ULTRASTRUCTURE AND MOLECULAR ANALYSIS OF ERYTHROPOIETIC CELLS IN β-THALASSEMIA

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SUMMARY: This study concerns the evaluation of β -thalassemia alleles in 20 individuals with homozygous β -thalassemia major, using gene amplification and dot-blot hybridization with synthetic oligonucleotide probes.

In addition to the genetic and molecular screening, erythropoietic cells in the peripheral blood samples of the same patients (12 non-splenectomized and 8 splenectomized) were studied by transmission electron microscopy. The molecular lesions causing β -thalassemia major have been delineated in Antalya (a Turkish city on the Mediterranean coast) by using polymerase chain reaction technique, which preferentially amplifies β -globin DNA sequences containing the most frequent β -thalassemia mutations in this region.

The Mediterranean population in Antalya carries six different β -globin mutations; four of these had been cumulated into IVS-I domain of β -globin gene, which account for nearly 95% of all abnormalities.

On the other hand, most of the erythroblastic cells showed many intracytoplasmic alterations corresponding to that of mutations of β -thalassemia.

Key Words: β -Thalassemia, Blood cell, Hemopoietic cell, Ultrastructure, Gene amplification, Polymerase chain reaction (PCR).

INTRODUCTION

β-thalassemia represents a heterogeneous group of hereditary disorders characterized by reduced or non-production of β-globin chain. Molecular analysis of various β-thalassemia genes has led to the characterization of more than 100 different mutations (1-6).

In the last decade, the molecular basis of β-thalassemia has been largely elucidated. The large majority of the thalassemia defects is characterized by the point mutations, affecting critical functional areas of the β-globin gene and is frequently seen in the Mediterranean basin, Africa, South-East Asia and the Indian Subcontinent (7-12). β-thalassemia is the most common form of hemoglobinopathies in Antalya province, a region on the Mediterranean coast in Turkey. The incidence of β-thalassemia with increased Hb-A2 production is between the ranges of 6.7 % and 10 % (13-15) which varies greatly from region to region (16,17).

Although various ultrastructural abnormalities are reported in erythroblasts under light microscopy, electron microscopic examinations are very rare (18-21). This present study is therefore designed to;

a) investigate the ultrastructural abnormalities of erythropoietic cells in splenectomized and non-splenectomized β-thalassemia patients,

b) compare the relationship between the cellular structure and molecular analysis.

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MATERIALS AND METHODS

Electron microscopy

Erythropoietic cells in the peripheral blood of 12 non-splenectomized and 8 splenectomized patients with β-thalassemia major were studied under transmission electron microscopy (TEM) technique (19).

All the cases were homozygous β-thalassemia and the blood samples were taken before transfusion. 10 ml of anti-coagulated blood samples were obtained from 5 to 18 years old patients, centrifuged and then 6.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) was slowly layered over the buffy coat and the tube left in a vertical position for 30 minutes at 4°C.

The solidified buffy coat disk was removed from the test tubes, their frequencies are determined and placed in freshly prepared buffered 6.5 % glutaraldehyde fixation for 2 h at 4°C.

After this prefixation, the buffy coat disk was cut into small blocks about 1 mm^3 in size and post fixed in 1 % osmium tetroxide for 2 h at 4°C. The samples were rinsed through a graded series of alcohol and three times 100 % propylene oxide exchange was applied. Afterwords, they were embedded in Araldite CY212 epoxy resin.

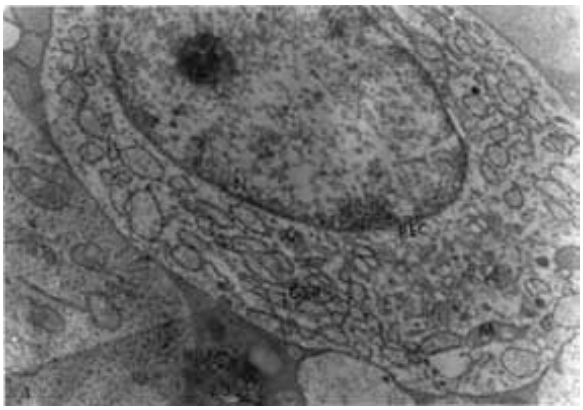
Sections were cut on a Nova ultramicrotome and examined by a JEOL 1200 electron microscope.

