INVESTIGATION OF WARBURG EFFECT IN MAMMALIAN CANCER CELL LINES BY STUDYING VARIOUS METABOLIC ENZYMES AND ASSAYS

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Submitted to Graduate School of Natural and Applied Sciences
in Partial Fulfillment of the Requirements
for the Degree of Master of Science in
Biotechnology

Yeditepe University
2017

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DATE OF APPROVAL: …. / …. / 2017

ACKNOWLEDGEMENTS

To begin with, I would like to thank my supervisor Assist. Prof. Dr. Hüseyin Çimen, for supporting, employing me and giving permission to work in his lab, and providing a chance to start my academic career with this master program. Additively, I am greatly thankful to Prof. Dr. Fikrettin Şahin for encouraging me and continuous material support, Assist Prof. Dr. Ali Ö. Aytekin, Assist Prof. Dr. Emrah İ. Nikerel, Assist. Prof. Dr. Bahar S. Özdemir for mental support.

I also thank my previous and current lab members Eray Esendir, Cansu Atılgan, Binnur Kıratlı, Fatih Aygenli, Ekin Sönmez, Ayşe T. Şahin, Cansu Dilege, Gülşah Ş. Özköse, İrem Soyhan, and İrem Baygutalp for helping and tolerating me when I was stressed. Besides, I am grateful to Selami Demirci, Safa Aydın, Mustafa Yoğurtçu, Taha B. Hayal, Hüseyin Abdik, Ezgi Boylu, Esra A. Çoban, Ezgi Abdik, Mine Altunbek, Pınar Akkuş, Cansu Ü. T. Tunç, Fatma Özen, Ayca Z. A. İlter, and Hande Nayman for both material and particularly moral support.

I am quite lucky person because I have unique, patient, supportive, indulgent, uplifting friends Ozan Soysal, Samet Koçum, Seçkin Yıldırımdemir, Selen Yıldırımdemir, Ömürcan Ateş, İpek V. Özcan, Alper Turgut, Tuğçe B. Turgut, Elif Yavaş, Şilan Ö. Coşkun, Kaya İşleyen, Gökcan Şahin, Polen Koçak, Bürge Ulukan, Merve Yıldırım, Gizem Uçankuş, Deniz Y. Öztaş, Deniz Uzunoğlu, Eyüp Yıldız, Oktay Akyürek, Kerem Kaya, Deniz Uras, Ebru Yılmaz, Cansu Gülerçin, Lila Sönmez, Ayşe Üstün, and Eda Olcaytuğ in my life.

As the latest and most significant gratitude, grateful, thankful feelings belong to my parents Hurşit Kanbağlı and Funda Kanbağlı, my grandmother Yıldız Tekin, my brother Caner Y. Kanbağlı, my grandparents Zeki M. Kanbağlı and Nevzat Kanbağlı, my uncle Erdal Kanbağlı. I can never pay my debt for my mother and my grandmother, who struggled with all difficulties and people in our life, encouraged me, provided a spiritual support also wanted to create me, a man who will be a role model for his country and will make the world a better place for humanity through science.

ABSTRACT

INVESTIGATION OF WARBURG EFFECT IN MAMMALIAN CANCER CELL LINES BY STUDYING VARIOUS METABOLIC ENZYMES AND ASSAYS

Tumor formations are the cause of the cancer death that constitutes second highest mortality rate with 21.3 per cent, published by TUIK in 04.01.2014. Studies have revealed that cancer cells alter their metabolic pathways including intrinsic and extrinsic pathways, also protein structures that initiate the 'molecular-protocol' of silencing to block several pathways, most commonly glycolytic, oxidative phosphorylation and apoptotic pathways, specifically, Caspase-9 (Cas-9) and Caspase-3 (Cas-3) are the molecular lynchpins. In this phenomenon, ATP synthesis has a vital role upon glycolytic pathway, which has recorded to be significantly preferred independently from oxygen levels by cancer cells to reach breakeven point with diminishing TCA cycle, and to generate abundant amount of ATP, in terms of energy requirement, also known as Warburg effect. In glycolysis, Glucose transporters (GLUTs), specifically GLUT4, are active structures responsible for glucose intake, hence, profiling differential expression level of this structure in proteomic prospects, considering various cell types, was the purpose of this study, as per to our means, tumor suppressor protein p53, crucial tumor suppressor and a proved GLUT4 down-regulator, additionally, respiratory chain (RC) complexes, in ATP synthesis by oxidative respiration of glucose structures, have investigated. Besides, Complex IV, which holds a significant role in electron transfer chain by reducing oxygen into the water molecules, was investigated. Immunoblotting assays performed against ETC complexes, GLUT4, Casp-3, Casp-9, p53 in PNT1A, DU 145, HeLa, Hep 3B, HEK 293T and SH-SY5Y cell lines to investigate variations under normoxic condition. ATP Synthase Activity Assay was applied to measure Complex V activity levels, and Rhodamine123 (Rh123) dye was applied to compare mitochondrial membrane potential with corresponding cell lines.

ÖZET

WARBURG ETKİSİNİN MEMELİ KANSER HÜCRE HATLARI ÜZERİNDEN ÇEŞİTLİ METABOLİK ENZİM VE TAHLİLLER İLE ARAŞTIRILMASI

TUİK'in 01.04.2014 tarihli raporunda açıkladığı üzere, tümor oluşumları ölüm oranlarında yüzde 21,3 ile en yüksek ikinci orana sahip olmakla birlikte; daha önce yapılmış çalışmalarda kanser hücreleri; iç ve dış metabolik yolaklarına ek olarak sinyal yolaklarından bazılarını azaltıcı veya komple etkisiz hale getirecek yönde görevli olan protein yapılarını değiştirebilme özelliklerine sahip olmakla birlikte, spesifik olarak glikolitik, oksidatif fosforilasyon ve apoptotik yolakları ile apoptozda yer alan Kaspaz-9 (Kasp-9) ve Kaspaz-3 (Kasp-3) yapılarını ve yolaklarını değiştirebilmektedirler. ATP sentezi, iki yolakta oldukça önemli rollere sahip olmakla birlikte, bunlardan birincisi olan glikolitik yolağın, oksijen varlığından bağımsız olarak, azalan TCA döngüsünün ürettiği enerji açığını kapatmak ve ATP üretmek için kanser hücreleri tarafından yoğunlukla tercih edilmesi durumu, Warburg etkisi olarak açıklanmıştır. Glikoz taşıyıcıları (GLUTs), özellikle GLUT4, glikoliz yolağında aktif bir şekilde glikozun hücre içerisine taşınmasında rol oynayan ve ayrıca farklı hücre hatlarında farklı ekpresyon seviyelerine sahip olduğunun gösterilmesi amaç edinilmiştir; tümor ve GLUT4 baskılayıcısı olarak da bilinen, tümor proteini p53 (TP53), araştırılmıştır. Ayrıca, glikoz moleküllerinin oksijenli solunum yoluyla ATP üretiminden başlıca sorumlu olan OXPHOS kompleksleri araştırılan önemli moleküllerden biridir. Ayrıca, elektron taşıma sisteminde oksijenin indirgenerek, su moleküllerinin üretiminden sorumlu olduğu için, kompleks IV molekülü araştırılmıştır. İmmün-emdirim analizleri, OXPHOS kompleksleri, GLUT4, Casp-3, Casp-9, p53 ile normal oksijen seviyesinde büyütülen PNT1A, DU 145, HeLa, Hep 3B, HEK 293T ve SH-SY5Y hücre hatlarında araştırılmıştır. ATP Sentaz Aktivite Analizi, Kompleks V aktivite seviyelerinin ölçülmesi ve Rhodamine123 (Rh123) boyası ise mitokondriyal zar potansiyel farklarının karşılaştırılması için kullanmıştır.

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α-KG	Alpha-ketoglutamate
µl	Microliter
Δψ	Electric otential, mitochondrial membrane potential
AKT	Serine/threonine kinase
AMPK	AMP-active protein kinase
APAF	Apoptotic protease activating factor
APS	Ammonium persulfate
ARF	Alternative reading frame
ATCC	American type cell collection
ATP	Adenosine triphosphate
ATM	Ataxia telangiectasia mutated
ATR	ATM and Rad3-related
BAK	BCL-2 antagonist killer 1
BAX	BCL-2 associated X protein
BCA	Bicinchoninic acid
BCL-X_L	BCL-2 related protein, long isoform
BCL-2	B-cell lymphoma protein 2
BID	BH3-interacting domain death agonist
BSA	Bovine serum albumin
CAD	Caspase-activated DNAse
CASP	Caspase

CD95	Fatty acid synthase receptor
DIC	Deacetylation inhibition cocktail
DISC	Death-inducing signaling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DR5	Death receptor 5
EDTA	Ethylenediaminetetraacetic acid
ETC	Electron transport chain
FBS	Heat inactivated fetal bovine serum
GLUT	Glucose transporters
GLN	Glutamine
GLS	Glutaminase
GLU	Glutamate
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HMIT	H^+ / myo-inositol transporter
HtrA2/Omi	Serine protease
LC/MS	Liquid chromatography / mass spectrometry
ICAD	Inhibitor of caspase-activated DNAse
IKK	IκB kinase
IMM	Inner mitochondrial membrane

MAB	Monoclonal antibody
M	Molar
ml	Milliliter
mM	Millimolar
MP	Membrane potential
MPTP	Mitochondrial permeability transition pore
MS	Mass spectrometry
Na_3VO_4	Sodium orthovanadate
NF-κB	Nuclear factor kappa B
OMM	Outer mitochondrial membrane
p53	Tumor suppressor protein
PFK	Phosphofructokinase
PI	Protease Inhibitor
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethanesulfonylfluoride
PTEN	Phosphate and tensin homolog deleted on chromosome 10
PUMA	BCL-2 binding component 3
PVDF	Immune-Blot Polyvinylidene difluoride
P/S	Penicillin-streptomycin
RC	Respiratory chain
RH123	Rhodamine123
RIPA	Radio-immunoprecipitation assay

RSLC	Rapid separation liquid chromatography
ROS	Reactive oxygen species
RPMI-1640	Roswell park memorial institute 1640 medium
SCO2	Cytochrome *c* oxidase 2
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SGLT	Sodium-dependent glucose transporters
SLC2A	Solute carrier 2A gene family
Smac/DIABLO	Second mitochondria-derived activator of caspase
TBID	Truncated Bid
TEMED	N,N,N′,N′-Tetramethylethylenediamine
TIGAR	Tp53-induced glycolysis and apoptosis regulator
TNF	Tumor necrosis factor
TRAIL	TNR-related apoptosis-inducing ligand
XIAP	X-linked inhibitor of apoptosis protein

1. INTRODUCTION

More than a century ago, in 1861, Louis Pasteur proved “Pasteur effect” via experimenting yeast in both in normoxic and hypoxic environments and determined that they had grown more in oxygen available conditions in contrast to hypoxic ones. A couple of decades later, in the 1890s, Richard Altmann and Carl Benda highlighted an organelle, mitochondria, which also known as “powerhouse of the cell” [1]. Later, in 1913, another research by Otto Warburg documented that, granules obtained from guinea pig liver extracts, upregulate iron-dependent enzymatic activity and influence oxygen transportation [2]. Keilin, Hartree, Weinhouse, Hogeboom, Krebs and numerous scientists contributed to the identification of complex function and structure mitochondria. Nevertheless, Warburg stated that cancer cells, even if healthy mitochondrial structures and normoxic conditions provided; perform an excessive amount of glycolysis, in other words, fermentation of glucose into lactate or “aerobic glycolysis”, to generate energy requirement [1,2].

The aim of this study is to indicate biochemical interactions, depending on glycolytic, apoptotic, oxidative phosphorylation pathways and activity assay and mitochondrial membrane potential, between different healthy and cancer cell lines may vary from each other, with respect to Warburg effect.

1.1. CANCER

Cancer is a very crucial, lethal and cureless disease that can arise in all tissues and cells of the body. The major characteristics of this disease are capability of proliferating ceaselessly and rapidly expanding upon other cells, tissues also entire body. Normally, healthy cells divide, grow and form into required cell type in the body and when these cells are aged or damaged, they die and were replaced by new cells. Since cancer cells can grow infinitely, they do not die even if they have injured.

Up to now, many of the seminal studies revealed that cancer cells can alter their numerous cellular functions and unlike normal cells, they can continue to grow even in the extreme or inefficient conditions. In 2000 and 2011, two of the seminal papers, Hallmarks of Cancer and Hallmarks of Cancer: The Next Generation, were published by Hanahan and Weinberg and shed light upon cancer biology to understand and increase knowledge about this disease. These scientists determined that tumors have ten different capabilities to divide and grow continuously (Figure 1.1) [3,4].

1.1.1. The Hallmarks of Cancer

The first hallmark about cancer biology indicates that these cells have an ability to maintain their proliferation without any influences or effects. Healthy cells have a strict control mechanism upon growth-promoting signals, which render cells to grow and divide via cell cycle, to provide a homeostasis of cell number with healthy and functional cellular activities. On the other hand, cancer cells modify this mechanism to induce unlimited cell cycle and growth via pairing growth signals up with specific cell-surface receptors that consist of intracellular tyrosine kinase domains. The origin of these signals, their initiator effect upon mitogenic signals, and activity levels, and durations upon dispersing tissue types remain crucially unclear in healthy cells. However, tumor cells may continuously divide, via expressing similar receptors that consist of intracellular domains, by dictating neighbor healthy cells to generate and translocate these signals to them via cancer stroma [5-10]. In addition, cancer cells can also become extremely sensitive to growth signals, due to the rate-limiting ligand formations, by over-expressing cell surface receptors, also

conformational changes within these receptors, induce cell cycle, unrestrictedly from growth signals, and contribute high sensitivity for growth signal too.

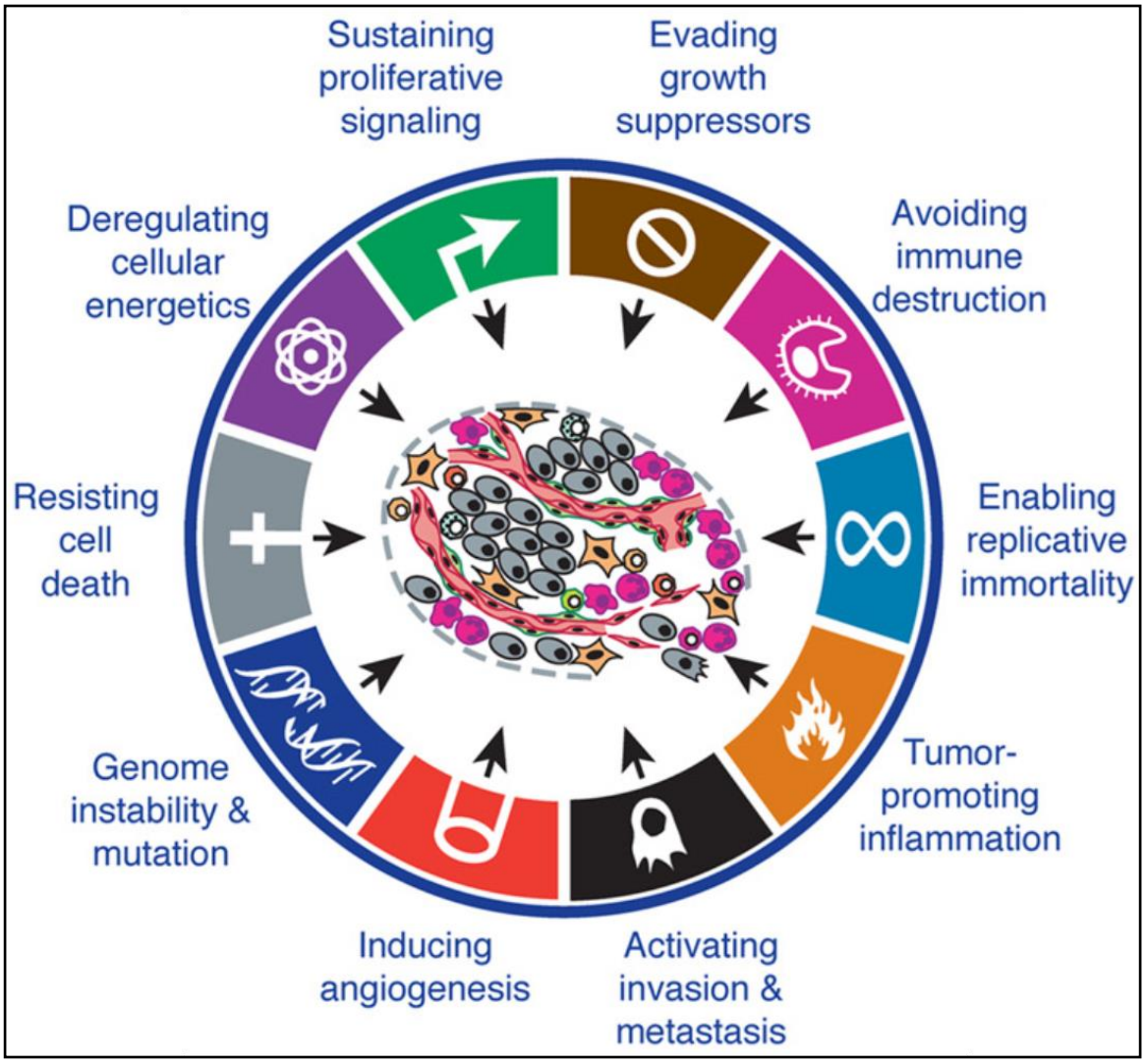

Figure 1. 1. Ten hallmarks of cancer documented by Douglas and Hanahan [4].

Many of the cancer cells, and nearly 40 per cent of the human melanomas, determined with the somatic mutations that lead conformational changes in B-Raf protein and lead cancers to activate mitogen-activated protein (MAP)-kinase pathway. Besides, phosphoinositide 3-kinase (PI3-K), and Akt/PKB are two of the excessively activated molecules with respect to somatic mutations [11-13]. Additionally, the negative-feedback mechanism also contributes to the maintenance of intrinsic cellular activities depending upon growth factor, and unlike normal cells, cancer cells capable of deregulation of this mechanism via

mutating *Ras* genes, which alter the activity of Ras GTPase, and responsible for temporary growth signal activation. In human cancers, healthy PTEN phosphatase deteriorates phosphatidylinositol produced by PI3-K, but methylated promoter of PTEN phosphatase, result in PI3-K enhancement and induces tumor formations [11,12]. Same as PTEN, mTOR kinase has a down-regulatory effect on PI3-K and Akt/PKB, and suppression render cells to tumorigenesis, which also mediates both cellular metabolism and dividing depending on PI3-K [14,15].

Previous studies stated that overexpression of oncogenes and proteins provide an enhancement in division and growth in tumors. However, a couple of years ago, several studies disproved that, Ras, Myc, and Raf oncoproteins can stimulate diversely upon cell death and/or aging mechanisms [16,17]. For instance, senescence of the cells may arise via elevated Ras oncoproteins, and cells may evade from aging and continue to divide via expressing the low amount of Ras oncoprotein. Moreover, various tumors can circumvent their apoptotic and/or aging mechanisms to survive under extreme oncogenic stimulants.

The second hallmark about cancer biology depends on activities of tumor suppressor genes and proteins, RB (retinoblastoma-associated) and p53 (TP53). These proteins mediate two major cellular mechanisms in the cell, which render cell to grow and divide, or initiate cell death (apoptosis) and aging (senescence). Retinoblastoma protein mediates proliferation by assembling both intracellular and extracellular signal and decides continuation of the cell cycle [18-20]. Mutations in RB proteins, enable limitless proliferation via cell cycle within the cancer cells. RB controls and translocates downregulatory growth signals from extracellular parts into the cell. TP53 (p53) regulates intracellular pathways about cell cycle, such as stopping cell cycle under insufficient levels of growth stimulants, oxygen, glucose, and nucleotide amount also irreparable defects in the genome. Additionally, p53 can also initiate apoptosis within the excessive amount of damage that impairs cellular activities.

Another tumor suppressor expressed from *NF2* gene, Merlin, modulates ligand formation between transmembrane receptor tyrosine kinases (EGF receptors) with cell-surface adhesion molecules (E-cadherins), reinforces cell-to-cell bindings modulated by cadherins, and prevent the release of the mitogenic signals by hindering growth factor receptor. Human neurofibromatosis arises with *NF2* mutations [21,22].

Epithelial polarity protein, LKB1, contributes intact tissue formation and coordinates epithelial configurations. Loss of LBK1, determined within many of human malignancies, and lead conformational changes via *Myc* oncogene, also overexpressed LKB1 in healthy cells, can obstruct *Myc*-induced mitogenic signals [23-25].

TGF-β is well known about extreme complexity and antiproliferative signaling pathway features. Together with these, this pathway deregulated to prevent inhibitory effects on cell division and cell growth by lethal tumors, also it initiates epithelial-to-mesenchymal transition (EMT) mechanism which highlights highly malignant tumor features [26-28].

The third hallmark of cancer biology relies on evading from cell death pathway. Initiation of programmed cell death, apoptosis, depending on not only extrinsic and intrinsic signaling but also can be triggered via various stress factors, as mentioned in the previous hallmark. This mechanism consists of numerous regulators and molecules that can stimulate both downstream and upstream pathways. Extrinsic and intrinsic pathways are two of the major classes of apoptotic machinery and each one of the pathway includes different caspases that lead execution of the cell. Stimulants that induce apoptosis, mediated via antiapoptotic proteins, which are Bcl-2 family members Bcl-2, Bcl-XL, Bcl-w, MCL-1, A1, that bind and repress the activity of the proapoptotic inducers, and proapoptotic regulatory Bax, Bak proteins that translocate in the outer mitochondrial membrane. If Bax and Bak recover from downregulation, they brake-down mitochondrial membrane structure and release cytochrome *c* and many other proapoptotic signaling structures to activate a caspase-mediated cascade of cell death. Besides, Bax and Bak have a same BH-3 domain, which is a protein-protein interaction motif, with Bcl-2 family members that regulate their formations also BH-3 containing proteins, known as BH-3-only proteins, related in dispersing abnormalities and capable of acting via Bcl-2 family member antiapoptotic or proapoptotic proteins [16,29,30]. There are numerous unidentified factors and signals that can induce apoptotic pathway, but a couple of them detected, such as tumor suppressor protein p53 which regulates cell cycle, often controls DNA damage, and expresses Noxa and Puma BH-3-only proteins in the case of crucial DNA damage and chromosomal defects. Lack of interleukin-3 (IL-3), insulin-like growth factor1/2 (Igf1/2), and some other survival factor stimulants can trigger apoptosis via BH-3-only family protein, Bim. Myc and a couple of specific oncoproteins also initiate cell death via excessive signaling, unless antiapoptotic proteins counteract [16,31]. Since p53

defect does not trigger apoptosis in cancer cells, this situation leads us to wonder that tumors may also downregulate proapoptotic proteins or upregulate antiapoptotic family members or survival factor signals.

