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Mitochondrial metabolism changes in cancer cells

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Aybolek Babayeva

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1) Vypracujte literární rešerši na téma: Změny mitochondriálního metabolismu u nádorových buněk. V úvodní části bakalářské práce se věnujte morfologii a funkcím mitochondrií.

2) V hlavní části bakalářské práce popište vlastnosti a znaky nádorových buněk a následně se zaměřte na změny v jejich mitochondriálním metabolismu. Zvláštní pozornost věnujte úloze sukcinát-dehydrogenázy.

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Vedoucí bakalářské práce: **Mgr. Pavla Staňková, Ph.D.**
Katedra biologických a biochemických věd

Konzultant bakalářské práce: **doc. RNDr. Tomáš Roušar, Ph.D.**
Katedra biologických a biochemických věd

Datum zadání bakalářské práce: **18. prosince 2021**
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L.S.

prof. Ing. Petr Kalenda, CSc. v.r.
děkan

prof. Mgr. Roman Kanďár, Ph.D. v.r.
vedoucí katedry

V Pardubicích dne 28. února 2022

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TITLE

Mitochondrial metabolism changes in cancer cells

ANNOTACE

Tato bakalářská práce se zabývá změnami mitochondriálního metabolismu u nádorových buněk. Úvodní část je zaměřena na morfologii a funkce mitochondrií. Hlavní část práce se zabývá vlastnostmi nádorových buněk. Zbývající část vysvětluje změny v jejich mitochondriálním metabolismu se zvláštním zaměřením na roli enzymu sukcinátdehydrogenázy. Část práce je věnována také mitochondriálním biomarkerům, využívaných pro diagnostiku a terapii mitochondriálních onemocnění.

ANNOTATION

This bachelor's thesis deals with changes in mitochondrial metabolism in tumor cells. The introductory part focuses on the morphology and functions of mitochondria. The main part of the thesis deals with the features of tumor cells. The remaining part explains the changes in their mitochondrial metabolism with a special focus on the role of the succinate dehydrogenase enzyme. Part of the work is also dedicated to mitochondrial biomarkers used for diagnostics and therapy of mitochondrial diseases.

KEYWORDS

Mitochondria, cancer, mitochondrial DNA, TCA cycle, succinate dehydrogenase, biomarker

TABLE OF CONTENTS

TABLE OF FIGURES 8
TABLE OF ABBREVIATIONS 10
INTRODUCTION 13
1. MITOCHONDRIA 14
1.1. Eukaryotic cell and the origin of mitochondria 14
1.2. Structure of mitochondria 17
1.3. Mitochondrial function 19
2. CANCER 23
2.1. Cancer cells versus normal cells 25
2.2. Energy metabolism in cancer cells 26
2.3. Mitochondrial mechanisms in cell communication and cancer formation 28
2.3.1. Cytochrome c 28
2.3.2. Reactive oxygen species (ROS) 30
2.3.3. AMP-activated protein kinase (AMPK) 31
2.3.4. Mitochondrial DNA (mt-DNA) 32
2.3.5. TCA cycle metabolites 35
3. ABNORMALITIES IN TCA CYCLE ENZYMES AND TUMOR FORMATION 37
3.1. Mechanisms of tumor formation 38
3.1.1. Succinate dehydrogenase 39
3.1.2. Succinate as a cancer metabolite 40
3.1.3. The relationship between succinate and HIF-1a in cancer 41
3.1.4. Prolyl hydroxylase domain (PHD) enzymes 43
4. MITOCHONDRIAL BIOMARKERS 45
4.1. Functional markers measured in blood cells 45
4.2. Biochemical markers of serum/plasma 46
4.3. DNA markers 47
4.3.1. Small- and large-scale deletions 48
4.3.2. mtDNA copy number 48
4.3.3. Cell-free mtDNA 49
4.3.4. Mitochondrial microsatellite instability (mtMSI) 49
4.3.5. Alterations in somatic mtDNA 50
4.3.6. MtDNA and mitochondrial RNA (mtRNA) methylation 50
CONCLUSION 51

