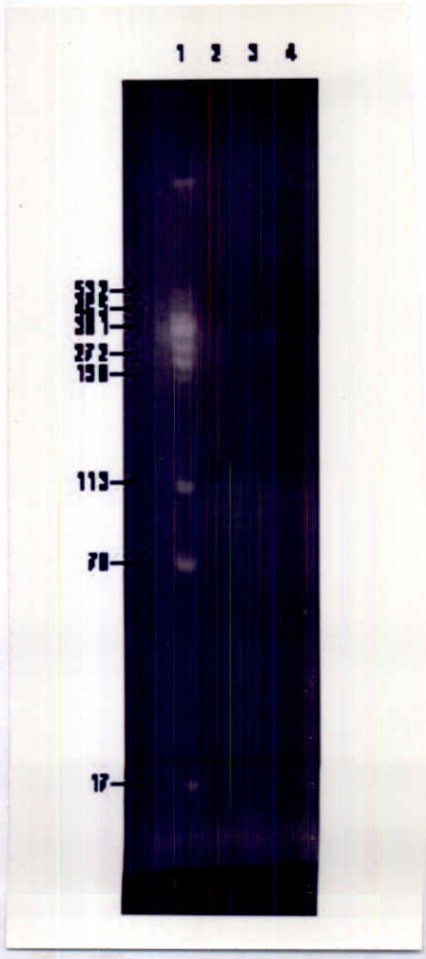


Photo. (3) Extracted DNA after Extra purification.

Lane 2 and lane 3 contained extra purification of extracted DNA from patient No. (7).while, A molecular weight DNA marker (17bp -532 bp) run in parallel, lane 1.

The gel again revealed non detectable band that might indicate excluded or break down of DNA during extraction.

(d) Digestion of the amplified products with restriction enzymes :

Each amplified product from patients or control was digested by some restriction endonucleases and visualized on 4% agarose gel. All patients and control DNAs were digested by NLa III, run on 4% agarose gel and visualized under U.V. Some of their products are shown in photo (4).

The end result of the NLa III digestion was represented in table (13). This table shows that 46 patients as well as control gave two band of 61 bp & 82 bp (means complete digestion). Three cases gave 3 DNA bands. One of them produced 3 bands of 61 bp, 82 bp and 143 bp, another one gaves 3 bands of 45 bp, 61 bp and 82 bp. and the last one gave 3 bands of 61 bp, 82 bp and 102 bp. For instance, the patient amplified product, which did not show band on agarose gel, was neither digested nor represented in such table

Another question was raised, whether the specimens which produced 3 bands (61, 82 & 143) gave such products due to incomplete digestion or due to point mutation. To differentiate between incomplete digestion and point mutation, the samples were reextracted by a phenol–chloroform, digested again and revisualized as shown in photo (5). The gel revealed the same pattern.

Table (13) Digestion of amplified product by restriction enzyme NLa III.

Restriction enzyme	Products	No. of DNA	Size of DNA
NLa III	Normal product (two bands)	46	1st band =61bp 2 nd band = 82bp
	Two bands normal & one small size (three bands)	1	1st band = 45bp 2 nd band = 61bp 3 rd band =82bp
	Two bands normal & one extra size (three bands)	1	1st band = 61 bp 2 nd band =82 bp 3 rd band =102 bp
	Incomplete digestion or point mutation (three bands)	1	1st band = 61bp 2 nd band =82 bp 3 rd band =143bp

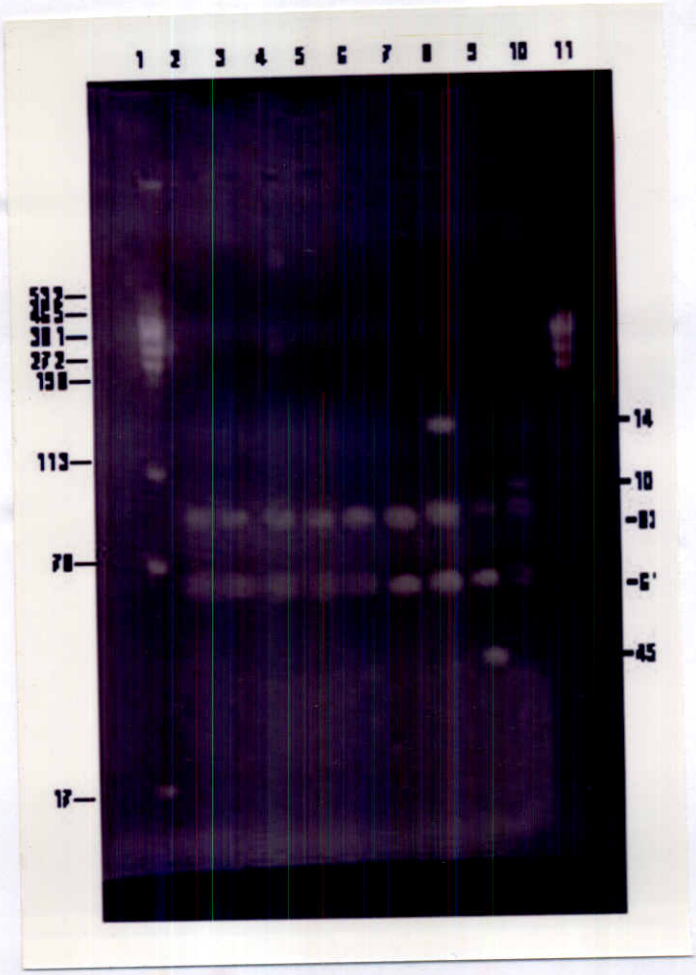


Photo (4) Digested DNA products of control and patients with Nla III of control and patients.