Autophagy is another form of cell death, that activated under the requirement of energy, and biosynthesis, also hyperactive under stressed conditions and deprived nutrients to maintain cell survival via autophagosomes and lysosomes, where execution induces. Usually, autophagosomes and lysosomes degrade small structures but they can destroy organelles too. This form of cell death mechanism, consists of numerous regulators and molecules that can stimulate both downstream and upstream pathways which coordinate autophagosome generation and transportation to lysosomes, same as apoptosis [32,33]. Recently, a study indicated that there is a strong correlation between apoptosis and autophagy, additively with cellular homeostases. Autophagy and apoptosis can be downregulated due to the suppressive effect of Akt, mTOR kinases, and PI3-K via survival factors but also they may initiate if the survival stimulants inhibited [33,34,35]. Besides, Beclin-1 is another BH-3-only family protein which binds with Bcl-2 and Bcl-XL same as other family members, also capable of inducing autophagy and apoptosis via liberating Bak and Bax, when stress-sensor-coupled BH-3 family proteins dislocate Beclin-1. Correlation between autophagy and apoptosis remains unknown but both cell death mechanisms inhibit tumorigenesis with or without correlating each other. Besides, not only genetic factors but also environmental, and stress conditions contribute to the effectiveness of autophagy mechanism upon cancer cells such as TGF-β, which is repressing and upregulating tumors depending on cellular stage [34,36-39].

Necrosis is an uncontrolled form of cell death that genetically mediated under certain conditions. Necrotic death liberates cellular structures and various stimulant factors, such as IL-1α, unlike apoptosis and autophagy, to assemble inflammatory cells which clean cellular debris but these factors, and inflammatory cells do not downregulate tumorigenesis, on the contrary, they enhance tumorigenesis, invasiveness, and metastasis [40-43].

The fourth hallmark of cancer biology highlights the immortalization and the regenerative modifications in cancer cells, during the cell growth and cell divisions. If cells can eliminate senescence mechanism and recover from crisis phase, which latter cell death in

most of the cases, they regenerate with a limitless replicative potential and become immortal. Limitless proliferation, mostly rely on telomeres which provides reassurance for the three prime (3'), and five prime (5') ends of chromosomal DNAs and consists of multiple tandem hexanucleotides repeats [44,45]. They gradually dwindle and become unable to prevent end-to-end DNA ligation, which alters cellular homeostasis. Unique DNA polymerase, Telomerase, continuously ligates telomere sequences to 3', and 5' ends and often lack in healthy cells. On the other hand, human tumors and various immortal cells overexpress this enzyme to prevent senescence mechanism, inhibit crisis phase, and/or apoptotic pathway. Studies revealed that very low percentage of the *in vitro* cultured cells can postpone senescence, survive until crisis phase and apoptotic pathways were induced, but telomerase-inhibited mice model, demonstrated precancerous cells upregulate senescence mechanism via downregulating tumorigenesis in genetically engineered mice which expected to regenerate multiple cancers [46-50]. Senescence mechanism, contribute to repress tumor growth and can be induced via dwindled telomeres or excessive oncogenic stimulants. Additively, studies upon precancerous cells revealed that, application of fluorescent *in situ* hybridization (FISH) technique highlighted telomere restriction, crisis phase, and end-to-end DNA ligations, due to the incapability of telomerase activation in the early stages of tumor formation. Thus, immortal cells also experience telomere dwindling, and telomere-dependent reactions before their transformations [51,52]. Inhibition of p53-dependent control mechanism upon genome stability, render premalignant cells to evade from telomere degradation and chromosomal breakage-fusion-bridge (BFB) cycles. Suppressed telomere activity enhances tumorigenesis independently from p53 and telomerase proteins in mice models [50]. Additively, human breast cancer cells and premalignant formations of human breast, determined with the overexpression and suppression of the telomerase, respectively [53,54]. However, a study documented that, telomerase contributes proliferation independently from telomere ligation, and unknown features of telomerase and its protein subunit TERT, had been demonstrated partially by both mice models and *in vitro* cell culture [55]. Apart from telomere ligation via TERT/telomerase, TERT functions as a cofactor stimulus of β-catenin/LEF transcription factor receptor, and forms a secondary signal, which activates WNT pathway, and lead hyperactive signaling. In addition, TERT is capable of binding with both chromatin upon the chromosomes and telomeres, thus not only telomerase but also TERT provide a homeostasis for telomere [56,57]. Upregulation

of cellular division and growth and inhibition of cell death [58], contribution to damaged DNA recovery [56], and RNA-mediated RNA polymerase activity [59] are the recently highlighted features of TERT/telomerase structures.

The fifth hallmark of cancer biology demonstrates the requisites of excretion of cellular wastes, carbon dioxide, and intake of supplementary molecules, glucose, oxygen, and other nutrition, within both healthy and cancer generation, impartially. Generation of vessels (angiogenesis), endothelial cells, and their aggregation inside the vessels (vasculogenesis) contributes reconstruction of the vascular system, in the early stages of embryonic development. Healthy cells suppress angiogenesis yet, the mechanism can be reactivated, until healing damaged tissues or scars, but cancer cells deregulate this process and keep "angiogenic switch" permanently through tumorigenesis, to maintain and sprawl tumor formations, and intake nutrition via angiogenesis. This mechanism is modulated by many initiator proteins, vascular endothelial growth factor-A, and inhibitor proteins, such as thrombospondin-1 (TSP-1), which binds to extracellular receptors that formed by vascular endothelial cells [60,61]. Homeostasis in the adult endothelial cell, and angiogenesis in embryonic phases are dictated via specific extracellular ligands, that expressed by *VEGF-A* gene. This complex mechanism coordinated by three disparate receptor tyrosine kinases (VEGFR-1, -2, -3) [62-64]. Besides, these ligands can activate ECM-degrading proteases, such as MMP-9, to initiate ECM degeneration [65]. The permanent activity of angiogenesis in cancer cells renders on proangiogenic signals, fibroblast growth factor (FGF) family members [61]. TSP-1, multifaceted angiogenic inhibitor, induces "angiogenic switch", and reactivates proangiogenic repressor stimulants via binding endothelial transmembrane receptors [66]. Blood leakage, excessive growth of endothelial cells, damaged vessels, immature capillary formation, irregular blood circulation, and uncoordinated vessel spread, highlights only a small part of the angiogenic features in cancer cells [67,68]. Additively, in situ carcinomas, precancerous cells, and noninvasive cancers determined with modified angiogenesis, such as continuous "angiogenic switch" [69,70]. One way or another, many of the cancer cells display angiogenic activity, independently from vascularization and/or intensity levels of this mechanism. Interestingly, promoter genes, structures, and/or factors can directly or obliquely modulate disparate hallmarks of cancers, such as activation of angiogenesis concomitantly with proliferative signaling supervised by oncogenes [60,61,71,72]. Aside from TSP-1,

endostatin and angiostatin, which are a type-18 collagen and a subunit of plasmin, are two other demonstrated inhibitor structures [73-75,66,76]. In addition, pericytes, support mechanically and physiologically endothelial cells in normal cells, in contrast, tumors usually observed with the deficient pericytes [77,78] and different cells that generated from bone marrows, may also contribute to enabling permanent angiogenic switch in many of the tumors [79].

The sixth hallmark of cancer biology stands on invasive behavior and metastatic activity of the cells. Shape and attachment between cells, also ECM is a crucial factor to survive. Most of the cancer cells usually downregulate and even inactivate E-cadherin, which normally coordinates adhesion of the cells, and highly malignant cancers upregulate N-cadherin, which acts in organogenesis in healthy cells, respectively [80,81]. Colonization of cancer cells begins with an easy invade, then spread into the vascular system and other tissues and cells, and finally, translocation onto disperse tissues [82,83]. Permanency, intensity and activity of a unique program, epithelial-mesenchymal transition (EMT), which known as an addition of new features to the modified epithelial cells to transform into cancer cells, modulated by the combinatory activation of several transcription factors, such as *Slug*, *Zeb1/2*, *Snail* and *Twist*, to increase apoptotic resistance, motility and all invasive and metastatic activity [84-88]. Numerous uncertainties remain unknown about these factors but highly invasive cancers determined with the EMT [89]. Besides, cancer cells can upregulate their malignancy and invasion feature via CCL5, which are released from tumors themselves that consist of CCL5/RANTES, expressed by mesenchymal stem cells (MSCs) [90]. Besides, cancer cells rarely perform the mesenchymal-epithelial transition (MET), a reverse EMT, to keep and increase their invasive characteristic when translocated into far tissue from primary tumor [91] and tumors can degrade ECM themselves via specific enzymes that are expressed by inflammatory cells or assemble pro-invasive inflammatory cells which degrade ECM [65,92,93].

The seventh hallmark of cancer biology strongly correlated with genome instability which can be caused by mutations. Alterations within the genome, such as inhibition of tumor repressor genes, contribute numerous traits and even can be inherited into sub clones of tumors via histone modifications and many other epigenetic processes [94-96]. Like p53, "*caretaker*" genes conserve genome and maintain DNA under control via inducing repair mechanism due to the determined DNA defect, recover DNA damage by themselves and

inhibiting mutant signals before DNA hurt [97-102] also these genes act very much alike tumor suppressor ones, and altered or mutated forms usually indicated in tumorigenesis [103]. In addition, comparative genomic hybridization (CGH) reveals genomic modifications, deletions, and achievements, and damaged control mechanisms within cancer genome [104] which differs from many distinct cancer cells.

The eighth hallmark of cancer biology points through the relationship between inflammatory cells and its up-regulatory effect upon tumorigenesis through multiple hallmarks, such as generation of survival, proangiogenic, and growth signals, and ECM-degrading enzymes, and liberation of various chemicals, such as reactive oxygen species (ROS) [43,90,93,105].

The ninth hallmark of cancer biology recapitulates the alterations in the energy generating processes, even in the appropriate environmental conditions, such as oxygen levels, nutrition, and/or pH value. Healthy cells generate ATP from two sequential procedures, glycolysis, and oxidative phosphorylation, but cancer cells usually prefer to perform glycolytic pathway, in massive [106-108]. Cancer cells enhance and support this reaction via increasing GLUTs and activating *Ras*, *Myc*, and other oncogenes. They also maintain damaged p53 in addition to the repressing, altering and/or activating oxidative phosphorylation for their own benefits [109,110]. Additionally, hypoxia render cells to generate ATP mostly from glycolysis via enhanced expression and activity of GLUTs and many other glycolytic enzymes, and increased HIF1α and HIF2α [109,110,111,112,113]. Besides, modified angiogenesis is the major cause of the various hypoxic conditions in the disperse cancer tissues [114]. Two specific isoforms of isocitrate dehydrogenase-1 and -2 (IDH-1, -2) enzymes can redirect energy generation in many cancer cells, including glioma [115]. Modifications in energy generation processes and their relation to other pathways increase the significance to determine unresolved hallmarks of cancer, depending on metabolic reactions.

The tenth hallmark of cancer biology demonstrates the mostly unidentified correlation between tumorigenesis and downregulatory features of the immune system. Generally, immune system participates in the execution of abnormalities and unusual growth of the healthy cells but cancer cells, especially solid forms of tumors, can evade or obviate immunologic responses and survive. However, several human cancers, colon, and ovarian

forms do not deregulate or obviate $CD8^+$ cytotoxic T lymphocyte (CTL) and natural killer cell (NK) mediated immune systems and their responses when excessively infiltrated with these anti-tumoral structures [116-118]. Additively, cancer cells may inhibit the CTLs and NK cells via TGF-β signals and/or many other immune-repressor stimulants [119,120] and downregulate cytotoxic lymphocytes via dictating myeloid-derived suppressor cells (MDSCs) and regulatory T cells (Tregs) [121,122]. As documented above, many studies contributed to increasing relation between tumorigenesis and inhibitory regulation of immune system but there are many unresolved factors remain unclear not only about immune system but also cross-talk between other hallmarks.

1.1.2. Several Cancer Types Employed in this Study

Prostate cancer is the fifth most lethal in the world, and the second most common cancer type among the men [123]. This cancer displays huge complexity and varies among oncogene expression, ploidy, biological features, genetics, and molecular pathways. Development of prostate cancer induces with the enzymatic activity of 5α-reductase, to form 5α-dihydrotestosterone from testosterone. This form of prostate cancer known as androgen dependent (AD) and may react with androgen, but the presence of androgen is not a necessity for the proliferation of androgen sensitive (AS) prostate cancer.

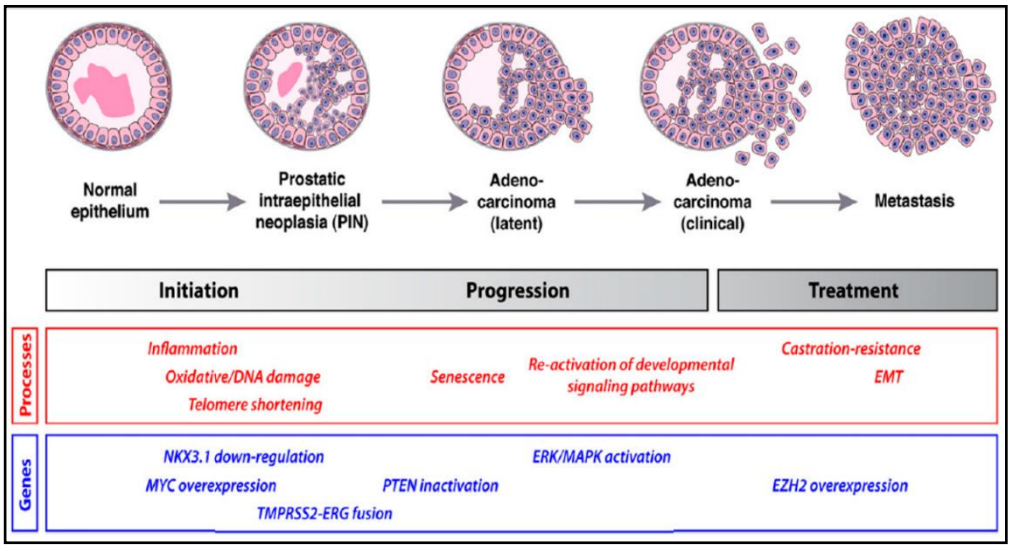

Figure 1. 2. Development of prostate cancer stages [124].

Besides, deletion and mutation of *PTEN/MMAC1* gene, which is a tumor suppressor gene in the 10q23.3 chromosome and has a downregulatory effect upon phosphoinositide 3-kinase (PI3-K)/Akt pathway, is the major cause of the prostate cancer. Inhibition of *PTEN/MMAC1* initiates metabolic alterations including cellular transformations via activated Akt and PI3-K. Finally, a mutation in a *p16* gene, which regulates cell cycle, was determined in DU 145 which was derived from the metastatic site of the brain [125,126].

Cervical cancer (CC) is the third fatal and the second highest diagnosed level among women in underdeveloped nations [123], also commonly arise women, who aged between 25 to 55, and strongly correlated with infection of human papillomavirus (HPV), which enhances invasive and malignant characteristics. Smoking, birth, age, and many factors contribute generation of the disease. HPV activates oncogenes and inhibits tumor suppressor genes to increase tumorigenesis. Cyclooxygenase-2 (COX-2), integral membrane protein (CD44), matrix metalloproteinase-9 (MMP-9), and immunosuppressive acidic protein (IAP) are some of the overexpressed molecules which enhance angiogenesis, proliferation, metastatic features, also COX-2 regulates apoptosis, cell cycle, degradation of ECM in CC [127]. HeLa is a member of human cervical cancer also the first obtained cancer cell line from cervical adenocarcinoma in 1952 and this cell line display overexpression of *c-Myc* oncogene [128].

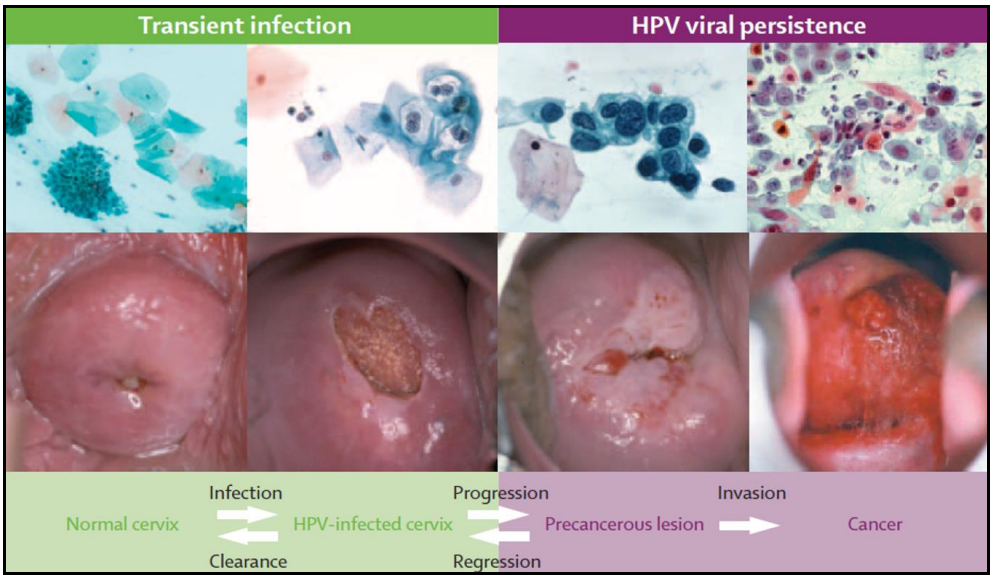

Figure 1. 3. Evolutionary stages of cervical carcinoma [129].

Hepatocellular Carcinoma (HCC) makes up 5.4 per cent of cancers worldwide. The peak incidence in Western populations is after 60 years of age, majoritarian on cirrhosis and significantly more frequent in men. 85% of HCC cases occur in regions where chronic HBV is epidemic (Subsaharan Africa, Korea, southeast China, and Taiwan). With an early onset as carrier ship, the most affected age group is 20-40. Half of these cases show the concomitant development of cirrhosis and the chance of survival is two years most. Besides, enlargement of the primary mass reaches an extent which depresses the normal liver functions. At the onset of HCC, which is a chronic liver disease, HBV, HCV, aflatoxin, alcohol or the synergy of these factors causes different states of chronic inflammation that triggers cytokine release and increases the number of regenerations thus allowing many mutations. The inflammatory cytokine IL-6 initiates JAK/STAT signaling but also acts as the regulator of HNF4-α as shown in animal studies. *p53* is suppressed, while beta-catenin is induced. The HCC occurs by the invasion of blood vessels within the liver tissue, the inferior vena cava and the portal vein leading to portal hypertension. In the advanced stage, the same mechanism allows further spread via the hepatic vein to the periphery, mostly the lungs [123,130].

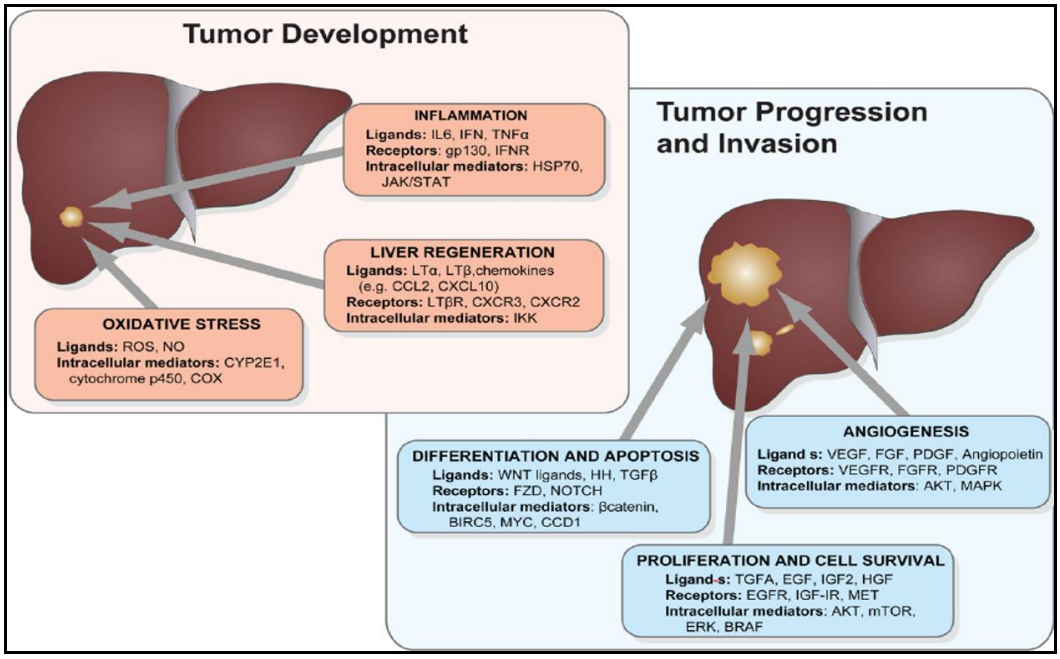

Figure 1. 4. Several factors that altered and/or inhibited by hepatocellular carcinoma phases [130].

Neuroblastoma is the major cause of the death and excessively develops in the children. This cancer can avoid multifaced treatments and forms as cranial solid tumor also contributes seven per cent of the mortality in patients aged above 15. Neuroblastoma can be diagnosed via elevated homovanillic acid (HVA), dopamine (DA), vanillylmandelic acid (VMA) levels and ratios of the DA/VMA and VMA/HVA. In addition, inheritance of neuroblastoma depends on mutated *Paired Homeobox 2b* (*PHOX2B*) and *anaplastic lymphoma kinase* (*ALK*) genes. Neurofibromin1 and tyrosine phosphatase *SHP2* (*PTPN11*) genes are also mutated. Besides, *PTPN11* moderates the regulation of growth factor signaling mechanism. Above all, development and proliferation of neuroblastoma mostly rely on the excessive expression of *MYCN* oncogene with respect to the recent studies [131,132].