REFERENCES 51

TABLE OF FIGURES

Figure 1. Cutaway drawing of a eukaryotic cell 15

Figure 2. Primary versus secondary endosymbiosis 16

Figure 3. Mitochondrion in longitudinal cut 18

Figure 4. Brief description of ATP generation pathway 21

Figure 5. The TCA cycle 21

Figure 6. Mitochondrial respiratory chain complex 22

Figure 7. Estimated annual number of new cancer cases globally for the year 2020 23

Figure 8. The hallmarks of cancer 25

Figure 9. Differences between normal and cancer cells 26

Figure 10. The Warburg effect in cancer cells 27

Figure 11. Crucial signaling functions of mitochondria 28

Figure 12. Role of cytochrome c in apoptotic processes of caspase-3 activation and DNA fragmentation 29

Figure 13. Impact of ROS on cellular structures 31

Figure 14. Role of AMPK as a tumor suppressor and an oncogene, regulated by a wide range of signaling cascades 32

Figure 15. A graphical illustration of the human mitochondrial genome, comprising protein-coding genes, noncoding RNAs and control regions 33

Figure 16. Map of human mtDNA and cancer types related to mutations in mtDNA metabolism 34

Figure 17. The TCA cycle as a signaling hub 35

Figure 18. Cancer-related mitochondrial dysfunction 38

Figure 19. The structure of complex II (succinate dehydrogenase) 40

Figure 20. Behaviors of malignant HIF-1α in tumorigenesis under hypoxic conditions. 42

Figure 21. The proposed mechanism of the interaction between mitochondrial complex II and FH in the HIF-α signaling pathway stability 43

Figure 22. Metabolic components controlling PHD activity 44

TABLE OF ABBREVIATIONS

Acetyl-CoA	acetyl-coenzyme A
ADP	adenosine diphosphate
AMPK	AMP-activated protein kinase
Apaf-1	apoptotic protease-activating factor 1
ATP	adenosine 5′-triphosphate
cf-mtDNA	cell-free mtDNA
cGAS-STING	cyclic GMP-AMP synthase and stimulator of interferon genes
CK	creatine kinase
CoA-SH	coenzyme A
COX	cytochrome c oxidase
DAD2	dopamine D2
DNA	deoxyribonucleic acid
EMT	epithelial-mesenchymal transition
ETC	electron transport chain
FAD	flavin adenine dinucleotide
FGF-21	fibroblast growth factor-21
FH	fumarate hydratase
GIST	gastrointestinal stromal tumor
GLUTs	glucose transporters
G6P	glucose 6 phosphate
GTP	guanosine triphosphate
HK	hexokinase
HNPGLs	head and neck paragangliomas
HREs	HIF response elements
HSP	heavy strand promoters
hTERT	human telomerase reverse transcriptase
IDH	isocitrate dehydrogenase
IL-1β	interleukin-1β

IMS	intermembrane space
KDMs	histone lysine demethylases
KGDHC	α-ketoglutarate dehydrogenase complex
α-KG	α-ketoglutarate
LDHA/B	lactate dehydrogenase A/B
LECA	last eukaryote common ancestor
LHON	Leber's hereditary optic neuropathy
L-2-HG	L-2-hydroxyglutarate
lncRNAs	long noncoding RNAs
LPS	lipopolysaccharides
LSP	light strand promoters
m1A	N1-methyladenosine
MCTs	monocarboxylate transporters
5mC	5-methylcytosine
MD	mitochondrial disease
MDDs	mtDNA depletion syndrome
m1G	N1-methylguanosine
mTOR system	mammalian target of rapamycin
MELAS	mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes
MERRF	myoclonus epilepsy with ragged-red fibers
MFF	mitochondrial fission factor
mtDNA	mitochondrial DNA
mtMSI	microsatellite instability
mtPTP	mitochondrial permeability transition pore
mtRNA	mitochondrial RNA
ΔΨm	mitochondrial membrane potential
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
nDNA	nuclear DNA
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells

ODD	oxygen-dependent degradation
2-OGDD	2-oxoglutarate
OXPHOS	oxidative phosphorylation
PCCs	pheochromocytomas
PDK1	3-phosphoinositide-dependent protein kinase 1
PCr	phosphocreatine
PEP	phospho-enolpyruvate
PFK1	phosphofructokinase-1
PGLs	paragangliomas
PHDs	HIF-1α prolyl hydroxylases
PPP	pentose phosphate pathway
ROS	reactive oxygen species
SDH	succinate dehydrogenase
SNPs	single-nucleotide polymorphisms
SUNCR1	succinate receptor 1
TCA cycle	tricarboxylic acid cycle
TET	ten-eleven translocation
TH	thyroid hormone
TME	tumor microenvironment
TMRM	tertramethylrhodamine
UCPs	uncoupling proteins
UCP1	uncoupling protein 1
VHL	von Hippel-Lindau

