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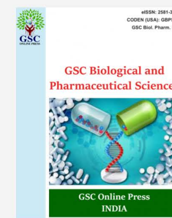

(RESEARCH ARTICLE)

Investigation of mitochondrial oxidative phosforylation enzymes in hematological cancers

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Abstract

It is known that cancerous cells are different from normal cells in their structure and functions. It is aimed to determine whether there is a change in the activities of mitochondrial oxidative phosphorylation (OXPOS) respiratory enzymes, the main energy production site of cells, in various haematological cancers and to use the obtained data for therapeutic purposes.

In this study, leukocyte mitochondria were isolated from blood samples obtained from patients with non-hodgkin lymphoma (NHL), hodgkin lemphoma (HL), chronic lymphocytic leukaemia (CLL), acute myelogenic leukaemia (AML), acute lymphocytic leukaemia (ALL), chronic myeloid leukaemia (CML), multiple myeloma and myelodysplastic syndrome (MDS) and controls. The enzyme activities of oxidative phosphorylation complexes I, II and IV were measured by spectrophotometric method.

Enzyme activity results were statistically evaluated according to various criteria. Enzyme activities were evaluated according to gender, chemotherapy or not and disease groups. As a result of these statistical evaluations, no correlation was found between the type of haematological cancer and gender ($p>0.05$). The difference in mitochondrial oxidative complex enzyme activities between patients receiving chemotherapy and those not receiving chemotherapy was insignificant ($p>0.05$). When the enzyme activities of the control group and all haematological cancer cells studied were compared, the difference was found to be statistically significant ($p<0.05$).

As a result, changes in the activities of OXPOS respiratory enzymes were found in various haematological cancers. The large number of therapies that specifically target mitochondria, the altered metabolic functions and dynamics of mitochondria in cancer cells make it a target for cancer therapeutics.

Keywords: Mitochondria; Cancer; Leukemia; OXPHOS enzymes

1. Introduction

The mitochondrial organelle has a DNA (mtDNA) that is independent of the nuclear DNA (nDNA) of the cell. The structure of mtDNA was fully elucidated in 1981 [1]. mtDNA is a circular, double-stranded molecule of 16569 bp. Each mitochondrion contains two to ten copies of mtDNA and plays a limited but important role in the biogenesis of organelles. The vital role of mitochondria in eukaryotic cells was demonstrated over a hundred years ago by Otto Warburg, who was the first to perform experiments on mitochondrial respiration [2]. In healthy replicating eukaryotic cells, mitochondria regulate important cellular processes such as proliferation, death, metabolic adaptation and $Ca2+$ homeostasis. Mitochondria are also an organelle in which important reactions occur, including fatty acid oxidation (FAO), the tricarboxylic acid (TCA) cycle, oxidative phosphorylation (OXPHOS), the first step of gluconeogenesis,

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ketogenesis, haem biosynthesis and Fe/S cluster formation [3]. They contain DNA that can change, mutate or be partially deleted by evolution. Mutation(s) occurring in mtDNA, whether spontaneous or induced by chemical mutagens, is a random process. However, the mitochondrial mutation rate is about 10 times higher than that of nDNA [4]. At the same time, mtDNA is a privileged target for certain mutagens and carcinogens [5]. Yamamoto et al. (1992)[6] suggest that some mtDNA mutations may be one of the endogenous factors that induce somatic mutations in the nuclear genome and etiologically contribute to human carcinogenesis [7, 8].

Given the numerous functions of mitochondria, it is not surprising that mitochondrial dysfunctions are implicated in a number of diseases, including cancer. It has long been recognised that mitochondrial dysfunctions contribute to the development and progression of cancer. Indeed, respiratory chain dysfunctions and mtDNA abnormalities appear to be a general feature of malignant cells [9]. In addition, mutations and deletions in mtDNA and abnormalities in the expression of proteins encoded by mtDNA are common findings in solid tumours and haematological malignancies. There are at least five mechanisms by which mitochondria may play a role in the development of the malignant phenotype through metabolic reprogramming of cancer cells. One of these mechanisms has been widely demonstrated to be associated with the overproduction of reactive oxygen species (ROS), which can lead to mutations in mtDNA due to alterations in subunits of the electron transport chain (ETC). Mutations in mtDNA lead to changes in the structure of enzymes in the oxidative phosphorylation complex encoded by this DNA. Extensive mitochondrial studies have shown that intrinsic alterations or limitations in OXPHOS favour aggressive disease spread in leukaemia [10, 11].