Table 1: Sequence of primers and allele specific oligonucleotides for the detection of β-thalassemia mutations by PCR.

Primers - Probes	Sequences (5' .... 3')
PRIMER A	CCAATCTACTCCCAGGAGCA
PRIMER B	CACTCAGTGTGGCAAAGGTC
IVS-I-110 (Mutant)	CTGCCTATTAGTCTATTTT
IVS-I-110 (Normal)	AAAATAGACCAATAGGCAG
IVS-I-6 (Mutant)	GCAGGTTGGCATCAAGGTT
IVS-I-6 (Normal)	AACCTTGATACCAACCTGC
IVS-I-1 (Mutant)	CCTGGGCAGATTGGTATCA
IVS-I-1 (Normal)	TGATACCAACCTGCCCAGG
-30 (Mutant)	GGCTGGGCAAAAAAGTCA
-30 (Normal)	GGCTGGGCATAAAAGTCAG
IVSI-3'END*	AAGGAGACCAATAGAACTG
GC clamp	AGAAAACATCAAGGGTCCCA
FSC-5* GC clamp	CTGTCATCACTTAGACCTCA
	CAACTTCATCCACGTTCACC

*These mutations were determined by denaturing gradient gel electrophoresis (DGGE).

Figure 1: Note the proerythroblast-like cell (PEC) showing an elliptical euchromatic nucleus (N) with well developed nucleolus (Nol) is seen as a very interesting finding. This cell type is different from normal erythropoietic cell lineage for some peculiarities like well developed granular endoplasmic reticulum (GER). Definitive other cellular organelles, mitochondrion (M), polyzomes and cytoplasmic composition were seen. X20.650, protocol no:225, non-splenectomized β-thalassemia major, homozygous (mutation: IVS-I-110).



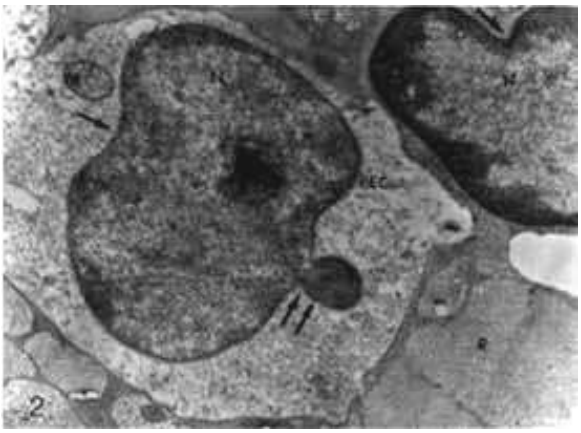
Molecular analysis

Blood samples were collected from 20 patients with β-thalassemia major from the department of pediatrics. DNA was isolated from white blood cells using the method described by Poncz *et al.* (22).

The method of gene amplification was performed according to the procedure of Saiki *et. al.* (23-25) with slight modifications (26, 27). Amplification was done in the region of the β-globin gene using by PCR with two primers, primer A and primer B (Figure 12 and Table 1). The amplified DNA samples were applied to zetaprobe nylon membrane using a Bio-Dot spotting apparatus. Amplification was in duplicate, one of the spots was used for hybridization to the normal probe and the other to the mutant probe. DNA primers and probes were synthesized using a DNA synthesizer in the department of prenatal diagnosis of Cagliari University, Sardinia.

All probes were labelled at the 5' end with the γ - P - 32-ATP (6000 ci/m mole-NEN-Dupont). Dot-Blots were hybridized with six probes located in amplificated region: IVS-I-110, IVS-I-6, IVS-I-1, IVS-I-3'END, -30, FSC-5 (Table 1). For each mutation, two probes (one specific for the normal allele) were used. After auto radiography, samples were hybridized only to the mutant probe which had the mutation on both chromosomes (homozygote) (Figure 11).