INTRODUCTION

Mitochondria are powerful biosynthetic, bioenergetic, and signaling organelles, which play crucial role in cell physiology through regulation of communication between cells and tissues. As a “power plant” of the cell, mitochondria regulate the energy transformation, that is further utilized for maintaining homeostasis in healthy cells and functional organisms. Cells that undergo series of metabolic alterations can end up transforming into cancer cells, which show abnormal division and proliferation, that can invade and destroy normal body tissues. Therefore, deregulated cancer metabolism has been recognized as one of the ten cancer hallmarks [1].

The main purpose of this review is to bring together the available information concerning alterations in mitochondrial function, which has been proven to have a clear impact on the physiology of tumor cells and particularly on its significance in signaling cell survival versus cell death and in the reprogramming of energy metabolism. Understanding these differences of mitochondrial structure and function in between normal cells and cancer cells will not only allow us to offer clinical markers for diagnoses and prognosis, but may also develop into a key target in cancer therapy.

1. MITOCHONDRIA

All living organisms require a continuous source of energy for their growth, movement, reproduction, and maintenance of viability. Heterotrophs, i.e. organisms that are unable to synthesize their own carbon-based organic compounds from inorganic sources, obtain their energy through a process called cellular respiration. Cellular respiration enables the regulated release of free energy from the energy substrates – protein, fat, and carbohydrates. The main center of the cell, that is responsible for the regulation of the cellular respiration and the generation of most of the ATP needed to drive the cell's biochemical reactions, is a powerful double-membrane structure known as mitochondria [2].

In addition to being the hub of the majority of biosynthetic pathways, mitochondria also control cellular and mitochondrial redox status, produce most of reactive oxygen species (ROS), control Ca^{2+} concentrations, and have the ability to trigger cell death through activation of the mitochondrial permeability transition pore (mtPTP). Furthermore, studies conducted over the past years have revealed connection of mitochondria to a wide range of cellular processes and pathobiologies, such as cell signaling, metabolism, cell death, aging, and cancer [3], [4].

1.1. Eukaryotic cell and the origin of mitochondria

The eukaryotic cell is characterized primarily by the presence of a structured nucleus and cytoplasmic organelles like mitochondria, which is not the case with prokaryotic cells (Fig.1).

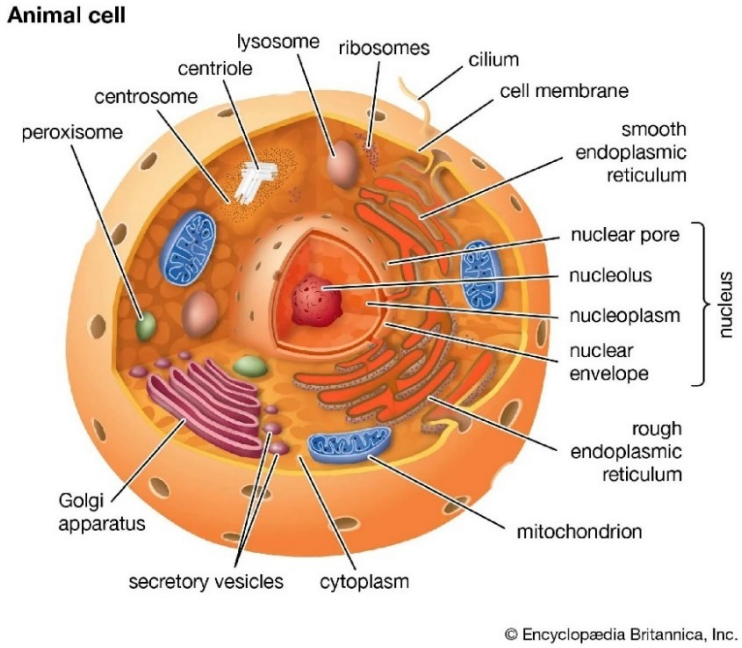

Figure 1. Cutaway drawing of a eukaryotic cell [5]

The nucleus is the cell's control center, which contains the genome, the genetic information storage [6]. Organelles are intracellular membrane-bound structures, typically specialized for a particular function within the cell. They possess characteristic sizes, shapes, compositions, and positions within cells, allowing them the regulation of the numerous cellular processes [7].