Research on the effects of these changes on cancer formation and development will shed light on clinical approaches and lead to satisfactory advances in cancer prevention and treatment. Based on this information, we carried out this study to determine the possible changes in the activities of oxidative phosphorylation complex enzymes in various haematological cancers.

2. Material and methods

2.1. Patient and control groups

A total of 108 (9 control and 99 hematological cancers diagnosed) individuals participated in this study [Table 1].

2.2. Leukocyte isolation

Leukocyte isolation was performed according to Burtis (1999)[12]. 10 mL of blood was collected from leukemia patients and healthy individuals in EDTA tubes. The leukocyte count was counted in an automatic blood count device. The samples were then stored at room temperature. Add 2 mL of a freshly prepared solution of dextran, 5 g/dL, in sodium cloride, 0.7g/dL. Mix gently by inversion. Allow to stand for 45 min for sedimantation of cells. Draw off supernatant with a pasteur pipet and discharge it into another plastic centrifuge tube. At 4 °C, centrifuge at 500 Xg for 10 min. Draw off supernatant and discard. Resuspend the buttom of White blood cells in 1.0 mL of cold sodium chloride, 0.9 g/dL. Shock treat the cells as follows: Add 3.0 mL of ice-cold distilled water and mix gently for 45 seconds. Immediately add 3.0 mL of cold sodium chloride, 1.8 g /dL, and mix. Centrifuge at 500 Xg for 10 min. Draw off supernatant and discard. These procedures were repeated in the same way until there were no red blood cells in the solution. Resuspend leukocyte cells in 1.0 mL of cold sodium chloride, 0.9 g/dL. Make a 500-fold dilution with 20 mL Isoton and count cells in this suspension on the Coulter counter at the white blood cell settings. Records the count (=W= cell count). Samples were frozen with liquid nitrogen and stored at -85 °C until studied.

2.3. The lysis of leukocytes and obtaining mitochondrial oxidative phosphorylation complexes

The lysis of leukocytes and obtaining mitochondrial oxidative phosphorylation complexes were performed using the method developed by Schagger (1996) [13].

The proccessing of leukocytes, also performed at 4 °C, 83 mM sucrose and 10 mM MOPS, pH 7.2, hypotone buffer 1-5X10^6 cells /500 µl were added. The homogenate was homogenised by 10 pulses with a glass Teflon homogeniser on ice. 500 µl of 250 mM sucrose, 30 mM MOPS, pH 7.2 solution was added to the homogenate. Centrifuged at 600Xg for 15 minutes at 4 °C The supernatant was taken with a pasteur pipette. The supernatant was centrifuged at 15000 Xg at 4 °C for 15 minutes. This time the supernatant was discarded with a pasteur pipette. Sediment was taken. 150 µl of 1 M aminocaproic acid, 50 mM Bis-tris pH 7.0 was added to the sediment. Resuspended with a spatula. 5 µl of 10% (w/v) dodecylmaltoside was added and a 15- min centrifugation at 100000 Xg, 4 °C. Then the supernatant was taken with a pasteur pipette. Protein was determined in the supernatant by Lowry method (1951)[14]. Supernatants were used for enzyme measurements and stored at -85 °C until studied.

2.4. Spectrophotometric measurement of oxidative phosphorylation complex enzymes

2.4.1. Complex I - NADH dehydrogenase (Enzyme I)

A 55 mM tris buffer, pH 8.0, containing 750 µl NADH and 130 µM 2,6-dichloroindophenol was prepared. A working volume of 900 µl of this buffer was taken and 100 µl of homogenate was added and absorbance change was recorded at 37 °C at 600 nm wavelength [15].

2.4.2. Complex II - Succinate dehydrogenase (Enzyme II)

- Solution I: 55 mM phosphate buffer pH 7.4 containing 0.1 mM EDTA, 0.1% BSA, 40 mM sodium succinate, 298 µM 2,6-dichloroindophenol was prepared.
- Solution II: 55 mM phosphate buffer pH 7.4 containing 0.1 mM EDTA, 0.1% BSA, 3.25 mM phenazine methosulfate was prepared.

0.4 ml of solution 1 and 0.4 ml of solution 2 were taken. 200 µl of homogenate was added and absorbance change was recorded at 37 °C at 600 nm wavelength [16].