Figure 2: Slight nuclear gaps (with single arrow) are seen in different conditions in the proerythroblast cells (PEC). Nuclear membrane is in the normal condition but a region of nucleus (N) that is similar to a drumstick-like structure (with double arrows) connected with the nuclear body. R=reticulocyte, Nol=nucleolus, M=mitochondrion. X20.650, protocol no:225, non-splenectomized β-thalassemia major, homozygous (mutation: IVS-I-110).



RESULTS

Molecular analysis

A total of 40 β-thalassemia genes from 20 homozygous individuals was studied using the PCR methodology and the mutations were characterized. The major part of the mutations involving in the G-A mutation at IVS-I-110 and in the T-C mutation at IVS-I-6 and the others are listed in Table 2.

Table 2: Frequencies of 6 different β-thalassemia alleles in Antalya population (40 chromosomes).

Mutation	Chromosoms	%
IVS-I-110 (G - A)	20	50.00
IVS-I-6 (T - C)	9	22.5
IVS-I-1 (G - A)	7	17.5
IVS-I-3'END (G - C)	2	5.0
-30 (T - A)	1	2.5
FSC - 5 (-CT)	1	2.5
	40	100

Electron microscopy

Most of the erythroblastic cells showed many intracytoplasmic changes. Different erythroblastic cell types, were basophilic, where polychromatophilic erythroblasts and normoblasts were found in predominantly non-splenectomized patients. Proerythroblastic- like cells were also observed in peripheral blood samples. The cells exhibited a fine structure corresponding to that of proerythroblasts and had an elliptical euchromatic nucleus, with well developed nucleoli which occupied three to four part of the cell area (Figures 1, 2, 3).

These cells had some new structural properties appearing as very well developed granular endoplasmic reticulum (GER) cisternae in contrary to the form of normal premature condition (Figure 2). Some of them showed very homogenous cytoplasm containing many ribosomes and had big nucleus with double membranes (Figures 1, 3).

Despite the fact that most of the erythropoietic cells had clear and preserved perinuclear space and the

Figure 3 : This electronmicrograph is showing a proery-throblast-like cells (PEC) with well developed granular endoplasmic reticulum (GER) cisternae and three early ortochromatic erythroblasts (EOE) attached to side by side (A,B,C). A relationship between them with daughter cell membrane and vacuoles (V). A pycnotic nucleus (PN) showing a geometrical shape of chromatin accumulations and reticulocytes (R) including "Heinz bodies" (HB with arrows) are seen. Many vacuoles degeneration and cell debris are seen in the intercellular area. x9.000, protocol no:225, Non-splenectomized β-thalassemia major, homozygous (mutation:IVS-I-110).

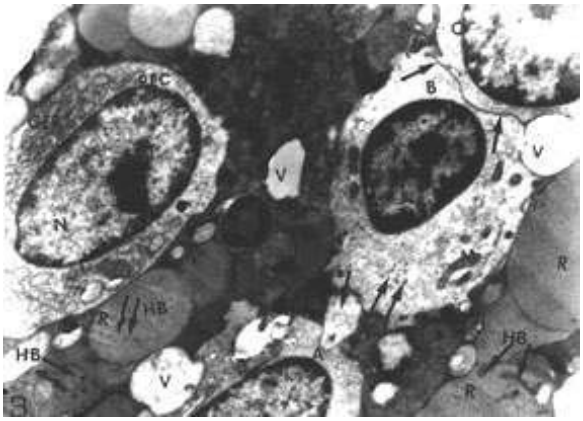


Figure 4: High magnification of figure 3 in partly early ortochromatic erythroblast (EOE), mature ortochromatic erythroblast (moe), reticulocytes (R), nucleus (N), pycnotic nucleus (PN), nucleolus (Nol), mitochondrion (M), vacuole (V) and Heinz bodies (HB, with arrows). X16.500, protocol no:225, non-splenectomized β-thalassemia major, homozygous (mutation:IVS-I-110).