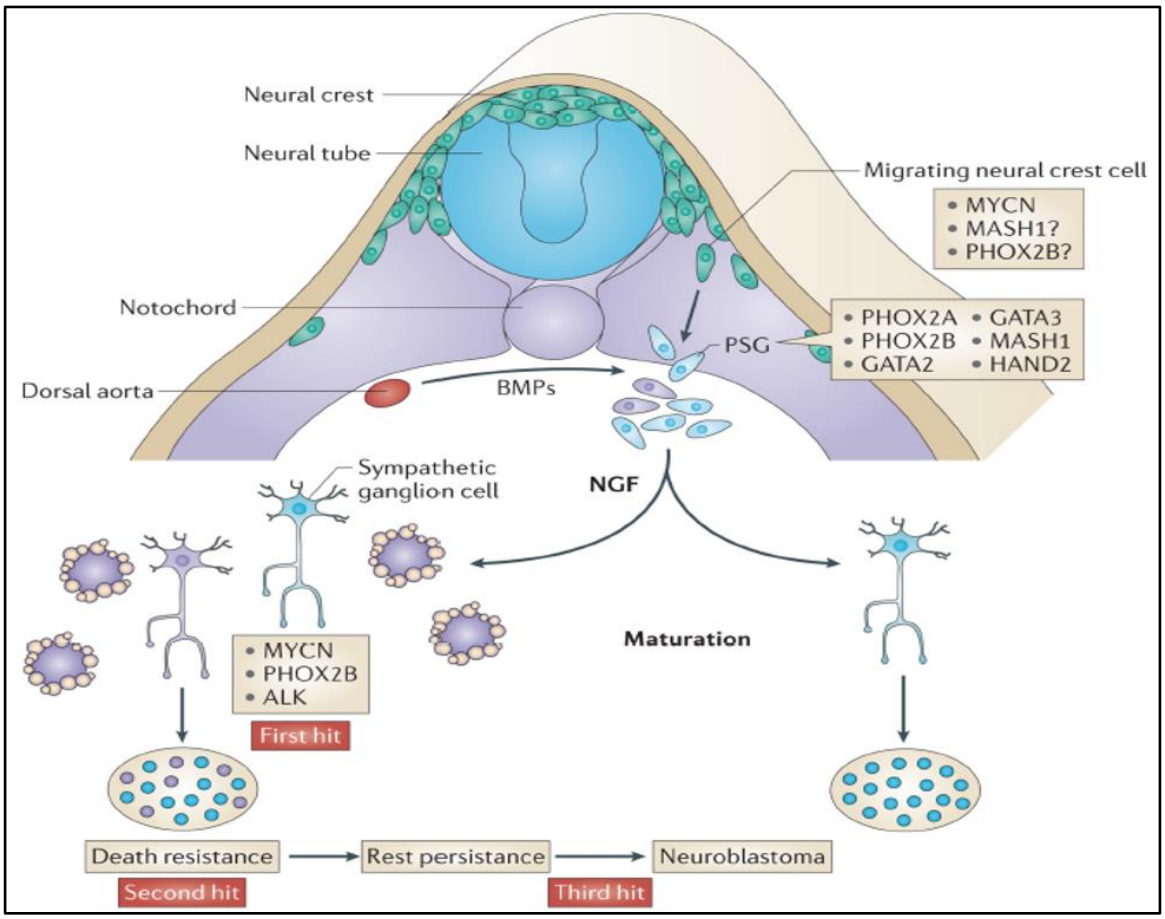

Figure 1. 5. Proliferation cascade of neuroblastoma from the neural crest [133].

1.1.3. Glucose Transporters (GLUTs)

Sugars, essentially glucose, and fructose, are the primarily depleted metabolic structures to generate energy. In addition, glucose is a very significant sugar in eukaryotic cells which contributes to glycogen, glycoprotein, and triglyceride production and metabolic energy production by oxidizing these molecules. Since glucose has a hydrophilic formation, it prevents their transportation into the cytosol due to the cell membrane's lipid bilayer and impermeable to hydrophobic structure. Facilitative glucose transporters (GLUTs) and sodium-dependent glucose transporters (SGLT) are two major hexose transporter families and consist of unique carrier proteins which regulate glucose uptake to produce energy, glucose-dependent complexes, and initiate many other processes mediated by this sugar [134,135]. However, these families differ in glucose intake. GLUTs act via concentration gradient system and independently from energy consumption, but SGLTs consume energy due to the sodium electrochemical gradient during the transportation of glucose [135,136].

Early steps of energy production begin with glucose intake and then glucose altered into pyruvate via glycolytic pathway. Later, pyruvate converted into acetyl-CoA and transferred and metabolized in mitochondria by oxidative phosphorylation and tricarboxylic acid cycle to generate ATP in healthy cells. Conversely, cancer cells cannot form sufficient amount ATP from mitochondrial pathways, thus, modify their metabolism and upregulate glucose intake and GLUT expression to obtain an excessive amount of ATP via glycolysis which also known as Warburg effect [137,138].

GLUTs are subfamily group of Major Facilitator Superfamily (MFS) membrane transporters and consist of approximately 500 amino acids. Until now, identified 14 GLUTs: GLUT1-12, GLUT14 and HMIT (H^+ /myo-inositol transporter), expressed by the *solute carrier 2A* (*SLC2A1-14*) family genes, and contain similar 12 transmembrane-spanning hydrophobic alpha-helices with intracellular carboxyl and amino termini with maintained tryptophan and glycine residues (Figure 1.6). Besides, GLUTs categorized into three groups (Figure 1.7): class one (I) (GLUT1-4, GLUT14), class two (II) (GLUT5, GLUT7, GLUT9, GLUT11) and class three (III) (GLUT6, GLUT8, GLUT10, GLUT12, HMIT), due to the location of their long extracellular loop, which regulates glucose intake via forming between first and second transmembrane domain including a glycosylation site in class one and class two.

Transporter	Tissues	Roles and properties
	SGLT transporters	
SGLT1	Small intestine, kidney.	Intestinal absorption of glucose from meal. Renal reabsorption of glucose.
SGLT2	Kidney.	Renal absorption of glucose from glomerular filtrate.
	Class I GLUT transporters	
GLUT1	Erythrocytes, brain (blood-brain barrier).	Basal glucose uptake.
GLUT2	Liver, pancreatic islet cells, small intestine, kidney.	Glucose sensing in pancreatic β-cells. Trans-epithelial glucose and fructose transport. High-capacity, low-affinity glucose transporter.
GLUT3	Brain (neuronal), testis.	Glucose neural transporter.
GLUT4	Muscle, heart, adipose tissue.	Expressed in tissues with insulin-stimulated acute glucose transport. In response to insulin, it is translocated to plasma membrane.
GLUT14	Testis.	
	Class II GLUT transporters	
GLUT5	Small intestine, testis, muscle.	Only fructose transporter.
GLUT7	Intestine, testis, prostate.	
GLUT9	Liver, kidney.	
GLUT11	Heart, adipose tissue, kidney, placenta, muscle.	GLUT11 has three isoforms: GLUT11a, GLUT11b, and GLUT11c, with distinct tissue distribution.
	Class III GLUT transporters	
GLUT6	Brain, spleen, leukocytes.	
GLUT8	Brain, testis, adipocytes.	
GLUT10	Heart, lung, brain, liver, skeletal muscle, pancreas, placenta, and kidney.	Mutations in GLUT10 were associated with arterial tortuosity syndrome. GLUT10 deficiency is associated with the upregulation of TGFB pathway in Loeys-Dietz syndrome.
GLUT12	Placenta, adipose tissue, small intestine and skeletal muscle.	In skeletal muscle, it is translocated to plasma membrane in response to insulin, like GLUT4.
HMIT	Brain.	Myoinositol transporter.

Figure 1. 6. Tissue-of-origin and characteristics of GLUT family and a couple SGLT family members [134].

In class three (III), this binding occurs between transmembrane domain nine and 10. Development of this loop causes bidirectional movement of glucose inside the membrane. Although, GLUTs share same structure, they have a different activity, capacity, and quantity due to the origin of the tissue [136,139-142,143,144].

Above all class one GLUTs, GLUT4 is the only carrier protein that transports not only glucose but also dehydroascorbic acid and glucosamine. Under basal insulin amount, this transporter consists of two chains of internalization between intracellular sections and proteins. After insulin connected to the receptor; GLUT4 relocates into plasma transmembrane which is regulated via cytosolic, phosphatidic acid-binding motif that formed between second and third helices, thus glucose intake significantly upregulated.

In addition, insulin-independent AMP-active protein kinase (AMPK) activation may induce GLUT4 establishment [134,141]. A couple of studies revealed that GLUT4 usually produced by insulin-dependent tissues, skeletal muscle, heart, adipose tissues. GLUT4 performs most of the glucose uptake in fat and muscle tissues of the body also 90% of glucose transportation in rat adipocytes [145,146]. Additionally, the origin of the tissue, maturation stage and diet can affect expression levels of GLUT4. A research study determined that low amount of GLUT4 in the early formation of myoblasts was upregulated with cell fusion.

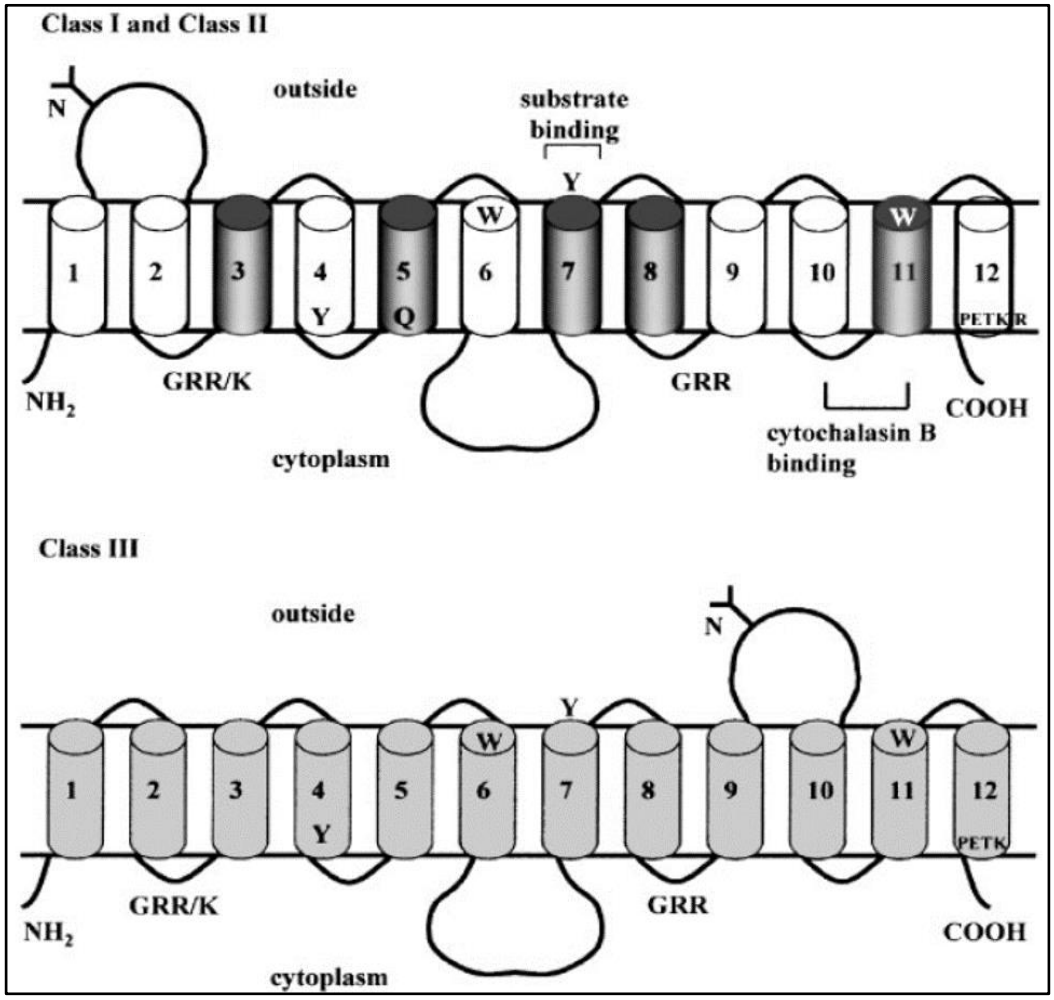

Figure 1. 7. All three classes of GLUT family members were shown with glycosylation (N) and transmembrane domain (TM) sites [136].

GLUT4 and GLUT1 have more common features than any other GLUT family members. Insulin-stimulated adipocytes and cancer cells express enhanced expression of GLUT1 [147]. Upregulation of serine/threonine kinase (Akt) in cancer cells has an initiative effect on both GLUT4 in adipocytes and GLUT1 in hematopoietic FL5.12 cells, to integrate them with transmembrane rapidly thus enhance glycolysis [148-151]. Nevertheless, repressive effect of *p53* on *GLUT* genes has been proven and both GLUT1 and GLUT4 transporters were transcriptionally down-regulated by wild-type *p53* in a tissue-specific manner [152,153].

1.1.4. Tumor Suppressor Protein TP53 (p53)

p53 (TP53), "guardian of the genome", is a tumor suppressor and transcription factor that manipulates numerous cellular events consisting cell proliferation, DNA repair, senescence, autophagy and apoptosis [154,155]. In addition, glycolysis and oxidative phosphorylation are the other significant pathways that are also controlled by p53. Ataxia telangiectasia mutated (ATM), and ATM and Rad3-related (ATR) are a couple of the cellular checkpoint kinases that phosphorylate p53, when irradiation, heat, drugs or diverse stimulants harm DNA structure. In normal cells, p53, often inspected via quick ubiquitin-dependent proteolysis by the murine double minute chromosome protein 2 (or E3 ubiquitin ligase; MDM2). However, phosphorylation of p53, repression of MDM2 and even post-translational modifications inhibit proteolysis and separate MDM2 and activate p53 [156-158]. Moreover, the cell remains in the checkpoint stage by p53 until DNA is repaired. However, if the damage is permanent, apoptosis is initiated by *E2F-1, MYC, RasV12* and other oncogenes which primarily activates Alternative Reading Frame (ARF) and then ARF segregates *MDM2* from *p53* [159].

The apoptotic pathway can be manipulated both transcriptionally and individually from gene transcription processes. Activation of Bid, Bax, PUMA, and many other pro-apoptotic proteins, by p53, plays key roles in both classes of the cell death pathways. Pro-apoptotic proteins, mediate mitochondrial (intrinsic) signaling pathway and enhancing expression levels of CD95 (Fas/Apo1) and DR5 receptors in death receptor (extrinsic) pathway to regulate extrinsic death stimulants. Moreover, Survivin, an anti-apoptotic protein, is another down-regulated structure by p53 [160,161].

In contrast to gene transcription manner, part of *p53* relocates itself upon mitochondrial membrane just after apoptotic signal arrives and activates Bcl-XL and/or Bcl-2 at the outer mitochondrial membrane (OMM) which leads to mitochondria to release Bid and Bax to induce their activity transcriptionally. Additionally, mitochondrial p53 also forms a complex with Bak via separating Mcl-1. Besides, not only mitochondrial p53 but also cytosolic p53 activates Bak, responsible for releasing cytochrome *c* and mitochondrial membrane permeabilization [162,163].

Energy metabolism is another p53 regulated mechanism in the cell. In normal cells, p53 represses glycolytic pathway and enhances oxidative phosphorylation pathway to generate more ATP from glucose [164]. Studies revealed that *p53* down-regulates GLUT1 and GLUT4, and phosphoglycerate mutase which transforms 3-phosphoglycerate into 2-phosphoglycerate [153,165]. Besides, GLUT3 is also obliquely suppressed by p53, via inhibition of nuclear factor kappa B (NF-κB) and IκB kinase (IKK) [152]. Conversely, p53-induced glycolysis and apoptosis regulator (TIGAR) levels are upregulated by p53 to decrease fructose-2,6-bisphosphate via activate phosphofructokinase (PFK) [166]. Moreover, *p53* enhances both *cytochrome c oxidase 2* (*SCO2*) expression via transcriptionally and phosphate and tensin homolog deleted on chromosome 10 (PTEN) expression, which suppresses phosphatidylinositol 3-kinase (PI3K) mechanism and glycolysis via down-regulating both HIF and *v-akt murine thymoma viral oncogene homolog 1* (*AKT1*) [166]. In addition, p53 also targets a catalytic enzyme, mitochondrial glutaminase (Glutaminase2, GLS2), that hydrolyzes glutamine (Gln) into glutamate (Glu) and then α-KG formed via deamination of Glu to generate more ATP in the TCA cycle [167,168].

Many disparate factors can cause inhibition, mutation and/or degradation of p53. Dysregulated p53 cause numerous disorders, metabolic alterations, tumor formations, genomic instability, anti-apoptotic function, immortality [169,170]. Cancer cells enhance glycolysis via overexpressing GLUT1, GLUT4, GLUT3, PFK, PGM molecules and activating PI3K via AKT and HIF; down-regulate mitochondrial respiration by inhibiting SCO2 and GLS2 structures. Besides, tumor cells become resistant to apoptosis and display genomic instability when p19 ARF inactivates p53. In addition, p53 is degradation determined in elevated levels of MDM2 or MDM4 [171,172].

1.2. MITOCHONDRIAL METABOLISM IN CANCER

Many of the studies have revealed that mitochondria generate a significant amount of energy. Aside from energy production, mitochondria tightly related to many diverse functions along with modulating cell death and cell signaling pathways; metabolite, reactive oxygen species (ROS) and biomolecule synthesis, also regulation of metabolism (Figure 1.8).

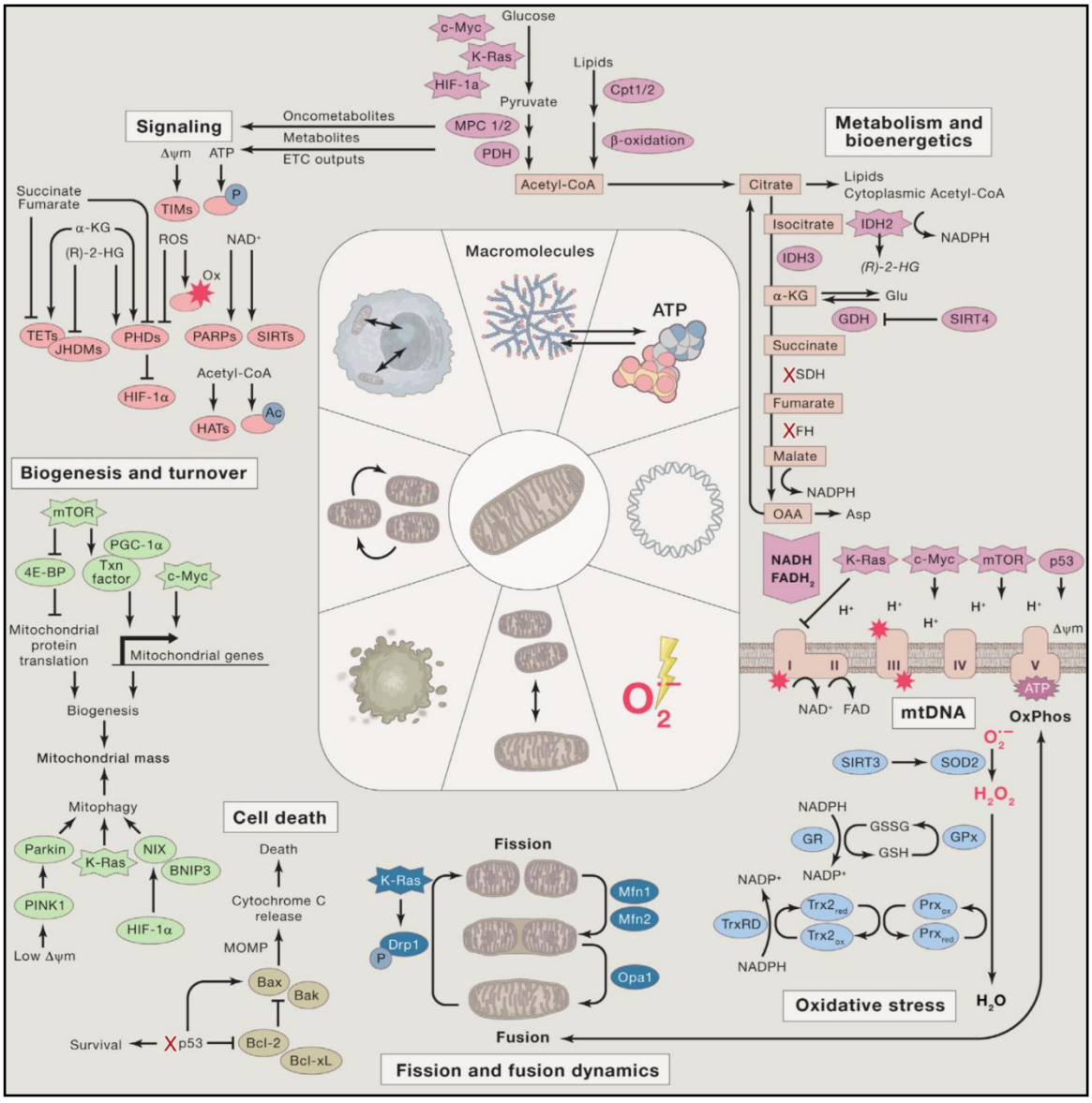

Figure 1. 8. Mitochondria-mediated pathways in cancer metabolism were illustrated [173].

Since it dictates and contributes to numerous reactions and interactions, healthy mitochondria have significant functions in adapting extreme conditions. Additionally, tumor cells do not affect from extreme conditions because of the conformational changes and functions in mitochondria. Moreover, not only environmental conditions but also genetic factors and tissue types play key roles in mitochondria.

1.2.1. Apoptotic Pathway

The programmed cell death, apoptosis, was first mentioned in a research paper that published by Kerr, Wyllie, and Curie in 1972 to define disparate pathway about cell death [174-176]. To keep cell populations under control, this process constantly performed during the growth, development and aging of the cells. Besides, apoptosis acts as a defense mechanism in cells when a mutation arises, an infection attack or cells damaged by extrinsic or intrinsic signals. There are few characteristics about apoptosis, disintegration of chromatin structures, diminution of cell, phagocytosis of deteriorating cells, contraction of cytoplasmic and nuclear parts are certain features of cellular self-destruction process [177,178]. In addition, fulfillment of this pathway relies on a couple of conditions such as the suitable amount of ATP and redox capacity consistent with caspase activation [179]. Despite the disparate signals, agents and cases, which can activate apoptosis, might not lead to the death of all these cells by cause of reciprocal of equal levels of agents or signals. Chemotherapeutic drugs or various irradiations, which are used in cancer treatments, result in mutated *p53* in cells leading to apoptotic cell death. On the other hand, different hormones, such as corticosteroids, can initiate apoptosis in specific or targeted cells, thymocytes, but leave others unharmed. Additionally, there are two main diversified conditions in between apoptosis and necrosis. In several conditions, intensity and/or kind of stimuli conclude the death pathway of the cell. Lessened levels of the harmful stimulants and agents such as drugs, irradiation, and heat can promote apoptotic cell death, while higher doses lead to necrosis in the cells. Moreover, apoptotic pathway is usually an organized and energy-dependent procedure by contrast with necrosis. To induce sequential events of apoptosis, many of cysteine proteases, also known as caspases (casp), needed to be activated. Moreover, chromatin condensation is another significant characteristic of apoptosis which also results in pyknosis [178].

Since apoptotic cells do not dispense their cellular components into other cells and tissues, and no inflammatory effect occurs because of the apoptotic pathway or apoptotic cells. Instead, neighbor cells rapidly phagocytosing them to prevent necrosis and anti-inflammatory cytokines do not generate by engulfing cells [180,181]. In some cases, these death pathways overlap in several biochemical networks identified as “apoptosis-necrosis continuum”, even when necrosis and apoptosis differ from each other with functioning and execution. Depletion of intracellular ATP molecules and down-regulated caspase structures are two of the significant features that modify continuing apoptosis into a necrosis [182-184]. However, specificity of the death signal, physiologic environment, developmental stage and origin of the tissue play a key role in both apoptotic and necrotic death pathways [182,185].

A great number of genes have been identified that are responsible for the extermination of the cells and engulfment of these apoptotic cells. More interestingly, molecular origin of death pathway has determined and evolutionarily protected by these genes. For a very long time, apoptosis has been accepted as an irrevocable protocol with the activation of caspases to lead cells to death, and engulfment genes to annihilate dead cells. However, excretion of dead cells by macrophages might play a key role not only clearance of cellular waste but also in unknown mechanisms. A research study by Hoeppner *et al.* revealed that down-regulation of engulfment genes in *C.elegans* embryos contributes to higher recovery rate when weak pro-apoptotic stimulus applied [186].