Some organelles are specialized and present only in specific cell types, but many organelles share physical similarities and provide essentially the same functions in all eukaryotic cells.

The so-called membrane-bound organelles are isolated from the rest of the cell by membranes, whilst others, like the ribosome, are not. The nuclear envelope and the endoplasmic reticulum are both continuous membrane systems that penetrate the cytoplasm. The secretion of proteins or other materials from the cell to the outside occurs through the Golgi apparatus, a stack of flattened membrane sacs and accompanying vesicles. The membrane-bound organelles known as lysosomes are designed for digestion and contain degradative enzymes [8]. First descriptions of these organelles were given in the 19th century with the advent of light microscopy and the development of cell theory. The identification of new organelles during the 20th century was made possible by electron

microscopy and subcellular fractionation. Furthermore, with help of radiolabeling the first contemporary research on their biogenesis was enabled [9].

The main driving factor behind eukaryotic cell evolution is endosymbiosis. According to the endosymbiotic theory, mitochondria and plastids were previously free-living prokaryotes that later evolved into organelles of eukaryotic cells. The theory was first referred to plastids and later involved mitochondria. The theory's key advantage is that it explains why organelles and prokaryotic cells are similar in terms of their physiology and biochemistry [10].

The term “primary endosymbiosis” describes the process by which the mitochondria and chloroplasts were first formed when initially an ancestral eukaryotic cell internalized a prokaryotic cell. Mitochondria and chloroplasts are encased in two membranes. The exterior membrane of mitochondria or chloroplast is established to be descended from the membrane of the host cell, whereas the inner one is descended from the bacterial progenitor. Algae in particular appear to have been absorbed by a number of protozoan lineages, as opposed to other single-celled eukaryotes. Therefore, by a process known as “secondary endosymbiosis”, several types of algae have chloroplasts that were obtained second-hand (Fig.2) [11].

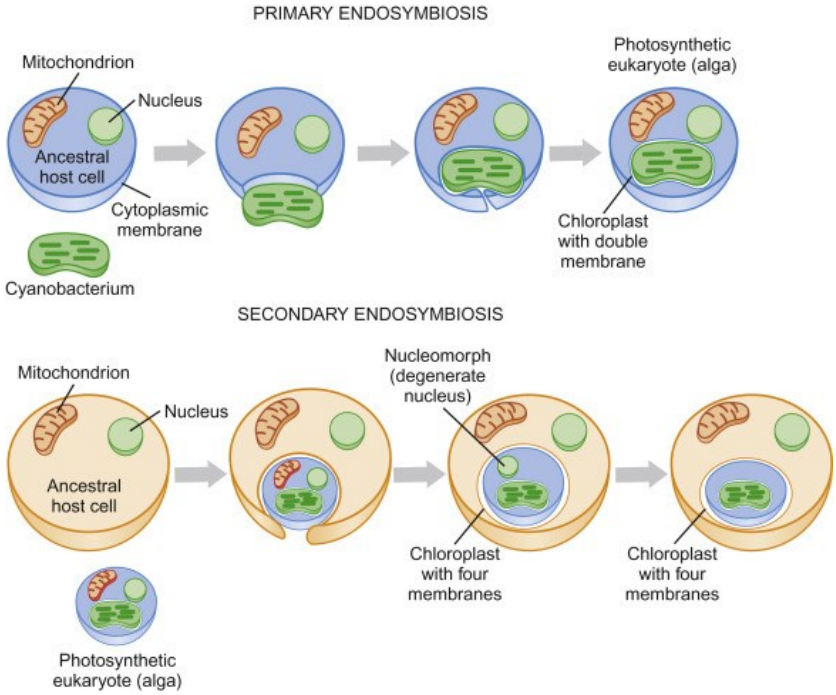

Figure 2. Primary versus secondary endosymbiosis. The initial independent cyanobacterium possesses both an outer and cytoplasmic membrane that are destroyed after symbiosis. The cyanobacterium is encircled by two membranes after the two cells connect, leaving the cyanobacterium in the host-cell cytoplasmic membrane. In contrast to primary endosymbiosis, secondary endosymbiosis happens when a photosynthetic eukaryotic alga is engulfed by an

ancestral host cell. The chloroplast of the alga already has two membranes, a nucleus, and other organelles. The other abducted organelles deteriorate and finally disappear since the host cell only requires the energy from the chloroplast. However, the membranes frequently persist, leaving the chloroplast with four as opposed to two membranes [11].