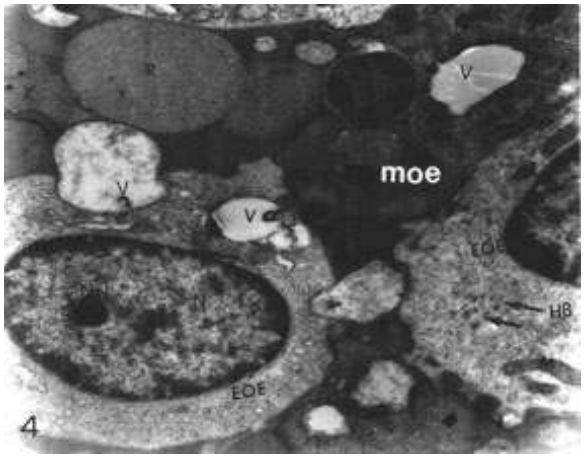
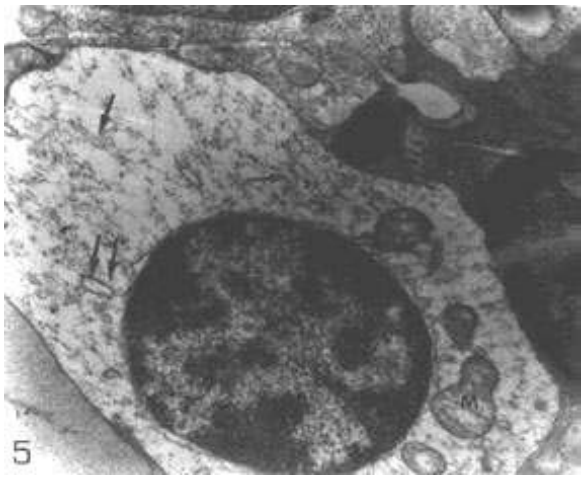


Figure 5: An intermediate cell form between polychromatophilic erythroblast and ortochromatophilic erythroblast is seen. The peculiarities of this cell variety are spherical heterochromatic nucleus (N) placed centrally, haemoglobin accumulation in the cytoplasm as a fine grain meshwork (with single arrows), spherical mitochondrion (M) and small endoplasmic reticulum profiles (with double arrows) in rare. X18.000, protocol no:225, non-splenectomized β-thalassemia major, homozygous (mutation: IVS-I-110).

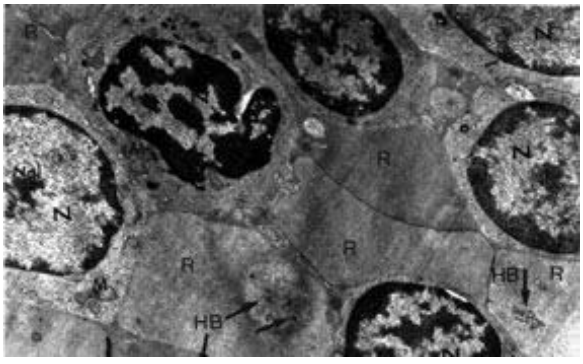


nuclear membrane, the same samples showed many large spaces and nuclear abnormalities. Nuclear invaginations were observed on the nuclear membrane in various sizes showing a slight communication between the cytoplasm and the nuclear matrix by means of nuclear pores. A region of nucleus, which was similar to the drumstick-like structure and connected with nucleus and surrounded with nuclear membrane, was observed in the form of proerythroblast-like cells (Figure 1). In addition to these, GER vesicles contained numerous polysomes and little mitochondrions were seen, but precise Golgi regions were not identified (Figure 1, 3).

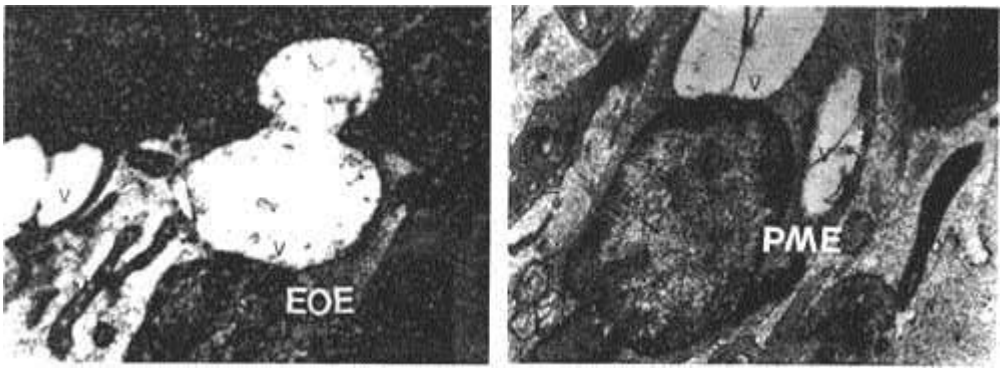
At the beginning of haemoglobin formation in erythropoietic cell series, heterogeneous inclusions of moderate density were characteristically accumulated and not surrounded by any membrane, which were derived from intracytoplasmic broken chains of hemoglobin systems. These structures were known as Heinz bodies (Figures 3, 4). It was possible to observe intermediate cell types, appearing at different developmental levels, between polychromatophilic and ortochromatophilic erythroblast cell types (Figures 5, 6).