2.4.3. Complex IV - Cytochrome c oxidase (Enzyme IV)

50 µM Cytochrome c was dissolved in 100 µM Tris pH 7.0 and reduced with 0.1 M dithionite. The reduction was continued until the A550/A560 ratio was between 9 and 6. After this ratio was obtained, 100 µl of homogenate was added to 900 µl of reduced cytochrome solution and absorbance change was recorded at 550 nm wavelength at 37 °C [17].

$$\text{Amount of volume activity:}= \frac{V}{\mathcal{E}.d.v}.\Delta E/\Delta T[\frac{U}{mL}.dk],$$

$$\text{Specific Activity}= \frac{V}{\mathcal{E}.d.v.p}.\Delta E/\Delta \mathrm{T}[\frac{\mathrm{U}}{\mathrm{mg}}]$$

V= Total volume of the experimental medium (mL)
$\Delta E/\Delta T$= Optical density difference per minute (nm/min)
Ɛ= Molar absorbance coefficient
v= Sample volume in the experimental medium (mL)
d= length of the path travelled by light (cm)
p= protein amount (mg/mL)
$\mathcal{E}_1$= 21mM^{-1} cm^{-1} (2,6-dichloroindophenol)
$\mathcal{E}_2$= 19.2 mM^{-1} cm^{-1} Reduced form (cytochrome c)

2.5. Statistical analysis

The data were uploaded to SPSS (Ver=22) programme and Kruskal-Wallis test, Mann Whitney U test and Chi-square test were used to evaluate the data. $P<0.05$ was considered as the significance level.

3. Results

A total of 108 individuals participated in this study. The characteristic features of the patient and control groups are shown in Table 1.

The distribution of the individuals according to gender is given in Table 1. Chi-square value was calculated as $\chi 2 = 9.56$. The value of this value at the $p<0.05$ level in the statistics table is $p=0.21$. Since this value is 0.213>0.05, there is no relationship between the type of haematological cancer and gender.

When the activity of enzyme I of the control group was compared statistically with the activities of enzyme I obtained from all haematological cancer types studied, it was found that the activity differences between them were significant ($p<0.05$) (Table 2).

When haematological cancers are compared among themselves in terms of the activity of enzyme I, the difference between NHL and CLL is significant, while the difference between them and other leukaemias is statistically insignificant. The difference between CLL and all other leukaemias except ALL is significant. When the enzyme I activity value of ALL is compared with the values of MM and AML, the difference is significant but the difference with other

leukaemias is insignificant. The difference in activity between HL and CLL, MM and AML is significant, while the difference with other leukaemia types is insignificant. The differences between the enzyme I activity values of MM and AML and the enzyme I values of CLL, ALL and HL are significant. Differences in enzyme I activity between cats and between other leukaemia types are insignificant. The difference between the enzyme I activity values of CML and CLL is statistically significant, while the difference between the enzyme I activity values of CML and other leukaemia types is insignificant (p>0.05) (Table 2).

Table 1 Distribution of control and patient groups according to sex

Cancer type	Male	%	Female	%	Total individuals	%
NHL	24	70.6	10	29.4	34	100
KLL	8	72.7	3	27.3	11	100
ALL	3	33.3	6	66.7	9	100
HL	17	65.4	9	34.6	26	100
MM	8	80.0	2	20.0	10	100
AML	2	33.3	4	66.7	6	100
KML	1	33.3	2	66.7	3	100
Control	5	55.6	4	44.4	9	100
Total	68	63.0	40	37.0	108	100

$\chi 2$:9.56, p=0.213, p>0.05

Table 2 Enzyme activities of mitochondrial oxidative phosphorylation complexes in haematological cancers

Cancer type	*n*	Age X±SH	Enzyme I	Enzyme II	Enzyme IV
NHL	34	50.32±16.03	332.63±25.04 ac	2.43±0.44 a	347.47±19.55 a
KLL	11	54.18±18.86	425.91±21.65 b	2.68±0.45 abc	296.51± 14.84 ac
ALL	9	33.77±14.98	388.83±31.17 ab	3.27±0.72 bc	249.31±14.31 b
HL	26	39.11±19.85	321.16±9.78 a	3.27±0.37 bc	269.64±10.15 bc
MM	10	51.20±15.01	256.47±23.95 c	2.19±0.40 ab	324.58±14.01 a
AML	6	34.16±17.31	252.15±29.34c	1.78±0.30 ab	336.05±11.37 a
KML	3	53.66±25.69	266.36±33.94 ac	2.49±0.43 abc	272.25±30.13 ab
Control	9	33.00±5.65	167.20±8.15 d	7.48±2.30 c	169.86±7.40 d

X: Average value; SH: Standart error; n= number of individuals; *: * Means with the same letters in each column do not differ significantly at p<0.05 level (abc). (Lettering done according to Mann-Whitney test)

When the control group was compared with the patient groups in terms of enzyme II activity, the difference between NHL, AML and MM was statistically significant, while the difference between the activities of enzyme II in the other leukaemia groups was insignificant (p>0.05) (Table 2).