The procedure of apoptosis relies on two main signaling pathways (Figure 1.9): the extrinsic (death receptor) pathway and the intrinsic (mitochondrial) pathways. It was demonstrated that these pathways are connected to each other by the effect of a molecule in one pathway into another [187]. Various biochemical alterations are displayed by the apoptotic cells: disrupted DNA, cross-linked and cleaved protein structures, functional phagocytosis are some of these modifications [188]. Caspases are commonly expressed proenzymes which also originated in an inactivated configuration in many of the cells. Activation and cleavage of the initiator proenzyme results in latter cleavage and activation of different pro-caspases which trigger sequential proteolysis and enhance apoptotic signals to lead accelerated cell death. Besides, cleavage and activation of an initiator caspase often cause an inevitable death of cells. Up to now, many caspases have been determined and classified into three major groups, initiators (casp-2, -8, -9, -10),

executioners or effectors (casp-3, -6, -7) (Table 1.2) and inflammatory (casp-1, -4, -5) types, respectively [189,190]. Additively, casp-11, responsible from cytokine maturation and apoptosis through the septic shock, casp-12, regulates endoplasmic-dependent apoptosis and cytotoxicity by using amyloid-β, casp-13, characterized to form as a bovine gene, *casp-14*, extremely generated in embryonic tissues rather than adult tissues [58,191,192].

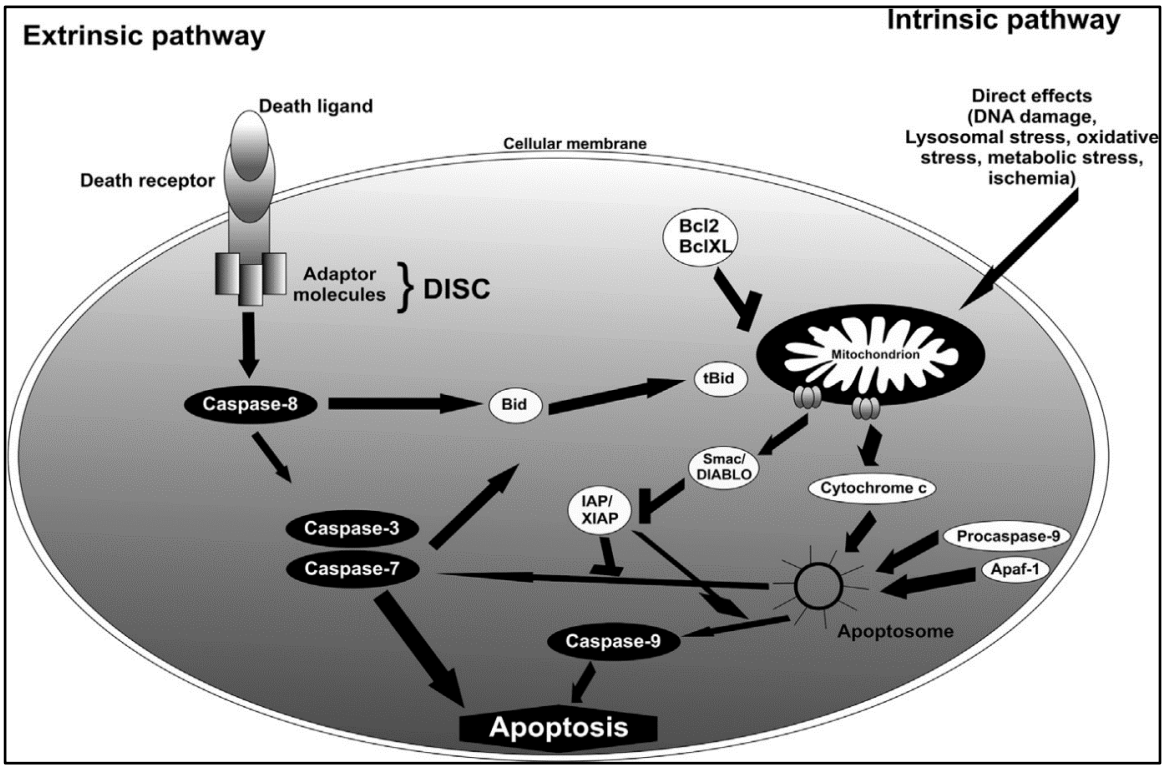

Figure 1. 9. Major functions of the death receptor (extrinsic) signaling and mitochondrial (intrinsic) signaling pathways that mediated apoptosis [193].

The death receptor (extrinsic) signaling pathway is triggered by death ligands when connected to the tumor necrosis factor (TNF) family receptors. TNR-related apoptosis-inducing ligand (TRAIL) receptors are one of the most distinguished and identified receptor types in this family. Ligation of these two structures aggregates death-inducing signaling complex (DISC) and leads activation of caspase cascade which has rich, cysteine domains [194]. Once initiator caspase, casp-8, cleaved and activated, executioner caspases together with casp-9, casp-3 are cleaved and activated in the following steps [195].

Table 1. 1. Mitochondrial (intrinsic) signaling pathway proteins and their abbreviations which contribute to the regulation of apoptosis [178].

Abbreviation	**Name of the protein**
Smac/DIABLO	2nd mitochondrial activator of caspases/direct IAP-binding protein with low PI
HtrA2/Omi	High-temperature requirement
IAP/XIAP	Inhibitor of Apoptosis Proteins/X-linked IAP
Apaf-1	Apoptotic protease activating factor
Caspase-9	Cysteinyl aspartic acid-protease-9
AIF	Apoptosis-Inducing Factor
Bcl-2, -10	B-cell lymphoma protein-2, -10
Bcl-x	BCL2 like 1
Bcl-XL/-XS	BCL2 related protein, long isoform/short isoform
Bcl-w	BCL2 like 2 protein
BAX	BCL2 associated X protein
BAK	BCL2 antagonist killer-1
BID	BH3 interacting domain death agonist
BAD	BCL2 antagonist of cell death
BIM	BCL2 interacting protein BIM
BIK	BCL2 interacting killer
Blk	Bik-like killer protein
Puma	BCL2 binding component-3
Noxa	Phorbol-12-myristate-13-acetate-induced protein-1
14-3-3	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein
Aven	Cell death regulator Aven
Myc	Oncogene Myc

Mitochondria mediated (intrinsic) signaling pathways can be initiated via diminished antiapoptotic B-cell lymphoma 2 (Bcl-2) family members, upregulated proapoptotic

proteins and receptor-dependent formations. Nonetheless, intrinsic pathways are also induced by infections, irradiation, changes in the pH, ATP, O_2, CO_2 levels, free radicals. One by one, all these factors can alter inner mitochondrial membrane structure and these modifications lead the mitochondrial permeability transition pore (MPTP) to open and release of the cytochrome *c* and pro-apoptotic structures that are second mitochondria-derived activator of caspase (Smac/DIABLO) and the serine protease HtrA2/Omi, from inner mitochondrial membrane into the cytosol [196-199]. The release of these proteins and activation of apoptotic protease activating factor-1 (Apaf-1) by cytochrome *c* contribute the formation of apoptosome [188,195,200]. Aggregation of apoptosome initially triggers activation of initiator casp-9 and executioner casp-3 in the sequencing steps [195,201,202]. During the activation of caspases, Smac/DIABLO and Htr2A/Omi inhibit X-linked inhibitor of apoptosis protein (XIAP) which blocks caspases [203-205]. Once BH3-interacting domain death agonist (Bid) protein, which is a member of Bcl-2 family, cleaved into truncated Bid (tBid) by casp-8; it induces permeabilization of the outer mitochondrial membrane by relocating itself to the mitochondrial membrane. Moreover, casp-3 might also play a key role in the transformation of Bid into tBid, to enhance apoptotic signals [195,202].

Table 1. 2. Specification of the executioner and initiator group of caspases [178].

Abbreviation	**Name of the protein**
Caspase-3	Cysteinyl aspartic acid-protease-3
Caspase-6	Cysteinyl aspartic acid-protease-6
Caspase-7	Cysteinyl aspartic acid-protease-7
Caspase-10	Cysteinyl aspartic acid-protease-10
PARP	Poly (ADP-ribose) polymerase
Alpha fodrin	Spectrin alpha chain
NuMA	Nuclear mitotic apparatus protein
CAD	Caspase-activated DNAse
ICAD	Inhibitor of CAD

After all inhibitory factors blocked and casp-3 successfully activated; it cleaves and separates caspase-activated DNAse (CAD) from an inhibitor of caspase-activated DNAse (ICAD), to break chromosomal DNA apart which is already in the nucleus and induce chromatin condensation. Lastly, casp-3 causes an irreversible damage to cellular signal transduction, intracellular transportation, cell division and cytoskeleton by targeting and cleaving gelsolin which is an actin-binding protein and regulates both signaling pathways and formation and polymerization of actin [206,207].

1.2.2. Respiratory Chain Complexes, ATP Synthase, and Mitochondrial Membrane Potential

Energy is one the most significant requirement that generated by mitochondria via oxidative reactions upon biological molecules to bestow energy, in forms of ATP. Cell growth, cellular signaling pathways, metabolic and biochemical reactions, and even death mechanisms require energy. However, mitochondrial activity in each cell differs from each other depending on tissue types, mitochondria amount, energy requirement and environmental features.

The mitochondrion is incumbent upon numerous catabolic and enzymatic reactions to produce energy from degradation of carbohydrates, fats, and proteins into simple sugars, fatty acids, and amino acids (Figure 1.10). Just after degradation, this organelle, induces β-oxidation and Krebs cycle reactions to obtain NADH and/or $FADH_2$ from previously degraded complex structures and then initiates oxidative phosphorylation via reduction of $FADH_2$ and NADH to obtain electrons, and process with molecular oxygen, to generate energy by “respiratory chain” (RC) or “electron transport chain” (ETC) complexes [208].

For mammals, ETC composed of two electron carriers, coenzyme Q and cytochrome *c*, and four different multiprotein enzyme complexes. Complex I (NADH dehydrogenase or NADH: ubiquinone oxidoreductase) which forms with 47 protein subunits and pumps out four hydrogen ions from mitochondrial matrix into the inner mitochondrial membrane, complex II (succinate dehydrogenase or succinate: ubiquinone oxidoreductase) that consist of four subunits and oxidizes succinate to fumarate and reduces ubiquinone, complex III (the bc1 complex or ubiquinone: cytochrome *c* oxidoreductase) which composed of 11 protein subunits and regulates reduction of coenzyme Q and divests four protons to

perform the same process as complex I, complex IV (cytochrome *c* oxidase, cyclooxygenase or reduced cytochrome *c*: oxygen oxidoreductase) that forms with 13 protein subunits and translocate four protons same as complex I and III, and regulates trans-passage of electron from cytochrome *c* to oxygen, to harvest two water molecules. All these structures are formed in the mitochondrial inner membrane and expressed transcriptionally by both mitochondrial and nuclear genes, except complex II which is encoded only by nuclear genes [209-213].

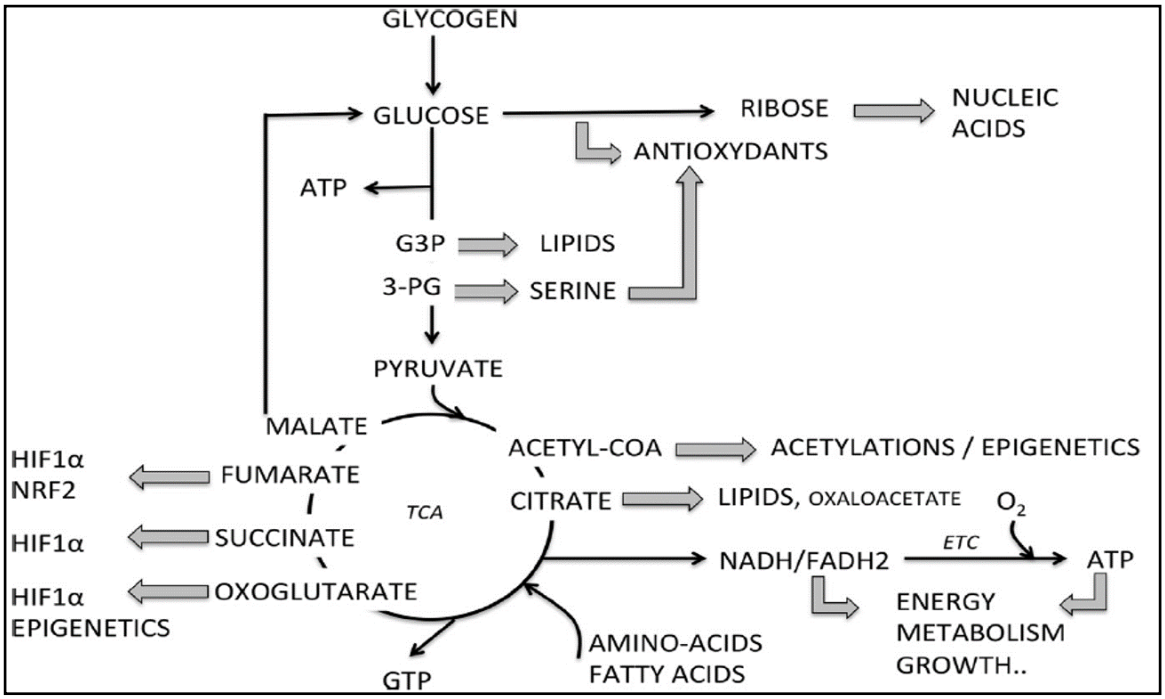

Figure 1. 10. Catabolic and anabolic reactions during the adenosine triphosphate (ATP) production [208].

Up to now, RC complexes played a significant role during the energy generation, but ATP synthase or complex V, is also required to produce the final product ATP. ATP synthase is another crucial oxidative phosphorylation complex that consists of 17 protein subunits and two sub-complexes, F_1 and F_0 catalytic sites on the mitochondrial matrix and hydrophobic membrane, respectively.

The cascade movement of the electrons from complex I to complex IV, initiates the release of the protons, which was obtained via oxidation of NADH by complex I, or $FADH_2$ by

complex II, from mitochondrial membrane into the inner membrane, and produces an electrochemical gradient that consumed by the ATP synthase, to assemble ADP and inorganic phosphate and create adenosine triphosphate (ATP). Moreover, oxidative phosphorylation complexes, accumulate and form “respirasome” or “super-complexes” at the inner mitochondrial membrane parts (Figure 1.11) [214-217,213,218].

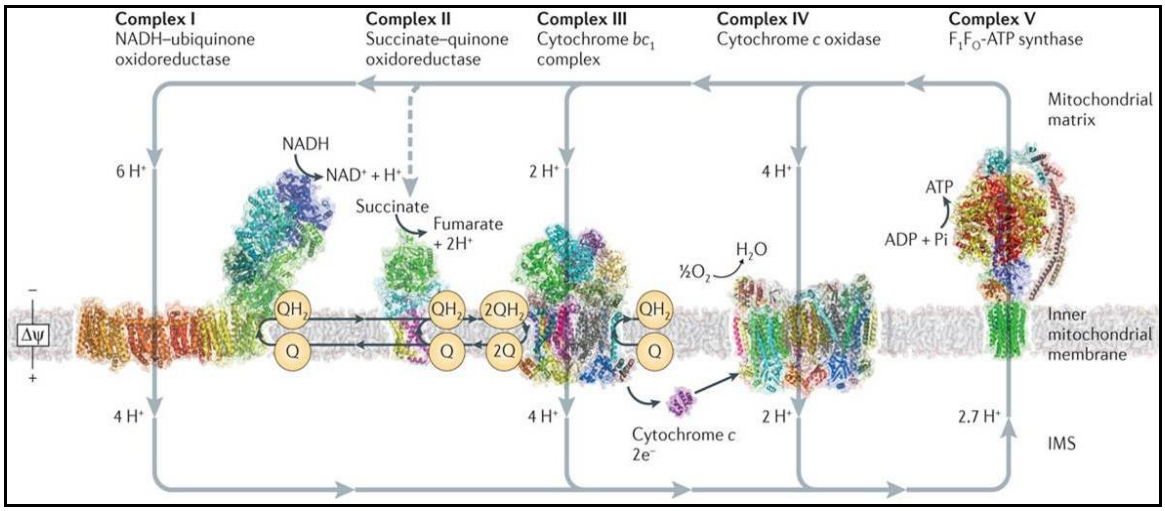

Figure 1. 11. Crystal structures for electron transport chain and ATP synthase complexes [219].

Electrochemical gradient, in other words, electrochemical transmembrane potential, is another significant and complementing feature that plays an important role in mitochondria-dependent reactions, such as ROS production, ion balance, cell death pathway, signaling pathway, metabolic transport and mainly in ATP synthesis. The electric potential ($\Delta\psi_m$, mitochondrial membrane potential) and chemical potential (ΔpH) are two types of the electrochemical gradient. An intact and functional mitochondrion, generates energy depending on two conditions, at stage four, “leak respiration state”, $\Delta\psi_m$ is high, and respiration is slow, thus ATP is not processed, but, at stage three, $\Delta\psi_m$ is low and respiration is high thus ATP is excessively generated. Additionally, these two statements can only be used for isolated mitochondrial organelles and cannot be applied to explain upon healthy and total cellular formations. Under normoxic conditions, ETC complexes provide $\Delta\psi_m$ via translocation of protons to generate ATP molecules, in healthy cells. However, under hypoxic conditions, this process is regulated by complex V due to its

hydrolytic activity, and adenine nucleotide translocators (ANT1-4) via exchanging ADP from the cytosol into the mitochondrial matrix. Depleted and/or decreased $\Delta\psi_m$ (mitochondrial membrane potential), also known as proton leak, most commonly result from dysregulation or inhibition of RC complexes. Besides, decreased $\Delta\psi_m$ levels in the mitochondria, induce ROS generation and may further cause tumorigenesis in normal cells [220-223].

1.3. MASS-SPECTROMETRY BASED MITOCHONDRIAL PROTEOMICS

The discovery of the mass spectrometry (MS), has been very helpful for specific identification of cellular structures of the cells, especially proteins. Along with these developments, mitochondrion and proteins, significantly transcribed and expressed from mitochondrion, had been proven to a crucial regulator not only on energy production but also upon number of cellular processes.

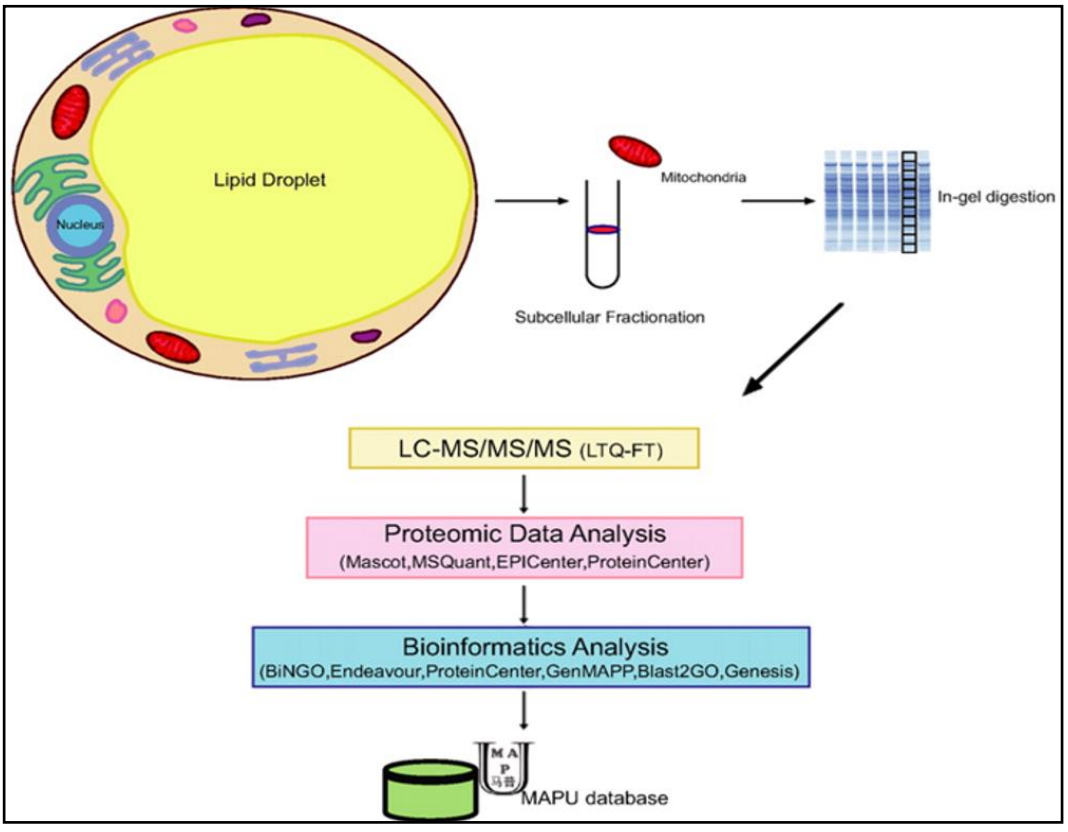

Figure 1. 12. Overall workflow in mass spectrometry-based mitochondrial analysis [224].

Globally highlighted protein analysis by MS documented the furthest efficiency, characterization and success rate upon mitochondrial proteins, among the protocols applied so far.

Although the experimental procedures by MS, to determine protein identities, seems effortless, yet it is extremely arduous when considered the fact that mitochondrial proteins represented in numerical quantity, also display inconsistent dynamic structures [225].

Together with the developments in both MS, tandem MS (MS/MS), and separation techniques contribute mitochondrial proteome and lead numerous studies to reveal mitochondrial proteins, 46 from human placenta [226], 80 in SH-SY5Y cell line [227], 615 from human heart [228], 399 in mouse models included with abundant structures [229], 2533 from multiple mouse organs [230], 1130 in 3T3-L1 cell line [224], 689 and 1162 from rat models [231,232], 297 and 3881 in mouse models [233,234]. Almost 75 per cent of the documented proteins are contaminants, and protein correlation profiling (PCP) is the organelle-specific profiling technique depending upon organelle separation which was applied in 2006, for mouse models to determine 297 mitochondrial of the 1404 various organelle proteins [233] and 334 specifically mitochondria-linked among 2533 proteins from multiple mouse tissues [230].