There was a relationship between the cell destructive and intracytoplasmic vacuolisation and cellular precipitants forming as local dense masses in cytoplasm (Figures 3, 4, 7, 8). In the late stages of erythroblasts, the nucleus showed a condensed

Figure 6: Various hemopoietic cell types appearing different developmental levels, of erithro-and leucopoietic cell series are seen in this electronmicrograph. Nucleus (N), nucleolus (Nol), mitochondrion (M), reticulocyte (R), Heinz bodies (HB, with arrows). X12.500, protocol no:226, non-splenectomized β-thalassemia major, homozygous (mutation:IVS-I-6).



Figures 7 and 8: High magnifications of the early ortochromatic erythroblast (EOE) (7) and premature erythroblast (PME) (8) are observed. Large vacuoles (V), elongated mitochondrion and electron dense cytoplasm are seen in these cells. Heterochromatic nucleus (N), reticulocytes (R), dense bodies (with single arrows). 7: X18.000, 8: x25.000, protocol no:226, non-splenectomized β-thalassemia major, homozygous (mutation: IVS-I-6).



heterochromatin and the accumulations of haemoglobin increased indicating that the electron density of the cytoplasm associated with the cellular maturation (Figure 9). The cellular devastation begined with the isolation of nuclear membrane and matrix following pycnosis.

When cellular degeneration begined, the intra-cytoplasmic inclusions, large vacuoles (Figures 4,5,7,8), surface cytoplasmic fringe-like processes increased. These cellular degenerations were frequently observed in homozygous patients showing both IVS-I-110 and IVS-I-6 mutation in splenectomized or non-splenectomized subjects.

Typically, the karyorrhexis and karyolysis were demonstrated in the necrobiotic cell cycle. These were due to a pathological specialization of the erythroblast's cell organelles, and cytoplasmic vacuolisation was related with the cellular permeability, resulting in a discontinuance of their normal function (Figures 4,5,7,8).

Many cellular debris and fragments of platelets, degenerated blood cells were found in different size accumulations, which were surrounded by reticulocytes (Figure 10). The highest vacuolisation and cellular degeneration were observed in the patients showing IVS-I-110 and IVS-I-6 point mutations.

DISCUSSION

The majority of the β-thalassemia patients suffering from anaemia often requires regular blood transfusions. This is mainly due to the high frequency of the IVS-I-110 (G-A) mutation. Other β-thalassemia variants causing severe disease in the homozygote forms are the IVS-I-6 (T-C), the IVS-I-1 (G-A), the IVS-I-3'END (G-C), the -30 (T-A) and the codon 5 (-CT) (Table 2).

Figure 9: This electron micrograph is showing two types of orthochromatic erythroblasts, that are at early (EOE), premature (pme) stages. Reticulocytes showing in different stages of development that, premature (PMR) and mature (MR) reticulocytes. Interestingly, Heinz bodies (HB, with arrows) are seen in premature reticulocytes. Nucleus (N), degenerative cell remains (DC). X7,500, protocol no:224, Non-splenectomized β-thalassemia major, homozygous (mutation:IVS-I-6).