Each lane contained 10 µl of digested DNA products with Nla III. . Lane 2 even 4 contained DNA from control, while lane 5 to lane 6 contained DNA from patients. On the other hand lane 7 contained DNA from patient N0.7. Lane 8, lane 9 and lane 10 contained DNA from patients No. 31, 12 and 21 respectively . A molecular weight DNA marker (17 bp -532 bp) run in parallel, lane 1and lane11.

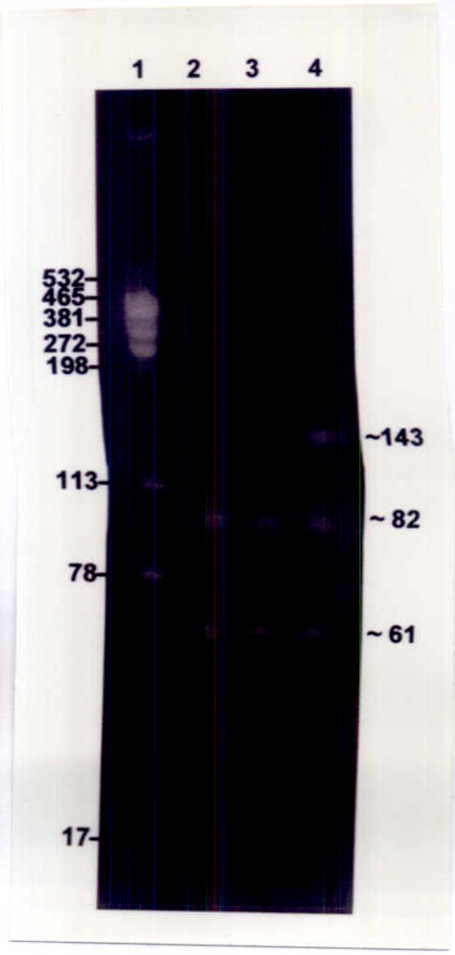


Photo. (5) Amplified DNA after Extra purification

Lane 2 and lane 3 contained extra purification of digested DNA product from patient No. 21. A molecular weight DNA marker (17 bp -532 bp) run in parallel, lane 1.

The amplified product of patients or controls was digested by BsmA1, run on 4% agarose gel and visualized under U.V. and some of their products are shown in photo.(6).

The end result of the BsmA1 digestion was represented in table (14). This table shows that 46 patients as well as all controls gave three bands of 25 bp, 38 bp and 80 bp (mean complete digestion). Three cases gave four bands the first one of 25bp, 38bp, 66 bp and 80 bp the second one of 25 bp, 38 bp,57 bp and 80 bp, the third one of 25 bp, 38 bp,80 bp and 143 bp, . For instance, the patient's amplified product, which did not show band on agarose gel, was neither digested nor represented in such table.

Table (14) Digestion of amplified product by restriction enzyme BsmA1

Restriction enzyme	Products	No. of DNA	Size of DNA
BsmA1	Normal product (three bands)	46	1st band =25 bp 2 nd band = 38 bp 3 rd band =80 bp
	Three normal band & one small size (four bands)	1	1st band = 25 bp 2 nd band = 38bp 3 rd band =66 bp 4 thband =80 bp
	Three normal band & one extra size (four bands)	1	1st band = 25 bp 2 nd band =38 bp 3 rd band =57 bp 4 thband =80 bp
	Incomplete digestion (four bands)	1	1st band = 25 bp 2 nd band =38 bp 3 rd band =80 bp 4 thband =143 bp