2. MATERIALS

2.1. INSTRUMENTS

- -86°C freezer (WiseCryo, WUF-500 -86°C ULT Freezer, USA)
- -20°C freezer (Arçelik, Turkey)
- Absorbance reader (BioTek, ELx808, USA)
- BD FACSCalibur Flow Cytometer: 4-color (BD Biosciences, 342975, USA)
- Centrifuge (Beckman Coulter Allegra 25R, USA and Hettich Mikro-22R, Germany)
- ChemiDOC XRS+ Gel Imaging System (Bio-Rad, 721BRO4012, USA)
- CO_2 incubator (Nuaire, NU-5841E, USA)
- G2 Semi-Dry Transfer system (Thermo Scientific, 22838, USA)
- Ice machine (Hoshizaki, FM-120DE, Japan)
- Laminar flow cabinet (ESCO, AC2-4E8 Labculture Class II Biohazard Safety Cabinet 2A, USA)
- LC system (Dionex-Thermo Scientific, Ultimate™ 3000 RSLCnano, USA)
- LC/MS (Bruker, Compact, USA)
- LC/MS column (Dionex-Thermo Scientific, Acclaim PepMap RSLC C18, USA)
- LC/MS ion source (Bruker, CaptiveSpray nanoBooster-Electrospray-UHR-Quadrupole-Time-of-Flight LC/MS, USA)
- LC/MS software (Bruker, Biotools, USA)
- LC/MS software (Bruker, Compass, USA)
- LC/MS software (Bruker, HyStar 3.2, USA)
- LC/MS software (Bruker, otofControl, USA)
- LC/MS software (Matrix Sciences, Mascot, USA)
- Magnetic Stirrer (Heidolph MR 3004, Germany)
- Mini-PROTEAN Tetra Cell Electrophoresis System (Bio-Rad, 165-8004 USA)
- Mini Trans-Blot Cell Blotting System (Bio-Rad, 164-5050, USA)
- Multiskan Spectrum (Thermo Lab. systems, USA)

- pH meter (Ohaus, Starter 3000, USA)
- Shaker (Stuart, Gyro-Rocker SSL3, UK)
- Vacuum centrifugation (Eppendorf, Concentrator 5301, Germany)
- Vortex (Stuart SA8, UK)
- Water bath (Stuart, SB540, UK)

2.2. EQUIPMENTS

- Cell culture flasks, T75, T25 (TPP, 90075, 90025, Switzerland)
- Cryovials (TPP, Switzerland)
- Filter 0.45µm, (Santorium Stedim Biotech, Germany), 0.22µm (TPP, Switzerland)
- Graduated Cylinder 1000 mL, 500 mL, 250 mL (Isolab, Germany)
- Hemocytometer (Sigma, #Z359629-1EA, Germany)
- Immun-Blot Polyvinylidene difluoride (PVDF) membrane for Protein Blotting 26cm*3.3m, 0.2µm (Bio-Rad, 162-0177, USA)
- Micropipettes 1000µl, 200µl, 10µl (Eppendorf Research, Q23872C, Q31661C, G16578, Germany)
- Pipette tips 1000µl, 200µl, 10µl (CAPP Expell Plus, Denmark)
- Polypropylene centrifuge tubes 2 ml, 1 ml, 0.5 ml (Isolab, 078.03.003, 078.03.002, 078.03.001, Germany)
- Serological pipettes 25, 10, 5 ml (SPL Life Sciences, 083.03.025, 083.03.010, 083.03.005, Korea)
- Falcon tubes 50 ml, 15 ml (Axygen, SCT-50ml-25-S, and Axygen, SCT-50ml-25-S, Germany)
- Whatman paper (Whatman™, 3030917, Germany)

2.3. CHEMICALS

2.3.1. Cell Culture Media:

- Dulbecco's Modified Eagle's Medium (DMEM) - high glucose (Gibco, 41966-052, USA)
- RPMI-1640 (Gibco, AA10491-10, USA)

2.3.2. Growth Supplements:

- Heat Inactivated Fetal Bovine Serum (FBS) (Gibco, 10500-064, USA)

2.3.3. Other Reagents:

- 2-β-Mercaptoethanol (Merck KGaA, 60-24-2, Germany)
- 2-Propanol >99.5% (Sigma Aldrich, 24137, USA)
- Acetic acid (Sigma Aldrich, 34851, USA)
- Acetonitrile (Sigma Aldrich, 49199, USA)
- Acrylamide/ Bis-acrylamide (29:1, 161-0156, Bio-Rad, USA)
- Amphotericin (PAN, P06-C1100, Germany)
- Ammonium bicarbonate (Sigma Aldrich, 40867, USA)
- Ammonium persulfate (APS) (Bio-Rad, 161-0700, USA)
- Bovine Serum Albumin (Santa Cruz Biotechnology, 2323, USA)
- Coomassie Brilliant Blue R-250 (Sigma Aldrich, 27816, USA)
- Deacetylation Inhibition Cocktail (DIC) (Santa Cruz Biotechnology, 362323, USA)
- Dithiothreitol (
- Dulbecco's Phosphate Buffered Saline (DPBS) (Gibco, 14190-250, USA)
- Ethanol >99.8% (Sigma Aldrich, 34870, USA)
- Formic acid (Merck-Millipore, 607-001-00-0, Germany)
- Glycine (MP Biomedicals, 808822, USA)
- Methanol >99.9% (Sigma Aldrich, 34885, USA)

- Penicillin-streptomycin (P/S) (Gibco, 15070-063, USA)
- Phenylmethanesulfonylfluoride (PMSF) (Sigma Aldrich, 78830, USA)
- ProSieveTM QuadColor™ Protein Marker 4.6 kDa-300 kDa (Lonza, 00193837, Sweden)
- Protease Inhibitor (PI) (Sigma Aldrich, P8340, USA)
- Rhodamine123 (Rh123) (Sigma Aldrich, R8004, USA)
- RIPA (Santa Cruz Biotechnology, 24948, USA)
- Sodium Chloride (Sigma Aldrich, 31434, USA)
- Sodium orthovanadate (Na_3VO_4) (Sigma Aldrich, S6508, USA)
- N,N,N′,N′-Tetramethylethylenediamine (TEMED) (Santa Cruz Biotechnology, sc-29111, USA)
- Tris-Base (Sigma Aldrich, T1503, USA)
- Trypsin-EDTA 0.25 per cent (Gibco, 25200-056, USA)
- Tween-20 (Merck-Millipore, 8221840500, Germany)

2.4. KITS AND SOLUTIONS

- ATP Synthase Specific Activity Microplate Assay Kit (Abcam, ab109716, USA)
- Bicinchoninic acid (BCA) Assay (Thermo Scientific, 23225, USA)
- Clarity Western ECL Substrate (Bio-Rad, 170-5061, USA)
- RIPA Lysis Buffer (Santa Cruz Biotechnology, sc-24948A, USA)

2.5. ANTIBODIES

2.5.1. Primary Antibodies

- Acetylated-Lysine (Ac-K^2-100) MultiMab^{TM} monoclonal antibody (Cell Signaling, 9814S, USA)
- Anti Caspase3 monoclonal antibody (Cell Signaling, 9665, USA)
- Anti Caspase9 monoclonal antibody (Cell Signaling, 9502P, USA)
- Anti GLUT4 monoclonal antibody (Cell Signaling, 2213, USA)

- Anti p53 monoclonal antibody (BioLegend, 628202, USA)
- Mitoprofile total OXPHOS rodent antibody cocktail (Abcam, ab110413, USA)
- HSP60 monoclonal antibody (Santa Cruz Biotechnology, A0313, USA)
- β-Actin monoclonal antibody (Cell Signaling, 3700, USA)

2.5.2. Secondary Antibodies

- Anti-mouse IgG antibody (Sigma Aldrich, A4416, USA)
- Anti-rabbit IgG antibody (Sigma Aldrich, A0545, USA)

2.6. CELL LINES

- PNT1A; human, prostate, epithelial, adherent, male (ATCC no: CRL-11609^{TM})
- DU 145; human, prostate; derived from metastatic site: brain, epithelial, adherent, carcinoma, male (ATCC no: HTB-81™)
- HeLa; human, cervix, epithelial, adherent, adenocarcinoma, female (ATCC no: CCL-2™)
- Hep 3B; human, liver, epithelial, adherent, hepatocellular carcinoma, male (ATCC no: HB-8064™)
- HEK 293T; human, kidney, epithelial, adherent, fetus (ATCC no: CRL-11268™)
- SH-SY5Y; human, bone marrow, epithelial, adherent and suspension, neuroblastoma, female (ATCC no: CRL-2266)

3. METHODS

3.1. CELL CULTURE TECHNIQUES

3.1.1. Cell Types and Culturing Conditions

All cell lines were received from ATCC (American Type Cell collection). PNT 1A cell line was cultured in RPMI-1640 media and other cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) media. All media were supplemented and with 10 per cent (v/v) Fetal Bovine Serum (FBS), one per cent (v/v) Penicillin and Streptomycin and 0.1 per cent (v/v) Amphotericin.

Each cell line was firstly taken from -86^{o}C freezer stock and seeded into T25 flasks with mammalian cell culture medium. Seeded cells were left within these T25 flasks to grow up. Depending on the character of the cell line, 12 to 48 hours later, their media was refreshed.

3.1.2. Cell Subculturing

When cells were bound with flasks, they were taken and washed with DPBS solution to get rid of the metabolic waste. When washing was done with DPBS, one or two ml of trypsin-EDTA enzyme was used, due to T25 or T75 flask types, to break cellular binding structures that produced in between cells and flasks. Next, the enzyme was inactivated with twice volume as completed media and centrifuged at 1500 rpm for five minutes. The supernatant part was spilled out and the pellet was dissolved in fresh media and seeded back into T75 flasks. For other experimental protocols, cells were collected with trypsin-EDTA after proliferation and centrifuged with DPBS.

3.1.3. Counting Cells

Cells were trypsinized from T75 flasks as described Section 3.1.3. 10µl of aliquots of the solutions loaded onto a hemocytometer and the middle area of the hemocytometer was identified as the cell counting area. The number of each cell line per milliliter was determined due to the formula "counted number of cells * dilution factor/mm^2 * chamber depth" by the inverted light microscope.

3.1.4. Cryopreservation of Cells

Cells were detached and counted as described Section 3.1.4. The total cell suspension was centrifuged at 1500 rpm for five minutes. The density of cell number per one cryovial tube was identified approximately two to three million cells per ml and cells were resuspended in a final volume of one and a half milliliter freezing media, that containing 10 per cent (v/v) of DMSO and 90 per cent (v/v) of FBS. Cells were then frozen at -86^{o}C and transferred to a liquid nitrogen box for the long-term storage.

3.1.5. Thawing Cells

Stock cells were taken from liquid nitrogen box and warmed up to 37^{o}C as fast as possible. The cell suspension was taken and placed into the 15ml sterile falcon tube and four point five ml of completed media was added drop by drop onto cell suspension and mixed continuously to prevent harming cells with a sudden increase in the osmotic pressure. Later, the solution in the falcon tube was centrifuged at 1500 rpm for five minutes to get rid of the DMSO containing freezing media. The cell pellet was dissolved in complete media and placed into cell culture plates. Cells were passaged at least twice before the experimental procedure applied.

3.2. BICINCHONINIC ACID (BCA) ASSAY APPLICATION

A Master Mix was prepared with Radio-Immunoprecipitation Assay (RIPA) lysis buffer, one per cent (v/v) PI, one per cent (v/v) Na_3VO_7, one per cent (v/v) DIC. Later, a master

mix was mixed with the pellets equally. Then, pipetting was performed and cells were exploded.

Samples were left on ice for 10 minutes and then centrifuged at +4^{o}C. Then again, samples were collected from a supernatant part of the Eppendorf tubes and the pellet was kept at -20^{o}C. Next, each sample was added into 96 well plates with BCA assay mix. 96 well plates were transferred into the incubator at 37^{o}C for 30 minutes. Finally, samples were measured at 562 nm and concentration values of the samples were measured.

3.3. SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Prepared samples were separated due to their molecular weight with SDS-PAGE protocol. Four per cent (w/v) polyacrylamide stacking gel and eight, 10, 12 per cent (w/v) polyacrylamide separating gels were prepared. Equal volume of proteins from collected cell pellets were prepared in 1:5 ratio with SDS-loading dye (250mM Tris-HCl, 30 per cent (v/v) glycerol, 10 per cent (v/v) SDS, 0.02 per cent bromophenol blue and five per cent (v/v) 2-β-merceptoethanol) and loaded into wells of the previously prepared gels. SDS-PAGE protocol was applied by using Bio-Rad Mini-PROTEAN Tetra Cell Electrophoresis System.

The stacking gel was constituted by using 0.4 per cent (w/v) SDS and 0.5 M Tris, pH: 6.8. Separating gels were made up with 0.4 per cent (w/v) SDS and 1.5 M Tris, pH: 8.8. After that, gels were prepared with four per cent (w/v) polyacrylamide stacking gel and eight, 10, 12 per cent (w/v) separating gels. Recipes of gels were shown in Table 3.1. When stacking and separating buffers were mixed with acrylamide and distilled water; 10 per cent (w/v) ammonium persulfate (APS) and N, N, N', N'-Tetramethylethylenediamine (TEMED) were added into falcon tubes to accelerate the polymerization protocol.

Firstly, separating buffer was poured into the casting gel and supposed to polymerize at the room temperature for 15 minutes and 2-propanol (isopropanol) was casted on top of the gel to get rid of the oxidation and let gel polymerize in a linear shape. Then, isopropanol was spilled out and the gel was washed with dH_2O and gently cleaned by thin Whatman papers. Stacking buffer was added on top of the separating gel, into casting gel. Later, a comb

which has 10-wells and 1.0 mm thickness were put on top of the casting gel and left for polymerizing for 30 minutes. When polymerization ends, the comb was taken out very carefully and the gel was washed with running buffer, pH: 8.5 (Tris-Glycine 0.25M, Tris-Base 1.92M Glycine, 1 per cent (w/v) SDS).

Gels were put into the special cassettes where thin glasses were positioned with facing inside of the cassettes. Then this cassette was placed into the electrophoresis tank and cassette was filled with running buffer until overflowed but half of the tank was filled with running buffer. After that, previously prepared protein samples from cell pellets were loaded into wells with gel loading tips with one µl of ProSieveTM QuadColor™ Protein Marker (Lonza). When samples were loaded into the wells, electrophoresis procedure was started with 20mA, 90V until proteins will pass through from stacking gel to separating gel and then increased to 35-40mA, 140V while running in the separating gel. Proteins kept running until SDS-loading dye front reached the second green level at the bottom of the cassette.

3.4. IMMUNOBLOTTING ASSAY

At the end of the running protocol, proteins transferred electrophoretically onto Bio-Rad PVDF membrane with 0.2 µm pore size by using semi-dry Thermo Scientific G2-blotter. One thick and one thin Whatman papers which were wetted with transfer buffer, pH: 8.3 (0.25M Tris-Base, 1.92mM Glycine, 20 per cent (v/v) methanol), PVDF membrane which was activated with methanol (HPLC grade) for five minutes, gel, one thin and one thick Whatmann papers which were wetted with transfer buffer were put into the G2-blotter in following order to make a gel sandwich model for transferring protein structures. Later, two sides of the G2 blotter device were locked in and the protocol was started for 20 minutes with 25V and 0.3A. After transfer procedure is done, PVDF membrane was blocked with five per cent (w/v) non-fat milk in TBS-Tween 20, pH: 7.4 for 30 minutes and then, monoclonal antibodies were used against GLUT-4, p53, Mitoprofile total OXPHOS rodent antibody cocktail, Casp-3, Casp-9, and Acetylated-Lysine by solving one µl of antibodies in four ml of TBS-Tween 20 solutions. After overnight incubation at +4^{o}C against monoclonal antibodies, membrane was taken and washed with TBS-Tween 20, pH: 7.4 for three times with 10 minute intervals and incubated again for 60 minutes against

Anti-mouse IgG and Anti-rabbit IgG monoclonal antibodies and washed twice with TBS-Tween 20, pH: 7.4, and once with TBS, pH: 7.4 with 10 minute intervals. Then, Clarity Western ECL Substrate solutions were mixed equally, 500 µl each, and poured onto membrane and images were collected by using ChemiDOC XRS+ Gel Imaging System. At the end of the imaging process, the membrane was washed with TBS-Tween 20, pH: 7.4 for six times with 10-minute intervals. The same protocol was applied against HSP60, β-Actin, and their secondary monoclonal antibodies. After image collection, the volumetric density of the protein bands measured with Bio-Rad Image Lab 5.2.1 software.

3.5. MEMBRANE POTENTIAL MEASUREMENT WITH RHODAMINE123

Mammalian cells were grown in T25 and T75 flasks with complete media. After cells were grown, they trypsinized and collected into Eppendorf tubes with centrifugation step as described before (Section 3.1.3). To measure membrane potential, a stock solution of Rhodamine123 was prepared as one mg/ml and then one µl of Rhodamine123 (Rh123) was dissolved in two ml DPBS. After, preparation of this solution, each cell line was mixed with 300µl of this master mix, covered with aluminum foil to create a dark environment and placed into the incubator, which was set to 37^{o}C, for 10 minutes. At the end of this time interval, samples were taken back into the laminar flow with aluminum foil. These samples were washed with DPBS and centrifuged twice, at 1500 rpm. Later, at the end of the last centrifugation, samples were taken and placed into the FACScan flow cytometry device for measurement.

3.6. ATP SYNTHASE ASSAY

3.6.1. Sample Preparation

Solution-1 was prepared with a Tube-1 chemical solution and deionized water (H_2O). Then, each cell line's pellet was solved with Solution-1. Freeze-thaw cycles were applied to each sample for five times. Once these cycles were done, all samples were centrifuged at 16000 rpm for 20 minutes. The supernatant was taken and the pellet was discarded. When supernatant parts of the samples were collected, concentrations were measured with BCA

assay to obtain their protein concentrations, which was adjusted to five and a half mg/ml. Then, detergent was added in 1:10 ratio of the total volume, to decrease the final protein concentration five mg/ml and samples were incubated on ice for 30 minutes. At the end of 30 minutes, samples were centrifuged for 20 minutes at 16000 rpm. Supernatants were collected again and pellets were discarded. Samples (50 µl) were added into each well with 50-100µg/50µl concentrations and they were kept on ice until protocol started.

3.6.2. Assay Method

Samples (50 µl) were added to the well including a Solution-1 as a control, null, and background reference. Then, samples were incubated at room temperature for 180 minutes.

Wells were emptied by turning the plate over and were shaken to get rid of the remaining solutions. Afterwards, wells were washed with 300 µl of solution-1 twice. Lipid mix (40 µl) was added to each well and incubated for 45 minutes at the room temperature. Then, 200 µl of reagent mix was added into the each well without emptying well which already including 40 µl of lipid mix. Lastly, the plate was placed into the spectrophotometer and kinetic program was applied to collect absorbance measurement for 180 minutes with one-minute interval readings at 30^{o}C and at 340 nm.

3.7. MASS SPECTROMETRY-BASED PROTEIN IDENTIFICATION

3.7.1. In-Gel Digestion Process

Protein samples were stained with coomassie brilliant blue R250 on SDS-polyacrylamide gel, then the selected bands were transferred into the Eppendorf tube with the scalpel. Washing solution, which was prepared with 50 per cent methanol and five per cent acetic acid, was first treated in destaining solution, that involved with 50 mM ammonium bicarbonate and 50 per cent acetonitrile until the blue color disappears. Later, the gel fragments were dried in 200 µl of acetonitrile, 10 mM dithiothreitol was added to gel fragments, and then 100 mM iodoacetamide was added into fragments to accomplish the “Protein Reduction and Alkylation” procedure. To digest proteins, trypsin solution (20ng/µl) was added and the sample was incubated for 16 hours at 37^{o}C. Mixing the

extraction solution, comprise of 50 per cent acetonitrile and five per cent formic acid, applied for the “Peptide Extraction” procedure, and then the final volume was reduced to 20 µl in vacuum centrifugation.

3.7.2. Protein Identification by Mass Spectrometry

Previously prepared samples were analyzed by nano-LC on a Thermo Dionex™ Ultimate™ 3000 RSLCnano system in a “Pre-concentration onto a Nano Column” set-up of 75 µm I.D. (Mobile phase A: 100 per cent water plus 0.1 per cent formic acid, mobile phase B: 100 per cent acetonitrile plus 0.1 per cent formic acid, loading solution: 95 per cent / 100 per cent), using and Acclaim PepMap RSLC C18, five per cent (v/v) water/acetonitrile and 0.1 per cent TFA). Flow rate of 0.300 µL/ min is applied to the device (gradient used: mobile phase B from five per cent to 40 per cent at 50 minutes, 95 per cent at 55 minutes five per cent at 65 minutes and then 10 minutes for conditioning. The separated peptide samples were analyzed in MS, CaptiveSpray NanoBooster-Electrospray-UHR-Quadrupole-Time-of-Flight, in high-resolution Bruker Compact mass spectrometry. HyStar 3.2 program for all system interface check, otofControl version 3.3 for MS control, Compass Data Analysis program for conversion of the mgf file as the analysis result and Biotools to access NCBInr and SwissProt data banks via Mascot 2.4.1 software, was used and identities were obtained.

3.8. STATISTICAL ANALYSIS

Represented data were collected from at least three independent experiments and showed as the mean +/- standard deviation (S.D). Then, paired *t*-test was performed to compare normal and cancer cell lines by Microsoft Excel 2016, also BioRad Image Lab 5.2.1 software was used to indicate volumetric band quantitation of obtained immunoblot results and latter Excel 2016 was used same as other data sets.

4. RESULTS

4.1. IMMUNOBLOTTING ANALYSIS

Immunoblotting was performed against Mitoprofile total OXPHOS rodent antibody cocktail (Respiratory chain complexes), Casp-3, Casp-9, GLUT4 and p53 to identify protein expression levels, in PNT1A, DU 145, HeLa, Hep 3B, HEK 293T, SH-SY5Y mammalian cell lines. After imaging process, the volumetric intensity of the expressed proteins measured via Bio-Rad Image Lab 5.2.1 software and calculated by Microsoft Office Excel 2013.

This experimental process was applied on corresponding cell lines against monoclonal antibodies for three main purposes. First, as previously demonstrated by Warburg, most of the cancer cells perform huge amount of glycolysis and GLUT activity independently from oxygen levels in the environment, also downregulation of p53 in tumors often correlated with enhanced glycolytic activity, thus the major reason of using these antibodies relies on determination and prediction of glycolytic activity.

Second, suppressive alterations of cell death mechanisms in tumors and relation between this phenomenon and mitochondria was aimed to be investigated to contribute literature by analyzing caspase-3 and caspase-9 upon corresponding mammalian cell lines.

Third, ATP synthase (complex V) and respiratory chain structures usually contribute and consist more frequently in ATP generation mechanism in the normal cells and in some cancer lines too, hence they have been employed for indicating this conflicted argument.