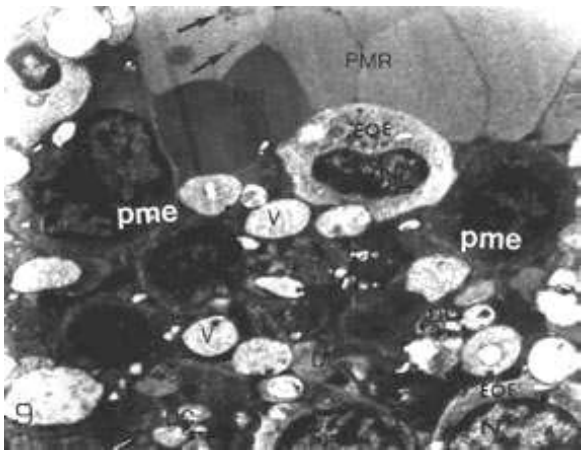


Figure 10: A degenerative area composed of different vacuoles (V), cell debris (DC) and amorphous substances surrounded by reticulocytes (R). N=nucleus, EOE=early orthochromatic erythroblast, M=mitochondrion, HB=Heinz bodies. X12.500, protocol no: 224, non-splenectomized β-thalassemia major, homozygous (mutation:IVS-I-6).

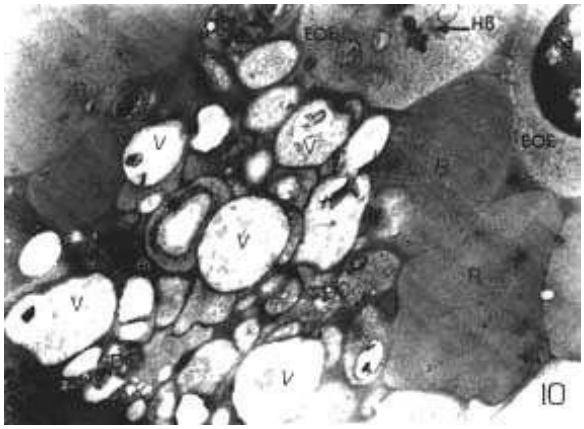
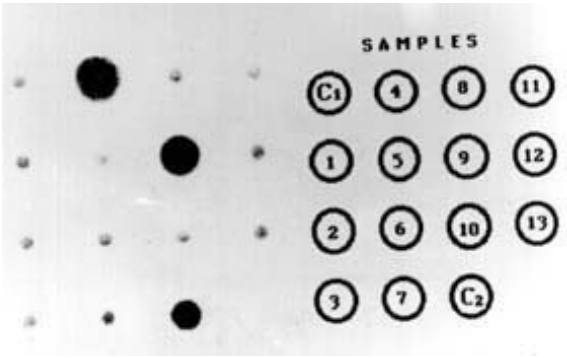


Figure 11: Dot-Blot hybridization of amplified β-globin gene. Two patients for the IVS-I-6 point mutations (samples 4 and 9) were evaluated as homozygous. The composition of the normal (N) and mutant (M) probes are listed below for this mutation (C1: normal control; C2: mutant control).
N:5'-AACCTTGATACCAACCTGC-3'
M: 5'-GCAGGTTGGCATCAAGGTT-3'



The most common three alleles which occur at frequencies above 5 %, account for 90 % of all β-thalassemia abnormalities, in agreement with the previously published data for Türkiye and for other Mediterranean countries.

The most predominant mutation for Antalya is G-A substitution at nt. 110 of the first intron (28).

Since defective haemoglobin synthesis appears in the erythroid cell lineage for β-thalassemia, we thought that proerythroblast-like cell (Figure 1) should not belong to another primitive cell lineage. In addition this cell type has well developed granular endoplasmatic reticulum (GER) cisternae which is the main stage for protein synthesis. In normal conditions, the erythropoietic cell series do not have this GER cisternae. Similar cell type is also observed in Figure 3. Pro erythroblast-like cells are frequently observed in homozygous patients showing IVS-I-110 but not for IVS-I-6 point mutation.