Furthermore, the availability of complete genome sequence data from both bacteria and eukaryotes provides information about the contribution of bacterial genes to the origin and evolution of mitochondria. Phylogenetic analyses based on genes located in the mitochondrial genome indicate that these genes originated from within the endosymbiotic alpha-proteobacteria in an archaeal-derived host cell. A number of ancestral bacterial genes have also been transferred from the mitochondrial to the nuclear genome, as evidenced by the presence of orthologous genes in the mitochondrial genome in some species and in the nuclear genome of other species [6], [12].

The transition from an autonomous endosymbiotic alpha proteobacterium to the mitochondrial cenancestor entailed many evolutionary changes such as genome reduction. In general transition has three stages pre-mitochondrial alpha proteobacterium, intermediate proto-mitochondria and mitochondrion last eukaryote common ancestor (LECA). The mitochondria of LECA, also known as the “mitochondrial cenancestor”, are completely integrated organelles in eukaryotic cells that were able to undertake aerobic respiration as well as a variety of other metabolic processes [13].

Till this day endosymbiotic theory for the origin of organelles offers the best explanation for similarities of chloroplasts and mitochondria to free living bacteria. Even though there are other noteworthy alternative theories, they usually tend to offer unstated premises, which make them more challenging to be accepted [10].

1.2. Structure of mitochondria

In the cells of animals, plants, and fungi, mitochondria are semiautonomous organelles that resemble chloroplasts of plant cells. They are usually located in the cytoplasm of eukaryotic cells and their size can vary between 0.5 micrometer to 1 micrometer. They have mostly tubular shape and are made of two specialized membranes, an interior and an outer membrane, which are structurally and functionally different from one another. One significant difference is their permeability characteristics. The inner membrane functions as an effective barrier to even small, charged molecules like ions and protons, while the outer

membrane allows unrestricted passage of the majority of molecules with a molecular weight of less than about 10,000 daltons [12]. The protein porin creates numerous channels, that play a significant role in filtering out big molecules in the outer membrane, while a collection of transport proteins is present in the inner membrane to facilitate the flow of various ions and metabolites from the cytosol to the mitochondrial matrix [14].

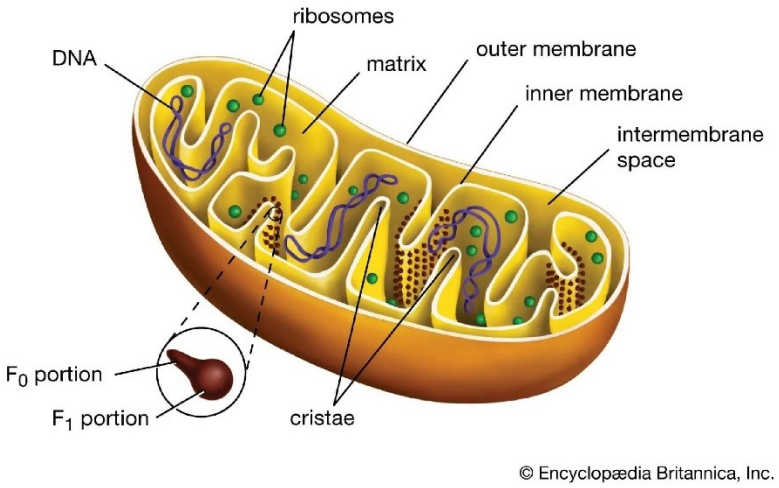

Figure 3. Mitochondrion in longitudinal cut [15]

Furthermore, the inner membrane is the site of the respiratory chain and ATP generation. The cristae, which are the folded structures on the inner membrane of mitochondria, serves as a residing place for transport proteins, electron transport chains, and enzymes that produce ATP. The major coupling factors F1 (hydrophilic protein) and F0 (hydrophobic lipoprotein complex) are also located in the cristae. Together these factors make up the ATPase complex that is activated by Mg^{+2} [16].