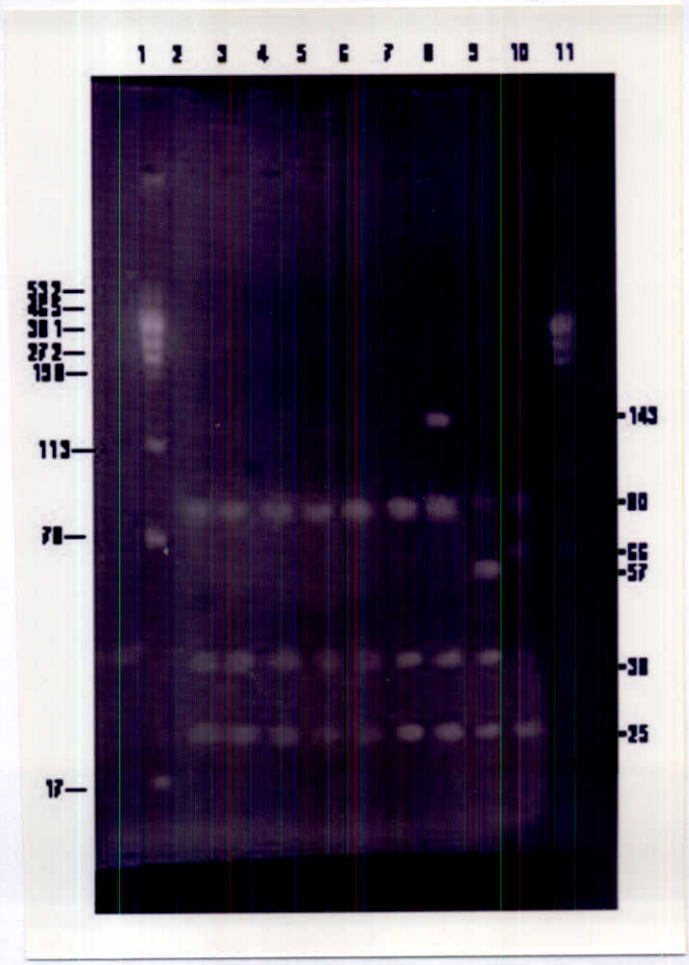


Photo (6) Digested DNA product of control and patient with BsmA1.

Each lane contained 10 µl of digested DNA products with BsmA1. Lane 2 even 4 contained DNA from control, while lane 5 to lane 6 contained DNA from patients. On the other hand lane 7 contained DNA from patient N0.7. Lane 8, lane 9 and lane 10 contained DNA from patients No. 31,12 and 21 respectively . A molecular weight DNA marker (17 bp -532 bp) run in parallel, lane 1and lane11.

The difference between patients with Intron-1 mutations, those without mutations and age of patients were non significant where, it recording (7.3±1.28) and (9.17±0.48) respectively as shown in table (15).

On the other hand, sex of patients, family history and consanguinity in patients with Intron-1 mutations and those without mutations were non significant and illustrates in table (15).

The hemoglobin concentration in patients with mutations recording (7.22±0.41) while, it recording (7.48±0.17) in patients without mutations but the difference between them were non significant. On the other hand, serum iron in patients with mutations recording (244.57±7.48) while, it recording (243.69±2.33) in patients without mutations where, the difference between them were non significant as represented in table (16).

The collective data in present study reaveled that the mean of TIBC in patients with mutations was (247.99±6.91) but it was (246.68±2.27) in patients without mutations, the difference between both groups were non significant.

Whereas the ferritin mean value (2031±12.09) in thalassemic children with mutations and recording (2029.93±4.22) in patients without mutations where, the difference between them were non significant as represented in table (16).

Table (15) Comparison between patients with Intron-1 mutations and those without mutations as regard personal data.

Parameter	With mutation N = 4	Without mutation N = 46	Test of significant	P-value
Age (Years) Mean ± SE Median Range	 8.37±0.89 8.75 6-10	 9.04±0.49 9 3-15	0.37	>0.05
Sex:	(No.) %	(No.) %		
Males Females	(3) 75 % (2) 25 %	(30) 65.2 % (15) 34.8%	X^2=0.15	>0.05
Positive consanguinity	(2) 50 %	(25) 54.3%	X^2=0.02	>0.05
Family history of Thalassemia	(2) 50 %	(14) 30.4%	X^2=0.64	>0.05

P >0.05 non significant.

Table (16) Comparison between patients with Intron-1 mutations and those without mutations as regard with hematological data.

Parameter	With mutation N = 4	Without mutation N = 46	Test of significant	P-value
Hb conc g/dl				
Mean ± SE	7.54±0.35	7.44±0.17	0.48	>0.05
Median	7.39	7.17		
Range	6.87 - 8.53	5.47 - 10.3		
Serum iron µg/dl				
Mean ± SE	239.46±7.06	244.41±2.33	0.94	>0.05
Median	236.70	242.66		
Range	225.38 - 259.06	205.22- 279.85		
TIBC µg/dl				
Mean ± SE	243.23±6.48	247.12±2.27	0.85	>0.05
Median	239.25	247.14		
Range	232.38 – 262.06	210.22- 280.85		
Ferritin ng/dl				
Mean ± SE	2020.05±7.76	2030.8±4.24	0.61	>0.05
Median	2020	2027		
Range	2002 - 2040	1980 - 2094		

P >0.05 non significant.

With regard to hemoglobin fractions the difference between patients with Intron-1 mutations and those without mutations were non significant and the mean value of HbA% in both groups recording (45.97±10.13), (49.20±3.33), HbF% in two groups recording (50.95±10.68), (47.40±3.29) and HbA2 was (3.07±0.71) and (3.40±0.25) in both groups respectively as illustrates in table (17).