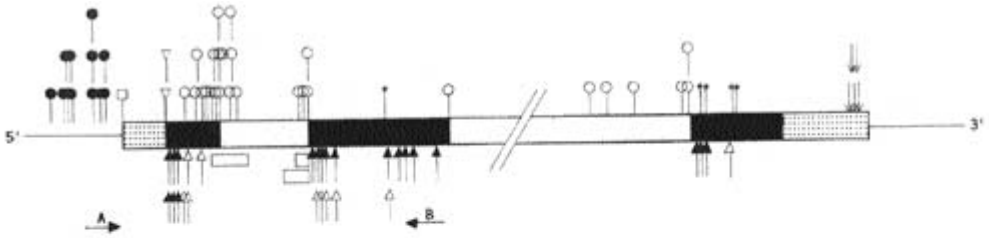


Figure 12: Region of the β-globin gene to be amplified using A and B primers (arrows indicate the location of these primers and the direction of amplification) and locations of β-thalassemia mutations in the human β-globin gene. Each of the specific mutations indicated along the linear map of the β-gene with a symbol corresponding to its effect on β-gene expression ♦: transcription; ♦:RNA splicing; ♦: cap site; ↓ : RNA cleavage; ♦:initiator codon; ♦: frameshift deletion; ♦: non sense codon; ♦: unstable globin; ▭: small deletion (Adapted from the references #32,33).

Major point mutations account for 50 % of β-thalassemia genes in Antalya is IVS-I-110 (G-A). This situation can be explained by high percentage (39.6 %) of consanguineous marriages in our population (29). According to these results, point mutation at IVS-I-110 may be responsible for ultrastructural properties which is shown in Figures 1 and 3 for genetic expression.

On the other hand, many ultrastructural changes are observed under transmission electron microscopic analysis. As the polychromatophilic and orthochromatic erythroblasts are often seen in the peripheral blood, high nuclear activity is determined in both cell types (30,31). Normoblasts are grossly disturbed and deformed, showing indentations and enfolding of plasma membranes with marked long cytoplasmic projection and large vacuoles including soluble substances. Cellular destruction is frequently observed on the free surface of erythropoietic cells. The cytoplasmic fragments including degenerated cell organelles, inclusions and lysosomal complexes are found. Heinz bodies and electron dense granules are frequently observed in normoblasts and reticulocytes. These ultrastructural findings suggest that, changes in the permeability and in the stability of cell membranes are also responsible for the haemolytic process in β-thalassemia patients (30,31).

Electron microscopic observations revealed that there are two different cell types of reticulocytes except cell types in the buffy-coat mentioned above. One of them exhibited a fine structure corresponding to that of early (immature) reticulocytes with Heinz body accumulations and very light cytoplasm including remains of organelle fragments.

The other type had darken cytoplasm exhibiting features of (mature) reticulocytes and no visible organelles fragments were observed.

The significance of the vacuolisation in erythropoietic series, homogenous and granular accumulations of substances appearing in the cytoplasm of normoblast cell populations have been discussed in relation to current concepts of haemoglobin formation and functions of cell organelles and degeneration processes. The first step of degeneration is the replacement of the nucleus peripherally by grouped vesicles, forming larger vacuoles. Cytoplasmic degeneration occurs during this process. Abnormal intracytoplasmic structures including the presence of excess iron in various forms, glycogen accumulations and abundant myelin figures are found commonly in erythropoetic cells of β-thalassemia.

Electron dense inclusions are abundant and are observed almost in all erythroblasts. Nuclei of normoblasts are surrounded by a nuclear membrane, showing condensed heterochromatin accumulations and including some euchromatic points are arranged at the periphery.

Although the proerythroblast-like cell type showed well developed GER cisternae for the mutation of IVS-I-110, there was no significant structural difference between IVS-I-110 and IVS-I-6 point mutations. So far, we are not able to determine the certain relationships between the other point mutations and ultrastructural abnormalities precisely. However, this study is currently being conducted for a large population in our region where the point mutations occur heterogenously.

As a conclusion, ultrastructural examinations suggest that there are potential connections between gene mutations and the cellular degeneration in erythropoietic cell maturation (21, 30).

Keeping this point in mind, we are thinking that the possible mechanism for denaturation process is hidden in the RNA processing and splicing stages. However, we are able to say that the stability of cellular membrane and organelles are changed due to the nucleocytoplasmic disconnection corresponding to genomic alterations and structural abnormalities.

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