There are two soluble submitochondrial compartments, the intermembrane space, and the matrix. The matrix houses both the enzymes in charge of the primary oxidative metabolic reactions and the mitochondrial genetic system. The key participants in the oxidative breakdown of carbohydrates, fatty acids and amino acids are the enzymes of the citric acid cycle [6].

Mitochondria carry its own ribosomes and cyclic DNA, which does not segregate during meiosis since it is outside the nucleus of the cell. The mitochondrial DNA (mtDNA) in humans is tiny (approximately 16.5 kbp), fully sequenced, and contains genes that specifically code for 13 proteins involved in oxidative phosphorylation [17]. Since mitochondria of sperm do not penetrate the egg or are degraded after fertilization, inheritance

is solely maternal and occurs through the mtDNA found in the mitochondria of the ovum [18].

Time-lapse microcinematography of living cells reveals that mitochondria are extremely changeable and mobile organelles, continually altering their shape and even fusing with one another and then separating again [19]. This highly dynamic nature of mitochondria is explained by its continuous fusion and division, which exhibits tightly controlled pattern. These dynamic behavior patterns regulate the amount, distribution, and morphology of mitochondria inside the cell, and as a result, they become crucial for a variety of mitochondrial functions including energy production, metabolism, intracellular signaling, and apoptosis [20].

1.3. Mitochondrial function

Cells rely on mitochondria for their energy supply, which is delivered in form ATP (adenosine 5′-triphosphate). ATP is an organic compound, which serves as a universal energy source for all biological systems. It is chemical composition consists of adenine, a nitrogenous base, ribose, the sugar molecule, and a chain of three phosphate groups bound to the sugar molecule. Energy in ATP is stored in between its phosphate bonds and is released as these phosphate groups breaks apart. The majority of ATP synthesis takes place during cellular respiration inside the inner mitochondrial membrane, producing about 32 ATP molecules for every glucose molecule that is oxidized [21].

Glucose is the primary source of metabolic energy in animal cells. Once it enters the bloodstream, glucose can be taken up by tissues with help of specific glucose transporters (GLUT1-GLUT4) located in cell membranes [22].

The first phase of glucose metabolism (glycolysis) takes place in the cytoplasm of the cell, where a 6-carbon molecule of glucose is oxidized to produce two 3-carbon molecules of pyruvate. In the presence of aerobic conditions, pyruvate continues its metabolism in the mitochondria and undergoes oxidative phosphorylation. Whereas under anaerobic conditions pyruvate remains in the cytoplasm, where it is transformed into lactate by the enzyme lactate dehydrogenase [23], [24].

Pyruvate dehydrogenase is a multienzyme complex located in the mitochondrial matrix, which helps to convert pyruvate to acetyl-CoA. Acetyl-CoA in its turn powers the tricarboxylic acid (TCA) cycle and generates six nicotinamide adenine dinucleotide

(NADH) + H^+, four flavin adenine dinucleotide (FADH2), and six CO_2 molecules per molecule of glucose [25], [26].

NADH + H^+ and $FADH_2$, that come from TCA cycle, serve as electron donors [27]. The TCA cycle is made up of eight steps, each of which is catalyzed by various enzymes. The first intermediate metabolite of the TCA cycle, acetyl-CoA, is coupled with oxaloacetate to produce citrate and to release coenzyme A (CoA-SH). With help of aconitase enzyme citrate is further transformed into isocitrate as a result of hydration and dehydration reactions. As isocitrate loses a molecule of C_2O, it undergoes oxidation, which results in the formation of α-ketoglutarate. The loss of a C_2O molecule of α-ketoglutarate ends with oxidization to succinyl CoA, which is further enzymatically converted to succinate. Succinate is then oxidized to form fumarate, which undergoes hydration reaction to produce malate. The cycle is ended with the oxidization of malate to oxalacetate (Fig.5) [28], [29] . In total two molecules of CO_2, a hydrogen atom and two molecules of NADH and $FADH_2$ are produced by this process. Later NADH molecule donates its electrons to the electron transport chain, which is located in the inner membrane of mitochondria. The electron transport chain (ETC) is made up of four protein machines (Complex I – Complex IV), which through series of redox reactions undergo conformational changes to pump protons from the matrix into the intermembrane space (IMS) [30]. The ATP synthase machine, also known as complex V, is a rotating turbine-like structure that powers the phosphorylation of ADP to ATP using the proton gradient produced by respiratory complexes I, III, and IV. Electron flow through these complexes enables generation of a mitochondrial membrane potential, which is the key for ATP production. This whole process is known as oxidative phosphorylation (OXPHOS) and it only takes place in the presence of oxygen. Complex I and II of ETC replenish NAD^+ and FAD, respectively, which then enables the proper functioning of TCA cycle. The only enzyme, which takes part in both the TCA cycle and the ETC, is the succinate dehydrogenase (SDH). We will discuss more about this enzyme in the upcoming chapters [31].