When the differences between the activities of enzyme II among haematological cancers were compared, the difference between NHL and ALL and HL was significant (p<0.05) (Table 2). Differences between enzyme II activities among other leukaemias were insignificant (p>0.05) (Table 2).

When the control group was compared with the patient groups in terms of enzyme IV activity, it was found that the activity differences between the enzyme IV activities obtained in all haematological cancer types studied were statistically significant (p<0.05) (Table 2).

When the differences between NHL, CLL, MM, AML and CML were compared statistically in terms of the activity of enzyme IV among the patient groups, the differences in the activity values of enzyme IV between ALL, HL and CML were insignificant. However, the difference between ALL and HL and other leukaemias was significant ($p<0.05$) (Table 2).

When we statistically compared the enzyme I, II and IV activity values within the same cancer type according to whether the individuals in the patient group received chemotherapy or not, the change in the enzymatic activities of all three enzymes was not statistically significant ($p>0.05$) (Table 2).

Since the number of individuals receiving chemotherapy was one (1) in the MM, AML and CML groups and there were no patients receiving chemotherapy in the CLL group, statistics were not applied to these groups (Table 3).

Table 3 Mitochondrial OXPHOS enzyme activities of individuals with and without chemotherapy in haematological cancers

Cancer type		n	Enzyme I	Enzyme II	Enzyme IV
NHL	Stage I	16	326.45±37.02	2.23±0.59	369.30±38.59
	Stage II	18	338.12±34.93	2.60±0.66	328.07±13.65
			P= 0.730	P=0.560	P=0.756
ALL	Stage I	6	401.27±44.29	2.62±0.22	247.39±21.09
	Stage II	3	363.94±37.18	4.58±2.15	253.14±14.85
			P=0.606	P=0.439	P=0.604
HL	Stage I	12	311.18±17.28	3.07±0.49	251.34±14.86
	Stage II	14	329.70±10.61	3.44±0.55	285.32±12.90
			**P=0.355	**P=0.777	**P=0.100
KLL*	Stage I		------	------	------
	Stage II	11	425.91±21.64	2.68±0.44	296.50±14.84
MM*	Stage I	1	412.48±0.00	1.74±0.00	261.20±0.00
	Stage II	9	239.14±18.46	2.23±0.44	331.61±13.54
AML*	Stage I	1	362.00±0.00	1.19±0.00	344.14±0.00
	Stage II	5	230.17±23.82	1.90±0.33	334.43±13.78
KML*	Stage I	1	334.00±0.00	3.33±0.00	331.00±0.00
	Stage II	2	232.54±4.88	2.07±0.16	242.88±11.62

*: Statistical test was not applied since the number of individuals receiving chemotherapy in CLL, MM, AML and CML was one (1); *Stage I: undergoing chemotherapy; **Stage II: no chemotherapy; **: Statistically significant ($p <0.05$)

4. Discussion

Free radicals formed as a result of basal electron leakage from the mitochondrial respiratory chain under normal conditions cause mutations in mitochondrial DNA encoding a total of 13 subunits of oxidative phosphorylation enzyme complexes. As a result of this mutation disrupting the protein structures, a vicious circle emerges in the chain due to more electron leakage due to electron accumulation in the complexes behind the disruption [18].

Defects in respiratory chain functions and mtDNA abnormalities are a general feature of malignant cells. In addition, mutations in mtDNA and abnormalities in the expression of proteins encoded by mtDNA are common findings in solid tumours and haematological malignancies. It is inevitable that research on the effects of these changes on cancer formation and development will shed light on clinical approaches and lead to satisfactory advances in cancer prevention, diagnosis and treatment.

In our study, analysis of mitochondrial oxidative enzyme complexes in peripheral blood samples from patients with hodgkin lymphoma, non-hodgkin lymphoma, chronic lymphocytic leukaemia, acute lymphoblastic leukaemia, myelodysplastic syndrome, acute myelogenic leukaemia, chronic myeloid leukaemia and multiple myeloma provides information about the activities of enzymes in a wide range of haematological cancers.