4.1.1. Protein Expression Levels and Relative Quantification of Glut4

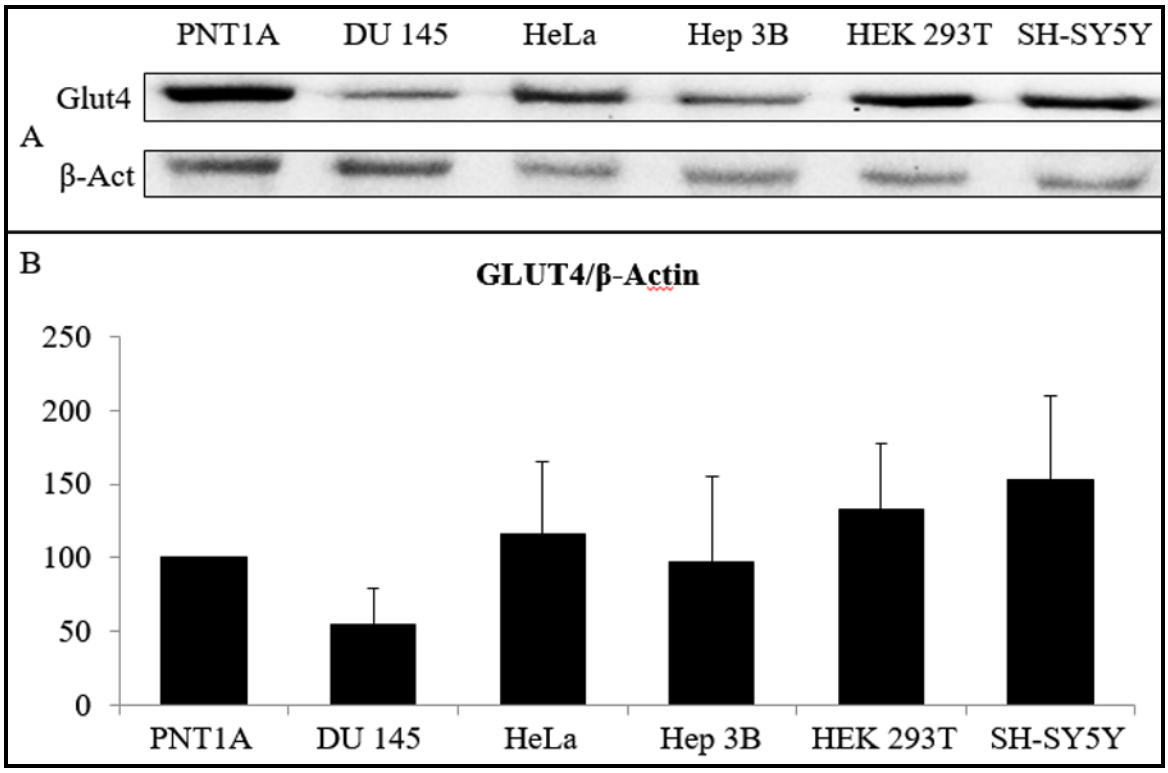

Figure 4. 1. Immunoblotting and relative quantification results of GLUT4 upon cell lines. Expression levels of **(A)** GLUT4, 50 kDa, and β-Actin, 45 kDa, in mammalian cell lines. GLUT4 was rabbit polyclonal, and β-Actin was mouse monoclonal antibodies and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(C)** Minimum three independent experiments were performed and normalized to β-Actin, during the relative quantification of GLUT4.

Cancer cells usually generate their energy by upregulating glycolytic activity which involves enhanced glucose uptake by GLUTs. The GLUT4 transporter was investigated to determine the diverse glucose uptake among various cell lines. The GLUT4 upregulated in HeLa, and SH-SY5Y cells. However, HEK 293T also had enhanced GLUT4 levels, and PNT1A expressed nearly two-fold more GLUT4 than DU 145. Additionally, Hep 3B and PNT1A obtained with the close expression of GLUT4 levels.

4.1.2. Determination of Expressed Protein Levels of p53

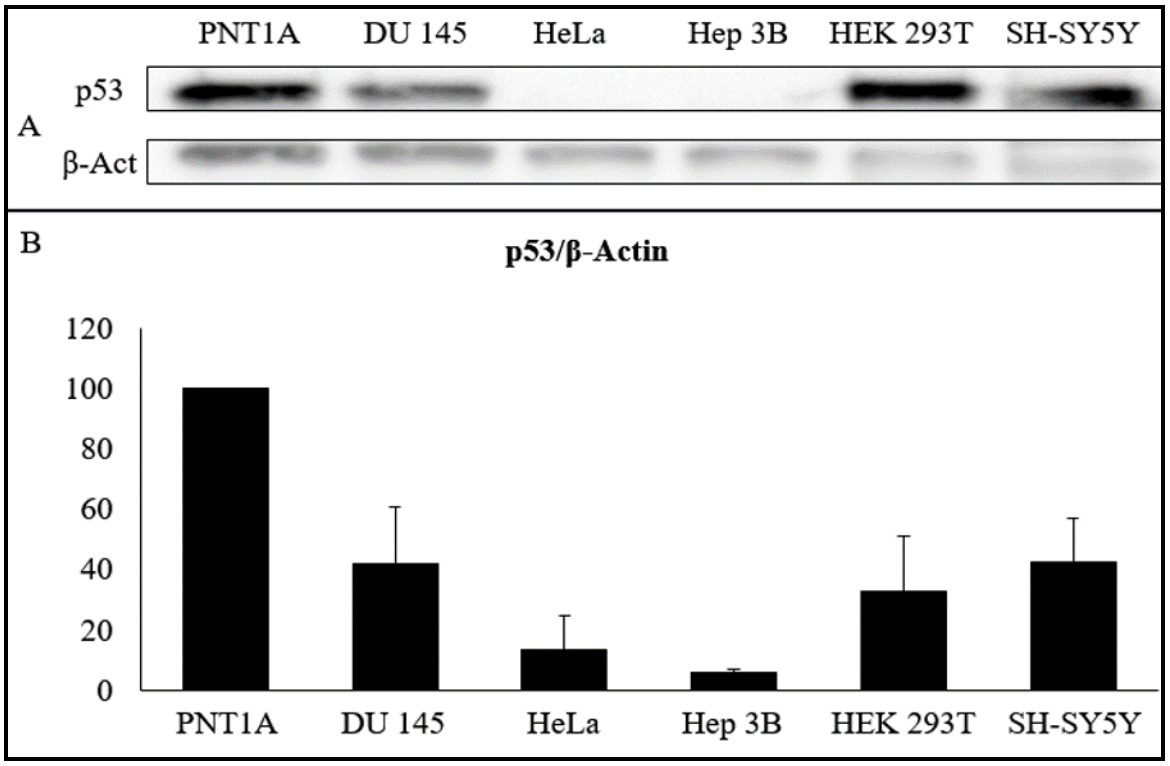

Figure 4. 2. Expression levels and volumetric intensity comparisons of p53. Representative immunoblotting analyses of **(A)** p53, 53 kDa, and β-Actin, 45 kDa, in mammalian cell lines. Both p53 and β-Actin were mouse monoclonal antibodies and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** Relative quantification of p53 levels measured and normalized to β-Actin in corresponding cell lines with three independent experiments.

p53, is a well-known promoter, due to the regulatory responses in many cellular mechanisms, including glycolysis, and cell death pathways, as well as some pathway-inducing, and inhibitory effects upon mechanisms too, hence experimented to demonstrate distinctness between healthy and cancer cell lines. All tumors and HEK 293T were displayed low p53 levels, specifically HeLa, and Hep 3B. PNT1A expressed almost three-fold more p53 than HEK 293T, and approximately two-fold more than DU 145. In addition, SH-SY5Y, and DU 145 identified approximate expression of p53 and higher p53 amount rather than HEK 293T.

4.1.3. Expression Levels of Protease Family Members Casp-9 and Casp-3

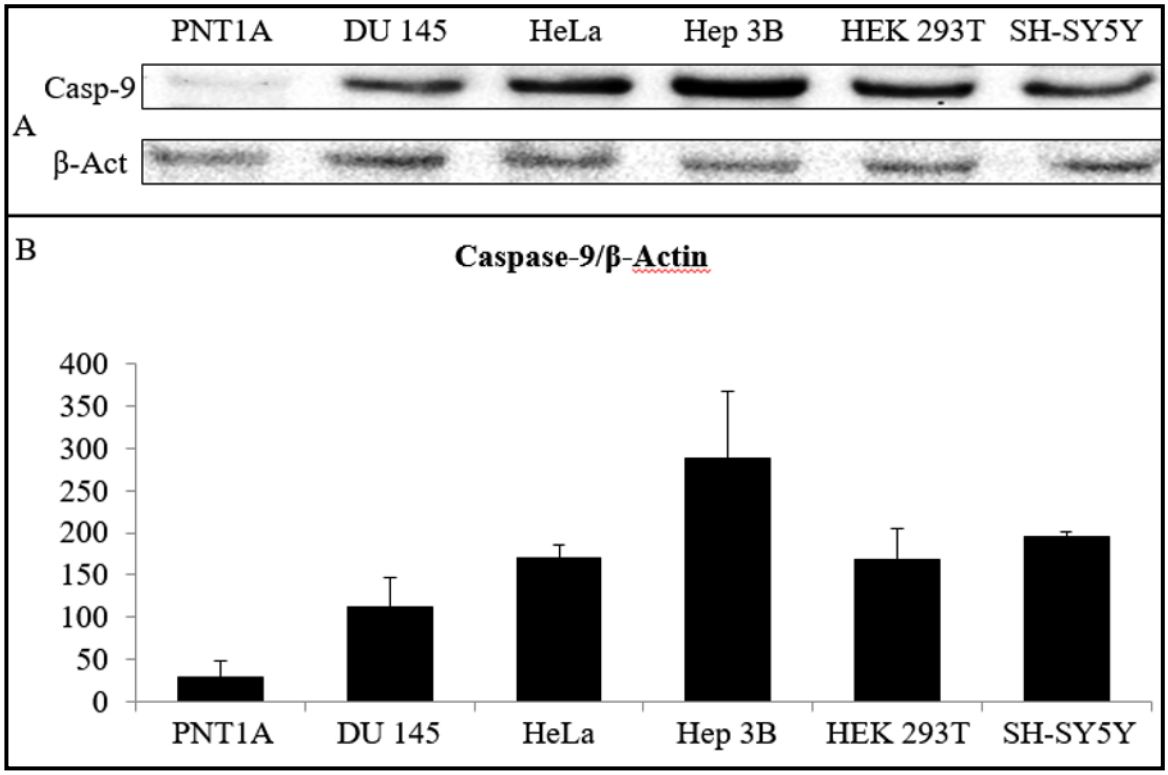

Figure 4. 3. Expression levels and volumetric intensity comparisons of casp-9. Protein levels from immunoblot analyses **(A)** Casp-9, 47 kDa, and β-Actin, 45 kDa. Casp-9 was rabbit polyclonal, and β-Actin were mouse monoclonal antibodies and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** The expression level of Casp-9 protease obtained at least three different replicates of Western Blot assays and normalized to β-Actin.

Casp-9 is an initiator class of caspase family that assemble with some other molecules to form apoptosome and latter activate caspase-3, also expected with altered expression levels in cancer cells. However, all cancer cell lines determined with high levels of Casp-9, especially in Hep 3B. Compared to PNT1A, Hep 3B expressed ten-fold, DU 145 expressed four-fold, HeLa and SH-SY5Y expressed six-fold, even HEK 239T expressed six-fold more Casp-9. Nonetheless, Hep 3B, HeLa, and SH-SY5Y expressed more Casp-9 than HEK 239T. Nonetheless, mitochondrial structures of cancer cell lines may be distorted due to the hyperpolarized membrane potential, which can induce caspase-9 activation.

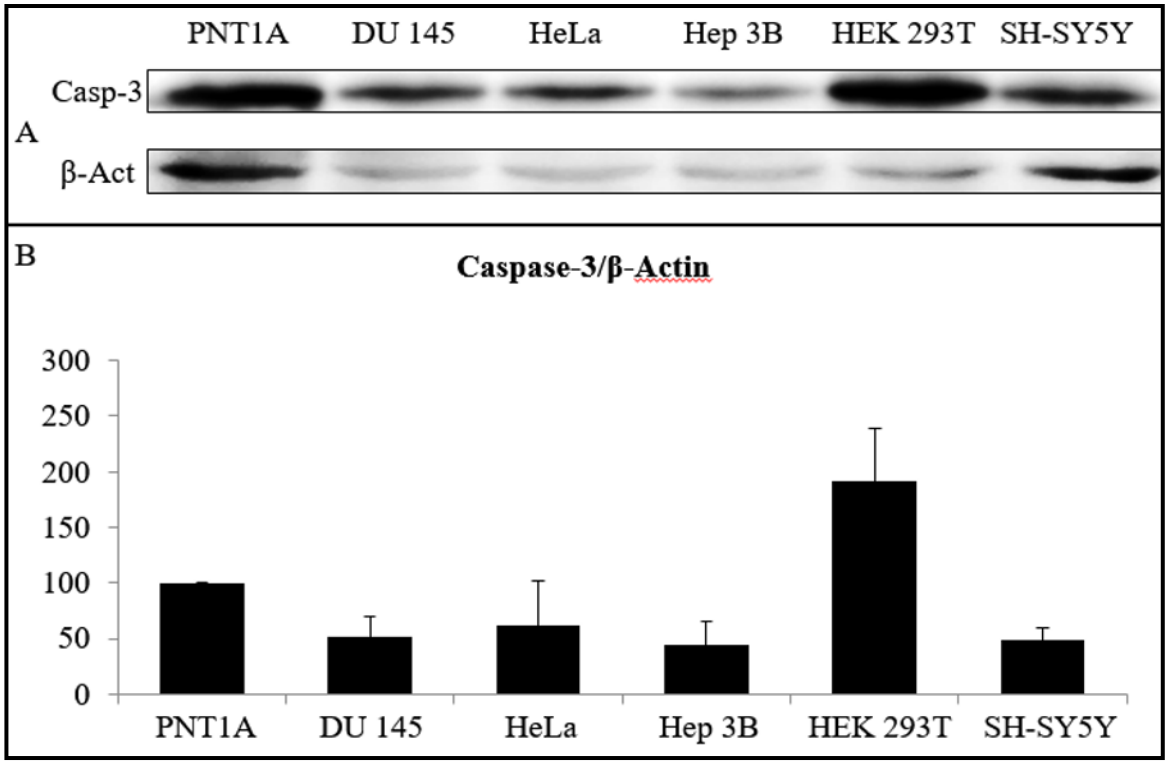

Figure 4. 4. Immunoblotting and relative quantification analysis of casp-3. Western Blot analysis of **(A)** Casp-3, 35 kDa, and β-Actin, 45 kDa, in healthy, and cancer cell lines. Casp-3 was rabbit monoclonal, and β-Actin were mouse monoclonal antibodies and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** Minimum three independent experiments were performed, and normalized to β-Actin, during the relative quantification of Casp-3.

In contrast to excessive Casp-9 levels, lower Casp-3, which is an executioner type of apoptotic mechanism that most probably causes normal cells to die also altered in cancer cells, levels were detected in all tumor cells. PNT1A displayed two-fold more Casp-3 than DU 145 cells, and HEK 293T expressed minimum two-fold more Casp-3 compared to DU 145, HeLa, Hep 3B, and SH-SY5Y cells. Together with these results, apoptotic activity of cancer cell lines significantly downregulated when compared to healthy cell lines.

4.1.4. Altered Expression Levels of ETC Complexes and ATP Synthase

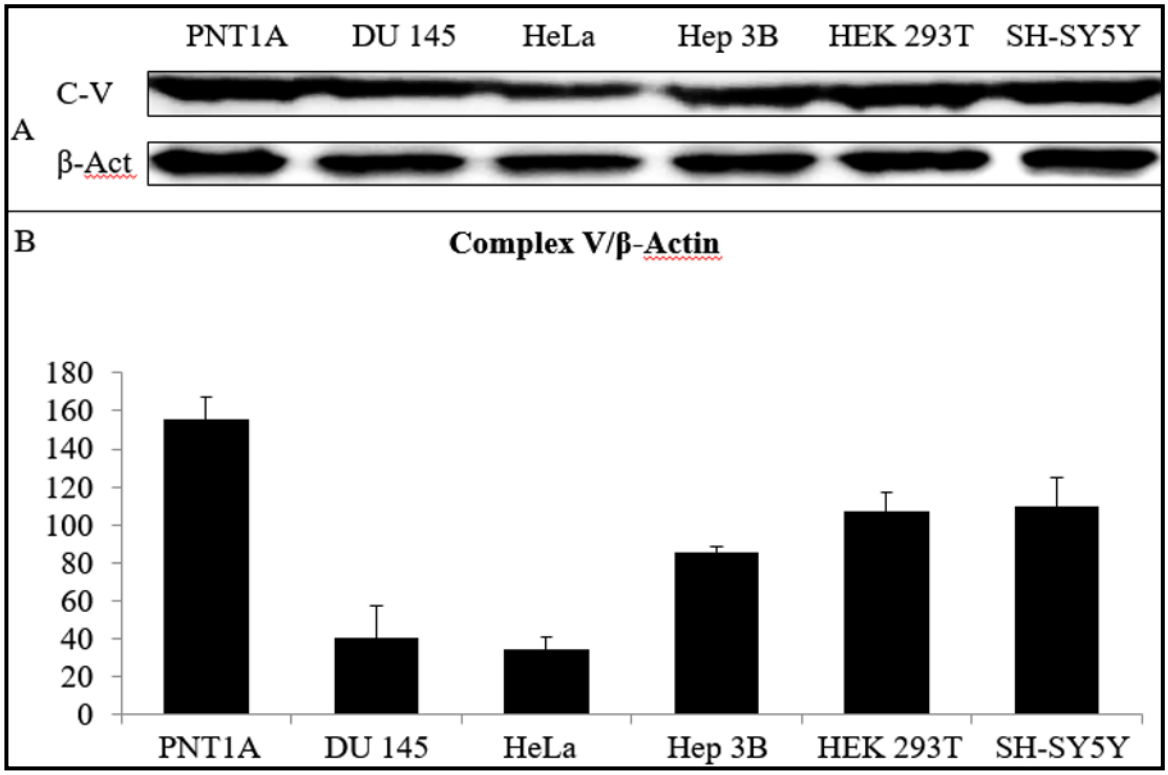

Figure 4. 5. Immunoblotting and relative quantification analysis of complex V. Expression levels of **(A)** Complex V (ATP synthase) alpha subunit, 55 kDa, and β-Actin, 45 kDa, in mammalian cell lines. Both Complex V, and β-Actin were mouse monoclonal antibodies, and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** Relative quantification of Complex V levels measured and normalized to β-Actin in corresponding cell lines with three independent experiments.

The conversion of the ADP to ATP through consuming electrochemical potential gained from proton movement is the final step of the complex V-mediated ATP production, also expressed variably in cancers depending on oxygen levels and studied to reveal this discrepancy. Highest Complex V levels were observed in PNT1A cell line. DU 145, HeLa, and Hep 3B identified with low expression levels. PNT1A, healthy prostate cells, expressed more complex V, approximately four-fold, than DU 145, prostate cancer cells. Surprisingly, SH-SY5Y cancer cell line expressed little higher complex V than HEK 293T cells.

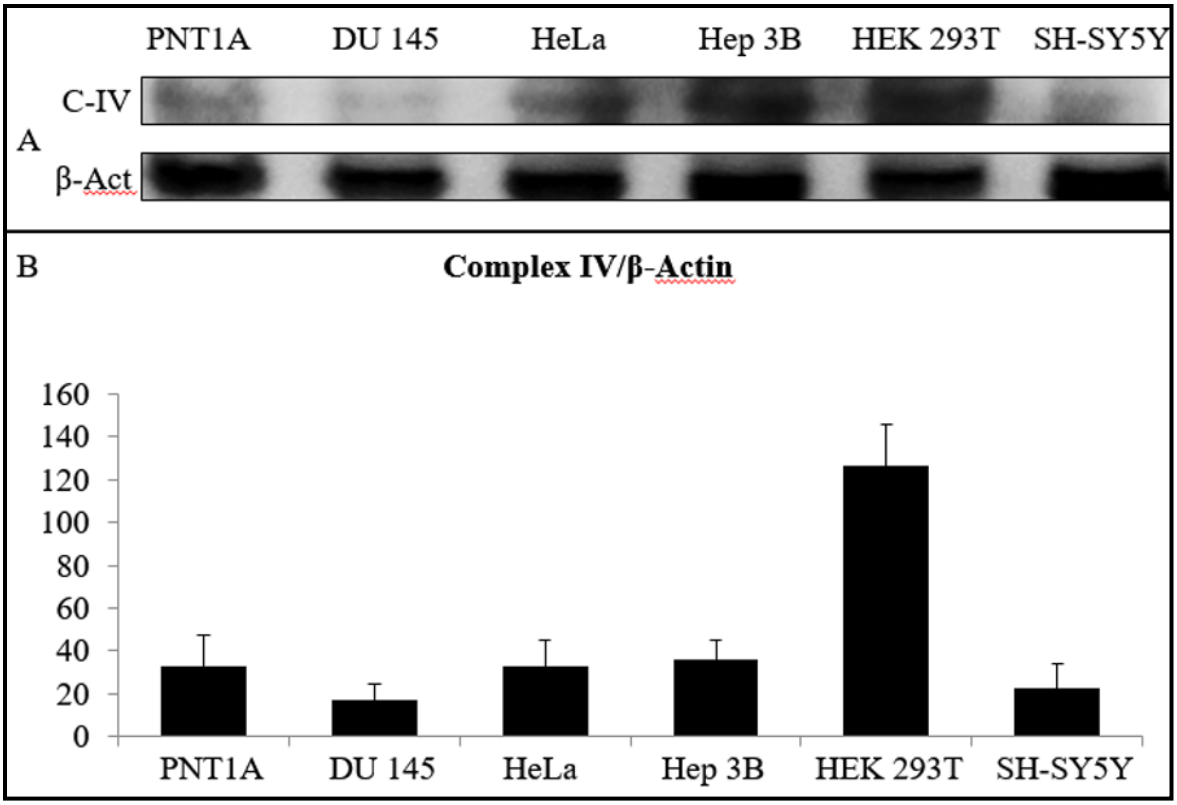

Figure 4. 6. Expression levels and volumetric intensity segregation of complex IV. Immunoblotting analyses of **(A)** Complex IV (Cytochrome *c* oxidase) MTCO1 subunit, 40 kDa, and β-Actin, 45 kDa, in corresponding cell lines. Both Complex IV, and β-Actin were mouse monoclonal antibodies, and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** The expression of Complex IV obtained at least three different replicates of Western Blot assays and normalized to β-Actin.

Generation of water molecules with oxygen and four hydrogens translocated and coordinated by complex IV and often results in lowered protein expression levels in cancers, so it was employed to obtain downregulation. Maximum Complex IV levels collected from HEK 293T. Besides, HEK 239T expressed more than three-fold of Complex IV, compared to PNT1A. There was a slight difference between HeLa and Hep 3B cell lines. However, HeLa and Hep 3B obtained with the higher Complex IV amount not only from DU 145, and SH-SY5Y but also from PNT1A.