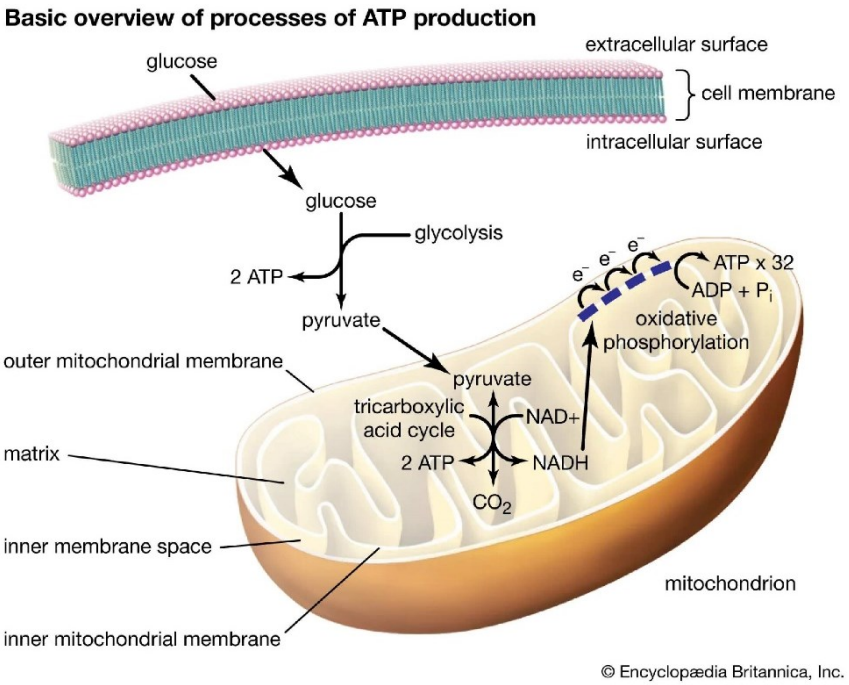

Figure 4. Brief description of ATP generation pathway**.** Glycolysis, the tricarboxylic acid cycle, and oxidative phosphorylation are the three mechanisms that regulate in ATP production. In eukaryotic cells, the TCA cycle and oxidative phosphorylation take place in the mitochondria of the cell [32].