Combined dysregulations in mitochondrial network function and dynamics may contribute to cancer development in many different complex ways by affecting metabolism and ATP production, redox homeostasis and the triggering of apoptosis [19]. Recent findings have shown that cancer cells benefit from high OXPHOS, including leukaemias, lymphomas, pancreatic ductal adenocarcinoma, melanoma and endometrial carcinoma [20-31], while mitochondria can modulate their morphology by regulating the intrinsic apoptotic pathway and participating in the resistance of cancer cells to apoptotic stimuli [32-42].

In this study, the difference between the activities of Complex I (NADH dehydrogenase) and Complex IV (Cytochrome c oxidase) enzymes belonging to the OXPHOS system in the eight haematological cancer types we studied and the same enzymes in the control group was statistically significant ($p<0.05$) (Table 2). There is increasing evidence that metabolic reprogramming required to meet the increased energy demand in cancers is linked to an increased ability to escape apoptosis [19,43]. These studies support our results. Because in our study, the numerical values of both complex I and complex IV were much higher than the control group. This may be to meet the energy requirement by increasing these complexes for increasing energy requirement in cancer cells ($p<0.05$) (Table 2). In our study, when the enzyme activity results of complexII were compared with the control group, the difference between the hodgkin lymphoma, multiple myeloma, acute myelogenic leukaemia groups was significant ($p<0,05$) (Table 2), while the difference with other leukaemia types was found to be insignificant ($p>0,05$) (Table 2).

An important study demonstrating the link between mitochondria and tumour formation is the finding of a mutation in the gene encoding the small subunit (cybS) of succinate ubiquinone oxidoreductase (complex II) cytochrome b in paraganglioma [44].

The reducing activity of Complex II has been shown to be associated with human cancer in renal carcinoma [45, 46] and breast cancer [47], where Complex II activity is lower compared to the corresponding normal tissues. In our study, when we evaluated the Complex II (Succinate dehydrogenase) enzyme activity and numerical values of the control group and patient groups, the enzyme activity value of the control group was higher in all cancer groups. According to this result, complex II activity seems to be decreased in haematological cancers. The decrease in the activity of complex II may be due to a change in nDNA. The results of other researchers given above support our data on complex II.

In a study conducted with children with acute leukaemia, it was investigated whether there was a relationship between gender and being ill, but no relationship was found [48]. In our study, no relationship was found between gender and being ill ($p>0.05$) (Table 1).

The induction of the Warburg effect in the microenvironment of Hodgkin and Reed-Stenberg HRS cells and the reverse Warburg effect in HRS cells may mediate drug resistance to chemotherapy drugs that impair OXPHOS function or affect cellular redox status, such as doxorubicin [49, 50]. Drugs commonly used in first-line therapy such as bleomycin, doxorubicin and vinblastine, which are part of the ABVD regimen [51-54], produce large amounts of reactive oxygen Barbato et al. OXPHOS in haematological malignancy species, causing oxidative stress and apoptosis [55], However, it also increases the expression of antioxidant enzymes that may contribute to chemoresistance in HRS and HL cells. In our study, when the enzyme activities of Complex I, II and IV were compared in patients receiving and not receiving chemotherapy, the difference between the activities before and after treatment was found to be insignificant. In other words, chemotherapy did not seem to affect enzyme activities ($p>0.05$) (Table 3). The lack of change in enzyme activities may be a result indicating drug resistance.

Mitochondria play many important roles in cell function and homeostasis, including ATP production, release of death-promoting factors upon apoptotic stimuli and various metabolic pathways. Contrary to conventional belief, functional mitochondria are essential for cancer cells. Different cancer cell types undergo different bioenergetic changes, some more glycolytic and others more oxidative, depending in part on the developmental state of the cell undergoing neoplastic transformation [56].

Ongoing trials in CLL, NHL, HL, MM, AML and other haematological malignancies indicate that targeting OXPHOS is a promising strategy to trigger a metabolic remodelling leading to chemosensitisation [57].

5. Conclusion

In our opinion, an important issue is to determine whether metabolic changes are the cause or the consequence of the malignant process. It is important to determine whether specific metabolic changes trigger the phenotypic evolution of cancer cells or simply follow them. Because in the first case targeting these changes may inhibit phenotypic progression, whereas in the second case metabolic flexibility may rapidly overcome therapeutic interventions.

Compliance with ethical standards

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Disclosure of conflict of interest

The authors declared no conflicts of interest with respect to the authorship and/or publication of this article.

Statement of ethical approval

Gülhane Military Medical Academy operates with the permission of the local ethics committee, decision numbered 2000-05/1.

Statement of informed consent

Informed consent was obtained from all individual participants included in the study.

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