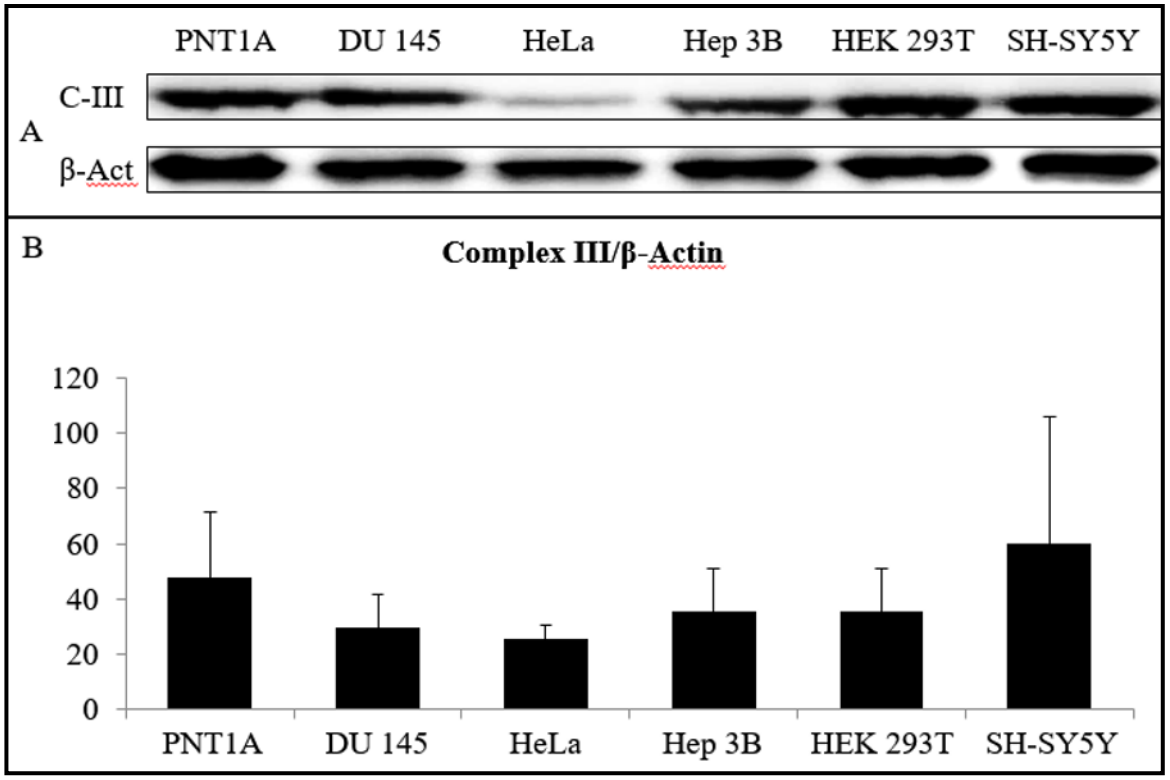

Figure 4. 7. Immunoblotting and relative quantification grades of complex III. Different expression levels of **(A)** Complex III (ubiquinol cytochrome *c* reductase)-Core protein 2, 48 kDa, and β-Actin, 45 kDa, in mammalian cell lines. Both Complex III and β-Actin were mouse monoclonal antibodies, and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** Minimum three independent experiments were performed, and normalized to β-Actin, during the relative quantification of Complex III.

Similarly, complex III also pumps four protons out from mitochondrial matrix to inner mitochondrial membrane and produces membrane potential in normal cells, therefore it was aimed to observe the different expression levels between normal and cancer lines. SH-SY5Y expressed more Complex III protein than all other cell lines. Additionally, the massive reduction was observed in HeLa cells. Moreover, almost no discrepancy was determined between Hep 3B, and HEK 293T cell lines, unexpectedly.

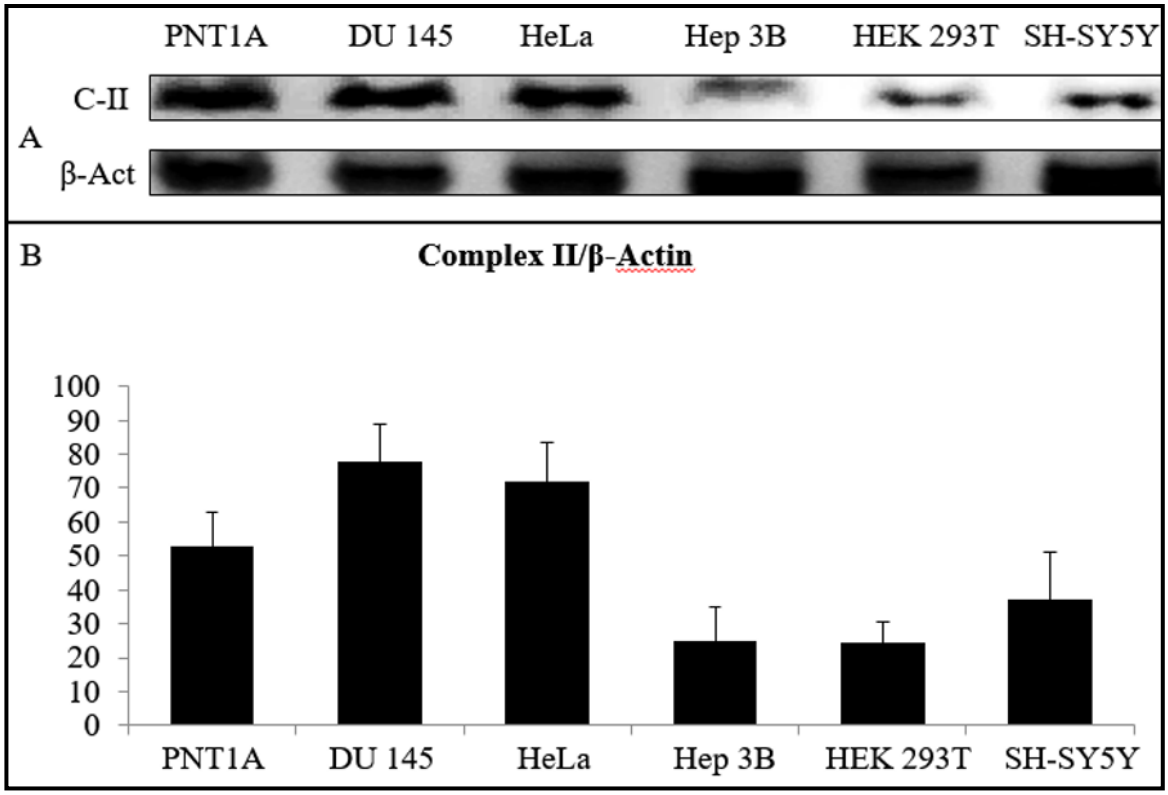

Figure 4. 8. Expression levels and volumetric intensity differentiation of complex II. Western Blot analysis of **(A)** Complex II, with traces of Complex III (Succinate dehydrogenase), 30 kDa, and β-Actin, 45 kDa in healthy and cancer cell lines. Both Complex II, and β-Actin were mouse monoclonal antibodies, and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** Relative quantification of Complex II levels measured and normalized to β-Actin in corresponding cell lines with three independent experiments.

Complex II-mediated ATP production renders on $FADH_2$ and/or oxidation of succinate to fumarate, also another ETC member that expected to altered extend of proteins in tumor formations. DU 145, and HeLa were highly Complex II expressed cell lines, even more than healthy PNT1A, and HEK 293T cell lines, but DU 145 has the greatest amount. DU 145 and HeLa represent at least, two and a half fold more Complex II from Hep 3B and HEK 293T, and partially two-fold more Complex II than SH-SY5Y.

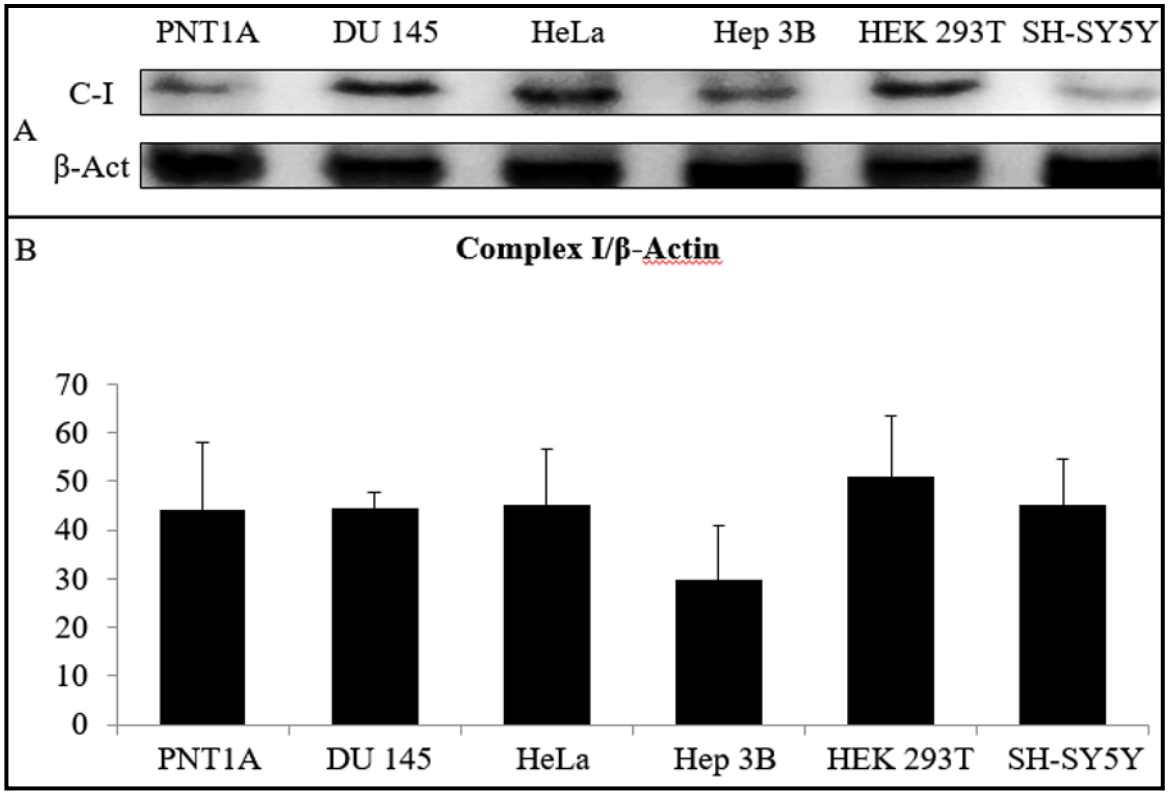

Figure 4. 9. Immunoblotting and relative quantification results of complex I. Different protein expression levels of **(A)** Complex I (NADH dehydrogenase) NDUFB8 subunit, 20 kDa, and β-Actin, 45 kDa, in corresponding cell lines. Both Complex I, and β-Actin were mouse monoclonal antibodies, and prepared with 1:4000 ratio (one µl in four ml TBS-Tween 20, pH: 7.4). **(B)** The expression of Complex I obtained at least three different replicates of Western Blot assays and normalized to β-Actin.

Energy production induces by ETC family member complex I which pumps four hydrogen atoms out from mitochondrial matrix to inner mitochondrial membrane, thus membrane gradient arises primarily in the mitochondria. Additively, distinct expression levels expected in cancer cells, when compared to healthy ones. All mammalian cell lines expressed almost same levels of Complex I. On the other hand, HEK 293T observed with greater, and Hep 3B observed with lesser Complex I expression among PNT1A, and other cancerous cell lines.

4.2. ATP SYNTHASE SPECIFIC ACTIVITY MICROPLATE ASSAY KIT

Samples for ATP Synthase Specific Activity Microplate Assay Kit collected from whole cell extracts with corresponding cell lines. The purpose of this kit is to identify the enzymatic activity of ATP synthase (Complex V). To accomplish this purpose, Complex V samples were loaded into wells of the microplate and immunocaptured by wells. Later, to measure activity levels of Complex V, generated NAD+, which was oxidized from NADH, interacts with ADP, which was transformed from ATP, render dwindle in absorbance values in 340 nm. Collected data were measured, and calculated by Microsoft Office Excel.

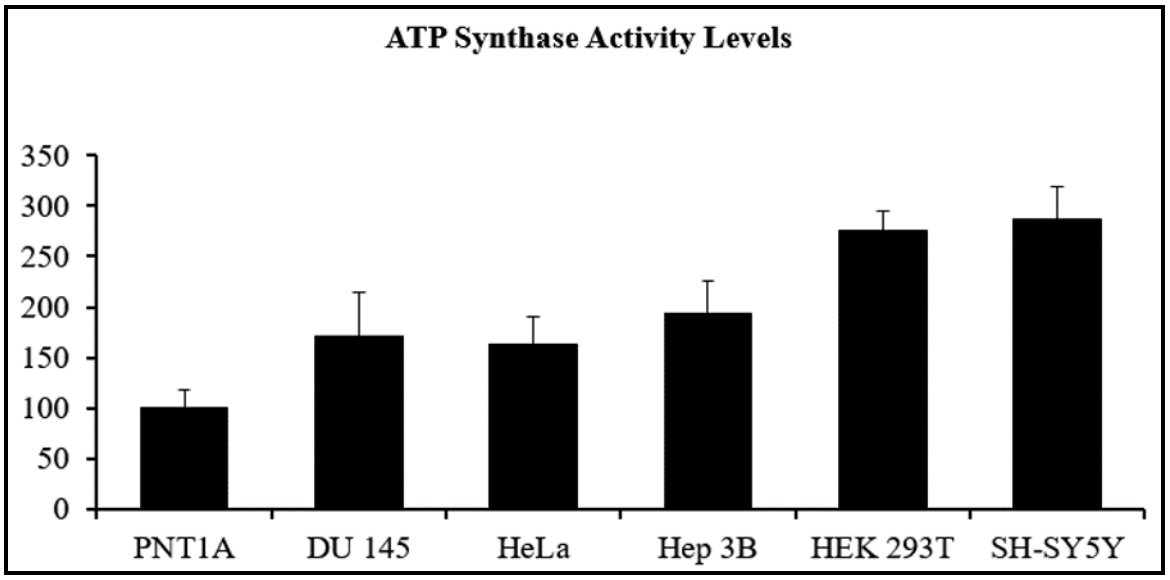

Figure 4. 10. Complex V comparison among employed cell lines.
Relative quantification of ATP synthase activity levels measured and normalized to PNT1A cells with corresponding cell lines with three independent applications.

SH-SY5Y and HEK 293T demonstrated the highest activity among all cell lines. Surprisingly, PNT1A, determined with the lowest Complex V activity compared to both HEK 293T, and cancer cell lines. Moreover, DU 145, HeLa, and Hep 3B cancers also noticed with lower activity levels than HEK 293T. To sum up, both healthy cells and cancer cell lines displayed various activity levels; even some cancer types may have higher complex V activity than healthy cell lines.

4.3. MITOCHONDRIAL MEMBRANE POTENTIAL MEASUREMENT

To observe the diversifications in mitochondrial membrane potential among PNT1A, DU 145, HeLa, Hep 3B, HEK 293T, and SH-SY5Y cell lines, Rhodamine123-related staining was performed and analyzed using flow cytometry. The negative control, (Neg. Cont.) specifies unstained cells without Rh123, and positive control, (Pos. Cont.) remarks Rh123 stained cells. Additively, y-axis, labeled as "Counts", refers counted cell numbers, and the x-axis, labeled as "FL-1-Height", represents the fluorescent intensity of Rhodamine123 dye. Each Rh123 experiment was performed through 10000 counts, by FACScan flow cytometer.

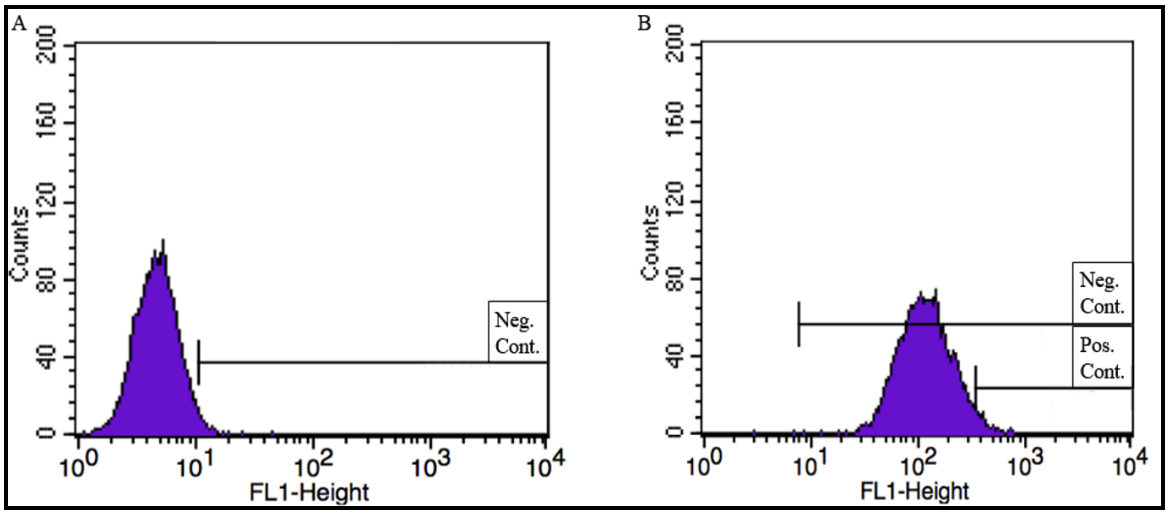

Figure 4. 11. Flow cytometry analysis of Rh123 uptake upon PNT1A cell line.
(A) The negative control, unstained cells, on the left, were measured first, then **(B)** positive control, cells that stained with Rhodamine123, on the right, determined. Documented image is representative of one of three different analyses.

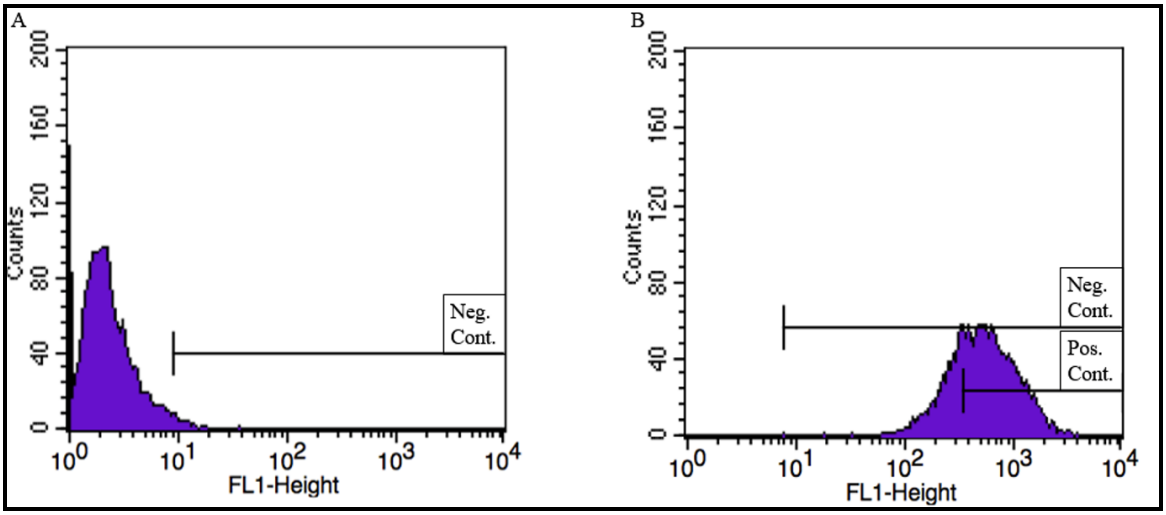

Figure 4. 12. Rhodamine123 intake analyzed by FACScan flow cytometer on DU 145. **(A)** On the left, unstained DU 145 cells, and **(B)** on the right, Rh123 stained DU 145 cells were evaluated. One experiment representative of three is shown.

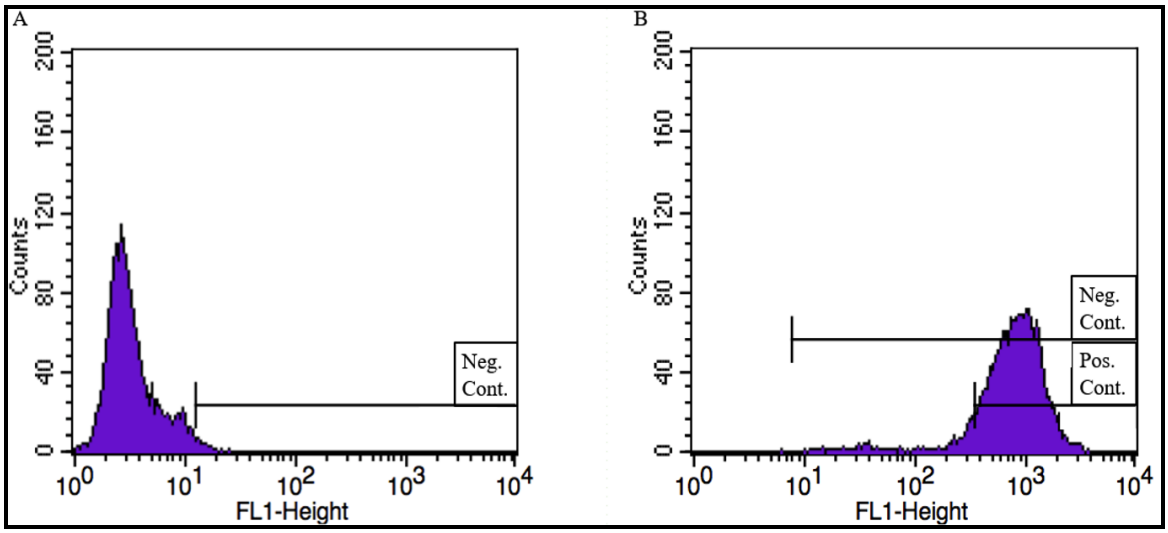

Figure 4. 13. Flow cytometry analysis of Rhodamine123 upon HeLa cell line. Rh123 unstained, and stained cells experimented on the left **(A)**, and right figures **(B)**, respectively. Highlighted figure is emblematical of one of the three independent experiments.

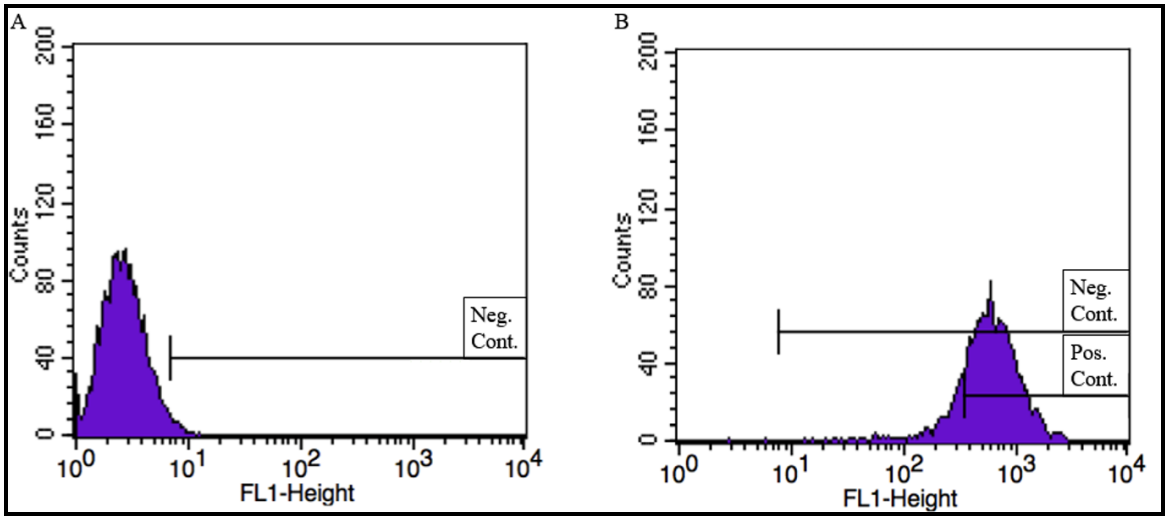

Figure 4. 14. Rh123 uptake analyzed flow cytometrically on Hep 3B.
First, **(A)** unstained Hep 3B cells on the left and **(B)** Rhodamine123 stained Hep 3B cells on the right, were analyzed. Displayed figure is depicting one of three different replicates.

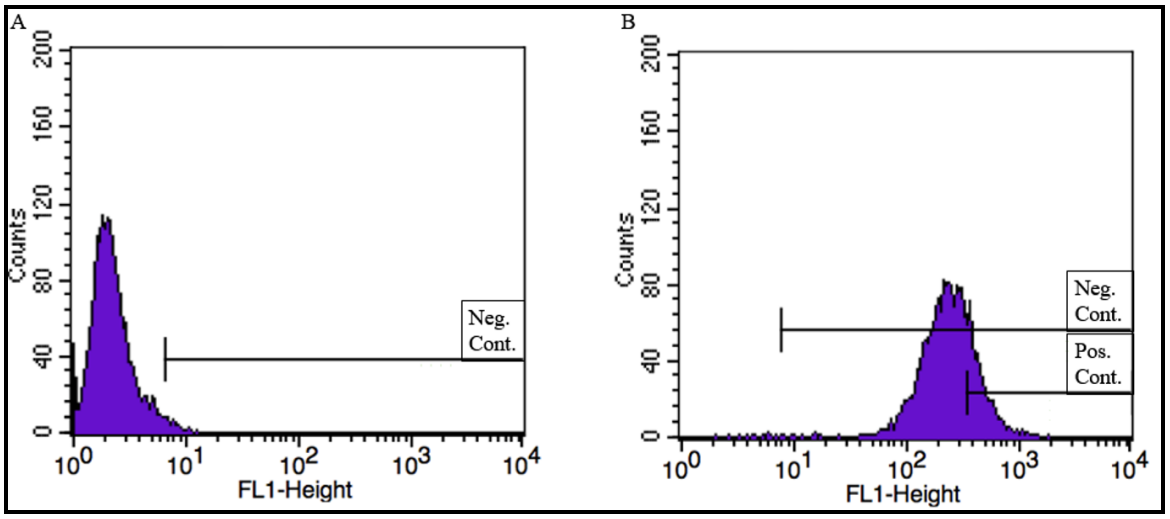

Figure 4. 15. Flow cytometry analysis of Rh123 upon HEK 293T cell line.
From left to right, **(A)** unstained, and **(B)** stained cells experimented, consecutively. Illustrated image is indicating one of three independent experiments.

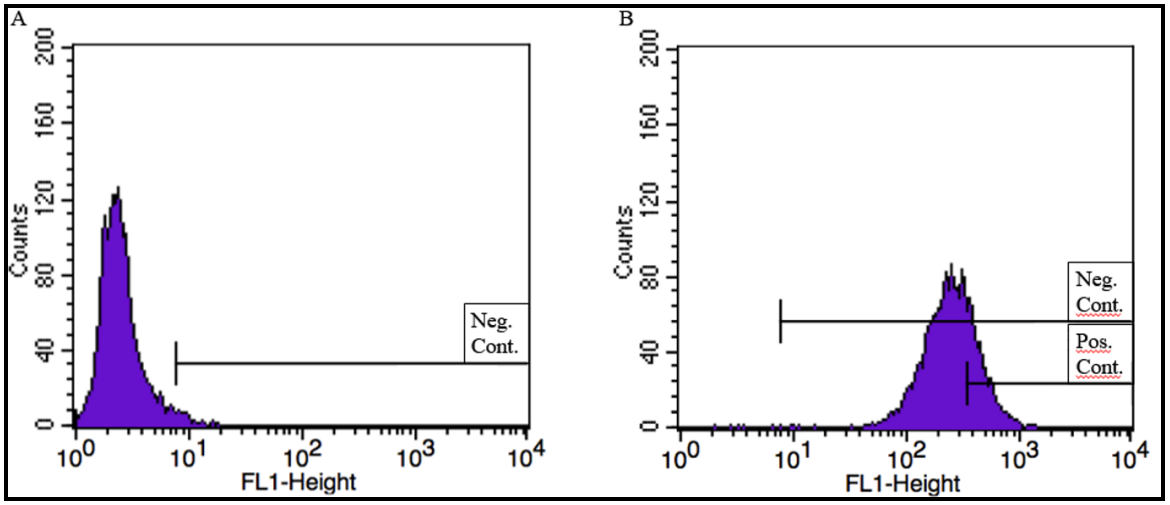

Figure 4. 16. Rhodamine123 intake analyzed via flow cytometry on SH-SY5Y. Antecedently, **(A)** unstained SH-SY5Y cells on the left, and then **(B)** Rh123 stained cells on the right, were indicated figure is descriptive of one of the three disparate applications.

Whole cell pellets from mammalian cell lines, stained with fluorescent and cationic Rh123 dye, which piles up in mitochondria as a superintending feature by membrane potential and then released immediately in case of membrane depolarization.

Flow cytometrically represented data from Figure 4.11, to Figure 4.16, with regarding to Rh123 analysis upon all cell lines, revealed that, 99.5 per cent of PNT1A, 100 per cent of DU 145, 100 per cent of HeLa, 99.96 per cent of Hep 3B, 99.63 per cent of HEK 293T and 99.88 per cent of SH-SY5Y from all cell lines were stained with Rhodamine123, sequentially.

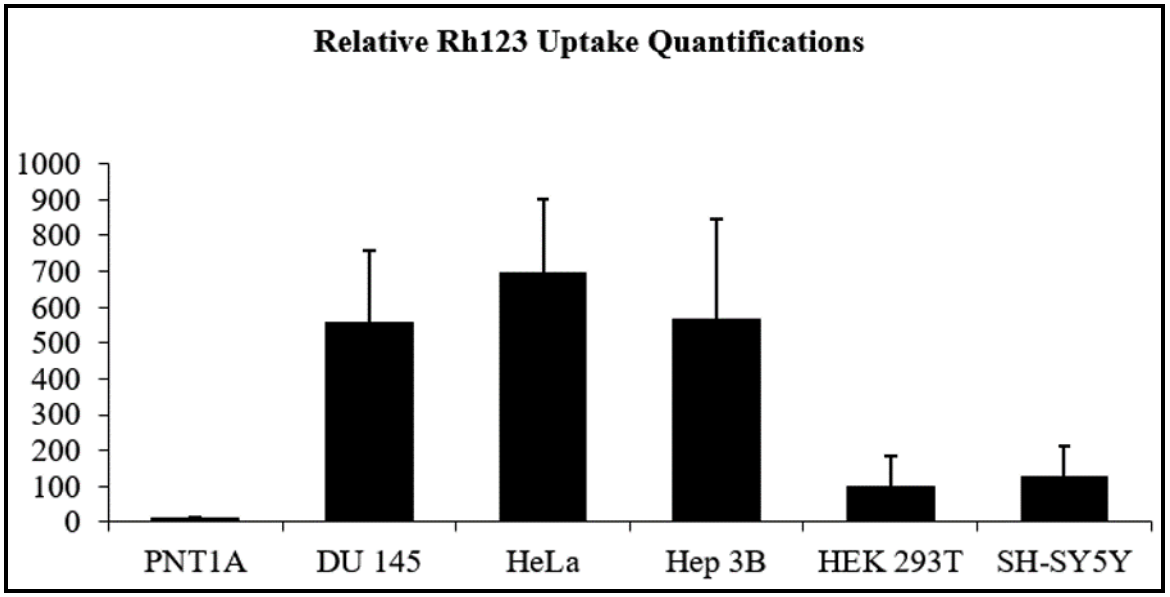

Figure 4. 17. Relative quantification analysis of Rhodamine123 uptake measured and normalized to PNT1A with corresponding cell lines, from three independent applications.

However, there was crucial contrariness on Rh123 intake between PNT1A, and DU 145, on behalf of DU145, which is healthy, and cancer subdivision of prostate cell line. Nonetheless, there was also significant dissimilarity observed between PNT1A, and HEK 293T and among cancer cell lines too. DU145, HeLa, Hep 3B tumors demonstrate highest Rh123 uptake, and PNT1A, HEK 293T cell lines with the lower Rh123 intake.

4.4. MS-BASED PROTEOMIC ANALYSIS

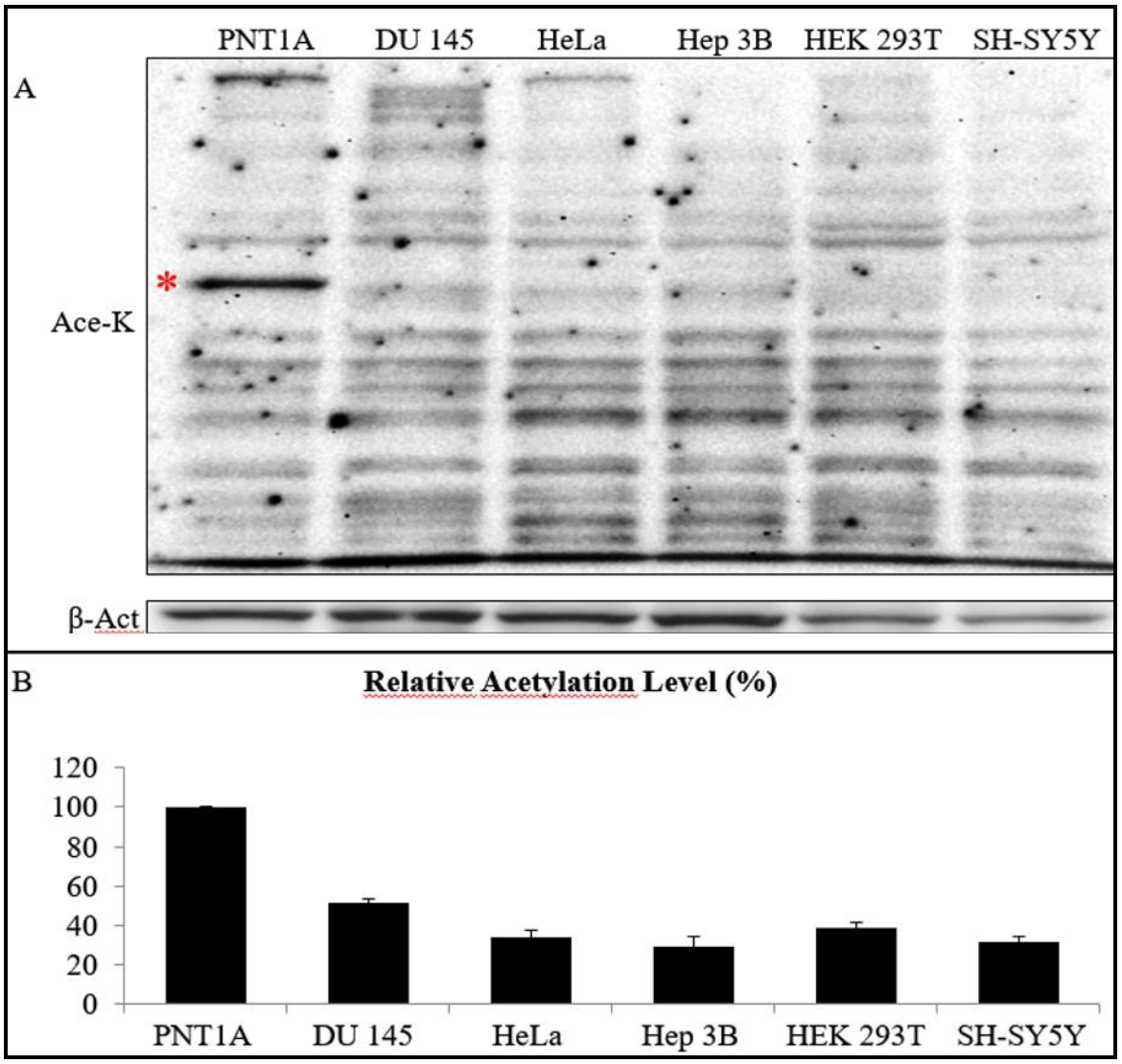

Figure 4. 18. Overall acetylation profile and levels illustrated on employed cell lines. **(A)** Total acetylation level from whole cell extracts and β-actin signal as loading control from corresponding cell lines. **Ace-K:** N-acetyl-lysine rabbit monoclonal antibody, and **β-Act:** β-Actin, 45 kDa, mouse monoclonal antibody. Immunoblotting results were collected by using Clarity Western ECL Substrate and ChemiDOC XRS+ Gel Imaging System. **(B)** Relative quantification graph of relative acetylation level normalized to loading control from three different immunoblotting analyses. The intensity of bands was measured with BIORAD Image Lab 5.2.1 software and then error bars were calculated with Microsoft Office Excel 2013.

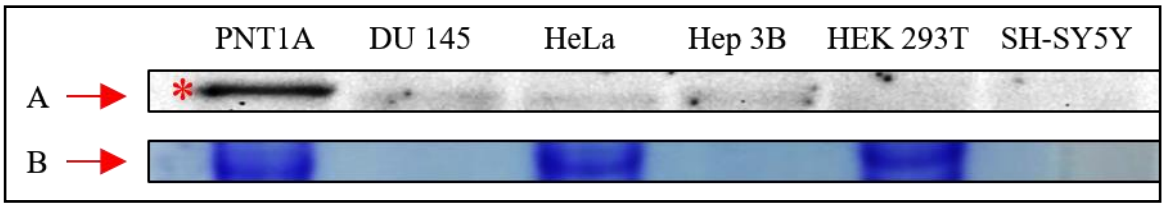

Figure 4. 19. Immunoblotting and SDS-polyacrylamide gel images illustrated. **(A)** Image of the protein band from all cell lines and **(B)** the protein band from triple loaded coomassie stained gel of PNT1A whole cell extract, corresponding to the specific acetylation signal (labeled with *) was in-gel trypsin digested, and analyzed with Bruker Compact ESI-qTOF mass spectrometry coupled with Dionex Ultimate 3000 RSLCnano.

Total acetylome profile was given in Figure 4. 18. As highlighted in **(A)**, acetylation levels and activities fluctuate not only between health and cancer cell lines but also within healthy lines and among cancer cell lines.

Table 4. 1. List of the candidate proteins obtained from MS analyses.
After MS-based proteomic analysis, many of the proteins were determined due to mass and Mascot scores. Three of the proteins which have the highest mass and Mascot scores were listed above.

Protein band from PNT1A: Protein Description	**Mass (Da)**	**Mascot Score**
Tubulin alpha-1B chain	50120	26189
Tubulin alpha-1C chain	49877	24968
Tubulin beta-5 chain	49639	23783

5. DISCUSSION

As aforementioned above, multifaceted mutations, oxygenation and nutrition levels, stress and environmental conditions, which lead gain and/or loss of functions, inhibitory and/or initiatory effects upon receptors, signals, genes, hormones, enzymes, transporters, trigger early tumor formations. Besides, development of cancer does not rely only on one hallmark but also these hallmarks are interconnected to each other.

In this manner, mitochondria have a vital and major role to compensate energy requirement, also have regulatory effects upon death mechanism, cellular transportation, biosynthesis, signaling pathways, cellular metabolism, and some other cellular systems. Damaged mitochondria, lead numerous modifications, deregulations and conformational changes in the cells, such as tumor growth, together with other hallmarks [235,173].

Energy generation in the form of ATP, mainly obtained from two pathways, glycolysis, and more often oxidative phosphorylation, in normal cells. Nevertheless, Warburg stated that cancer cells usually prefer glycolysis to produce ATP, even under well-oxygenated environments and normal mitochondrial structures [236]. However, recent studies [237] demonstrated, various cancer cells [238-240], and some solid tumor formations [241], produce ATP via OXPHOS, during the biosynthesis of glutamine, pyruvate, and lactate when normoxic conditions supplied [242,243,239,244] and healthy mitochondrial structure presented. Furthermore, they also generate ATP via OXPHOS when met with normal cells. Some cancer cells, DU 145, HeLa, Hep3B, and SH-SY5Y displayed higher respiratory activity rather than healthy forms [241].

Up to now, it was proposed that all cancer cells usually compensate energy requirement via glycolysis but special thanks to recent studies revealed that some cancer cells, specifically HeLa do not produce energy only from glycolysis under appropriately oxygenated conditions. This cancer line displayed not only two to four-fold increased glycolytic activity but also enhanced oxidative phosphorylation which provides approximately 60 per cent of the ATP requirement of the cell which may deregulate cell division and growth mechanisms and contribute for the upregulation of tumorigenesis.

GLUT4 expression levels from highest to lowest in SH-SY5Y tumor, HEK 293T normal line, HeLa and Hep 3B cancers, demonstrate the capability of glucose uptake independently from insulin signaling. Additively, less expressed GLUT4 in healthy PNT1A line and DU 145-cancer line, may rely on two factors. First, another GLUT family member may regulate glucose intake [151]. Second, the activity of glucose transportation is linked to existence of insulin hormone. Recently published research, highlighted the association between the repressive effect of intact p53 upon GLUT4 expression [153], and data analysis of p53 protein levels, sequenced from highest to lowest expression; PNT1A, DU 145, SH-SY5Y, HEK 293T, HeLa, and Hep 3B, respectively. Indicated results in this study, significantly match with highlighted study.

Central regulatory protein, p53 is an acknowledged transcription factor, influences divergent cellular mechanisms, such as tumor suppressing, pivotal in apoptosis pathway and maintenance of genome homeostasis. Unlike healthy cells, cancer cells alter almost all cellular processes, including p53-mediated mechanisms, thus mutated and suppressed the activity of p53, frequently determined in many of the tumor formations [143,169,171,164].

Detected low amount of oxidative phosphorylation complexes III [245], IV and V in cancer cell lines with contrast to healthy cell lines, may be caused by inhibition of OXPHOS expressing genes, and increased GLUT4 levels in tumors [147], as many other glycolytic activity promoters [246,247], might lead upregulation of glycolytic pathway to generate energy. Moreover, downregulation of Complex IV was often expected in tumors, yet, HeLa and Hep 3B determined with higher Complex IV expression than DU 145 and SH-SY5Y. In addition, there is a huge difference between expression levels of Complex IV and V, between normal cell lines. The reason of this diversity could rely on metabolic differences, the origin of the tumor, insulin-dependent glucose uptake.

Excessive casp-9 levels in cancer cells might be a result of down-regulation of p53 and inactivated intrinsic death pathway proteins. Suppressed cytochrome *c*, cannot activate casp-9 via ROS. Repressed casp-3 levels in tumors, even with high casp-9 levels, could be answered by the successful down-regulatory effects of numerous anti-apoptotic signals and proteins, mutated p53 or inhibited metabolic signals.

At the end of the ATP synthase (Complex V) activity assay, minimum and maximum activity values were collected from PNT1A and from HEK 293T, respectively. Complex V

was more active and outnumbered in normal HEK 293T cell line, and more expressed in healthy PNT1A prostate cells; rather than all tumor cell lines. Thus, activity assay and immunoblotting assay results revealed that not only expression levels, but also the activity of this molecule plays a significant role in the generation of ATP. Moreover, the alpha subunit of the Complex V might highly active in HEK 293T rather than PNT1, hence, ATP generation and energy requirement is mostly directed by electron transport chain complexes in PNT1A and HEK 293T cell lines. These results can contribute to understanding Complex V activity and expression levels, which can vary for each cell or tissue due to their characteristics, even in between healthy organisms.

With respect to the flow cytometry analysis of Rh123, all cell lines were successfully stained with this cationic dye. Since, hyperpolarized mitochondria enhances mitochondrial membrane potential via decreasing the efficiency of the electrochemical gradient is extremely common among cancer cells [248]. Relative analysis of mitochondrial membrane potential normalized to PNT1A highlighted massive variation between 10- to 60-fold. In 2013, Panov *et al.* documented that isolated DU 145 mitochondria had nearly two-fold higher MMP than healthy prostate [249], but our analysis upon prostate cancer demonstrated approximately 60-fold higher MMP with contrast to PNT1A, also determined three- to six-fold more mitochondrial efficiency on prostate cancers, compared with normal prostate cells [249].

6. CONCLUSION AND FUTURE PROSPECTS

Disperse protein expression levels of GLUT4, p53, respiratory chain complexes, casp-9, and casp-3 were authenticated upon normal and cancer mammalian cell lines. Moreover, down-regulation of respiratory chain complex-IV subunit MTCO1 might lead cancer cells to generate a lesser amount of energy, in the form of ATP, from oxidative phosphorylation pathway in the hypoxic conditions.

Cancer cells display hyperpolarized mitochondria, which latter render enhanced activation of pro-apoptotic components, immediately, as expected. High expression levels of casp-9, in contradiction with casp-3 explain the alterations in cellular survival metabolism among tumors.

ATP synthase activity and immunoblotting analysis can contribute to understanding Complex V activity, and expression levels can vary for each cell or tissue due to their characteristics, even in between healthy cell lines.

In agreement with both literature and demonstrated studies until now, cancer cells determined with downregulation of p53 levels, and upregulation of GLUTs liberated from extracellular signals as expected, which may lead them to obligate metabolic changes, such as enhance glycolytic activity depending upon dispersed extracellular conditions, lack of oxygenation and nutrition and signal levels [153]. On the other hand, some cancer forms practiced in this study demonstrate higher respiratory chain complexes which may lead us to reckon for raised oxidative phosphorylation activity to compensate energy requirement. Besides, higher complex V activity and expression levels, and increased mitochondrial membrane potentials in cancer lines support this theory. Lastly, highlighted excessive mitochondrial membrane potential in cancer cells also provide a huge caspase-9 expression level, which promoted and lead by these conformational modifications to upregulate apoptotic structures and induce apoptosis in normal cells.

For the future prospect, to prevent tumorigenesis, medicate cancer and increase the effectiveness the ongoing treatments, not only metabolic but also signaling, homeostatic and apoptotic contributions from mitochondria in various cancer developments must be paid sufficient attention. Because, as indicated in this study, both cancer and normal cell

lines differ from each other during the many cellular activities energy generation, metabolic uptake and activity, cell death and enable cellular homeostasis among different tissue types, which were observed with the altered mechanisms, significantly.

In short, the major and multifaceted effects of “the powerhouse of the cell”, mitochondria, should be investigated and characterized more specifically during the development of new and multifarious treatments for cancer and many other diseases. These include diverse cellular processes such as cell death mechanisms, oncogenes, bioenergetics and metabolic activities, and mitochondrial biogenesis.

Future studies upon identified acetylated protein in PNT1A cell line might be a promising candidate for the future aspect, during the development of the unique therapeutic drugs as personalized medicine upon employed cancer cell lines. The relation between signaling pathway, cell survival mechanisms, and mitochondrial post-translational modifications, should be investigated, most specifically by the acetylation process. In addition, since some cancer cells revealed enhanced oxidative phosphorylation, current treatments may be improved by targeting not only OXPHOS but also together with glycolysis.

Finally, in order to reveal mitochondrial characterization of different cancer cells and develop specific treatments, ADP/ATP ratio, oxygen consumption rate, transcriptional/translational activity of mitochondrial genes, altered metabolome, and proteome should be investigated.

Up to date, there is no applied treatment for mitochondrial diseases. According to our presented results, we conclude that there are differences in mitochondrial activities in between healthy and cancer cell lines, which needs to be evaluated further. The overall mitochondrial proteome and their post-translational modications, particularly acetylation, needs to be targeted as an enlarged theme of the hallmarks of the cancer. Particularly, mitochondria-involved signaling, apoptosis, and bioenergetics could be studied by using high-throughput approaches in order to develop seminal diagnosis and treatments. This would promise a hope for managing the patients suffering from mitochondrial diseases.

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