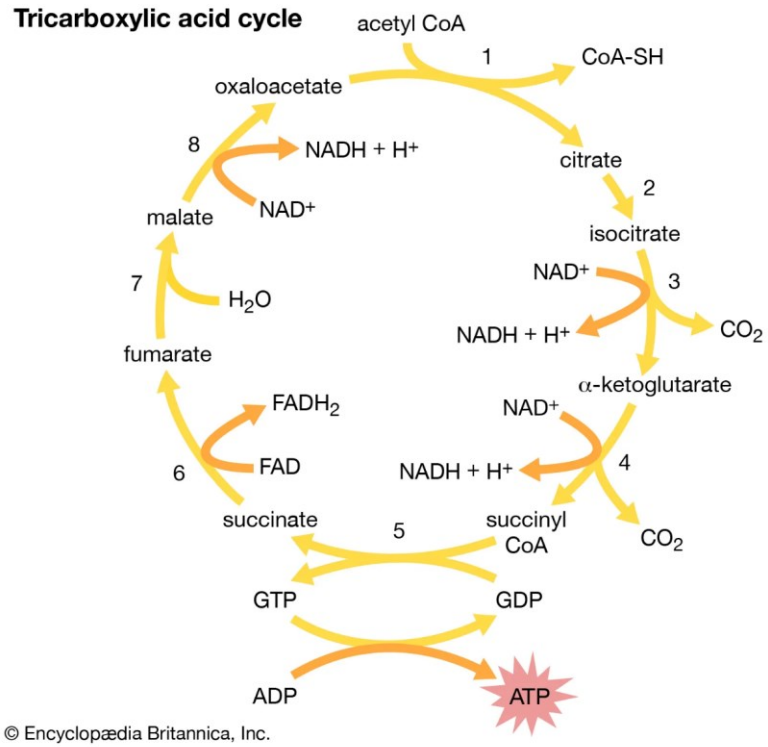

Figure 5. The TCA cycle [29]

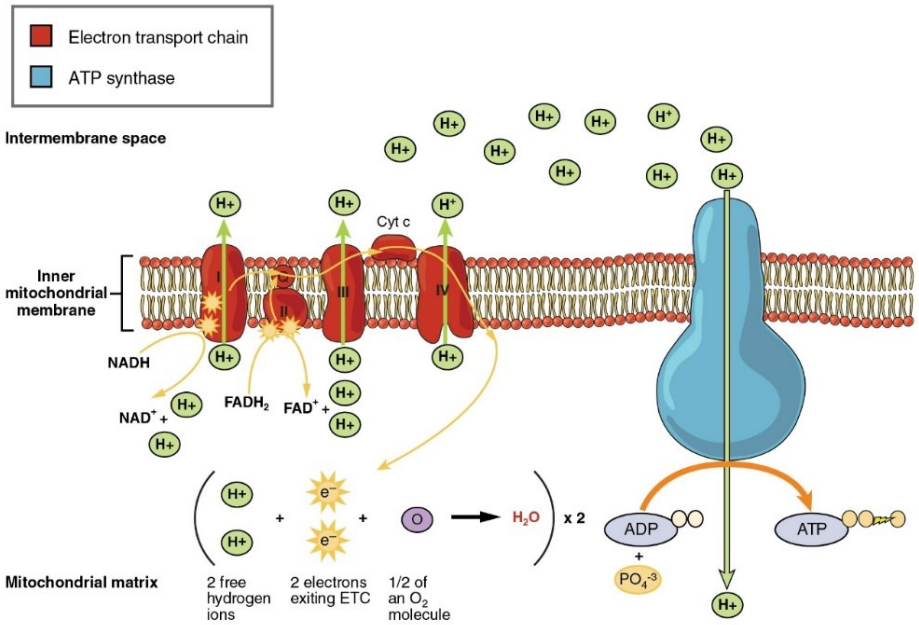

Figure 6. Mitochondrial respiratory chain complex. The inner membrane of the mitochondria houses the mitochondrial respiratory chain. It enables ATP synthesis by oxidative phosphorylation and is made up of four complexes and two coenzymes. Coenzyme Q10 (CoQ10) will receive two electron transfers from Complexes I (NADH: coenzyme Q oxidoreductase) and II (succinate dehydrogenase), respectively. The two electrons that were transported from complex I to complex II were produced by the oxidation of NADH and the oxidation of succinate to fumarate, respectively. Electrons can be transferred to complex III (CoQ10-cytochrome C oxidoreductase) with the use of CoQ10. When cytochrome C connects with complex IV (cytochrome C oxidase), the complex III will then transfer these electrons to it. In a molecule of H_2O, the complex IV decreases O_2. Proton pumps, such as Complexes I, III, and IV, enable the movement of protons in the opposite direction of the gradient from the matrix to the intermembrane space. Complexes I and III let the passage of four protons, but complex IV only permits the passage of two protons. The final complex of the chain, ATP synthase, will enable protons to pass once the intermembrane gap has been enriched with protons. This allows protons to flow in the gradient's direction. The synthesis of ATP from ADP will be made possible by this proton influx [32].

2. CANCER

With approximately 10 million deaths as per the year 2020, cancer is estimated to be the leading cause of death globally. According to the WHO, from the recent cancer cases for the year 2020, the most prevalent cancer types are estimated to be breast cancer, lung cancer, colon and rectum cancer, prostate cancer, non-melanoma skin cancer and stomach cancer (Fig.7), with lung cancer being the most common death cause [33].

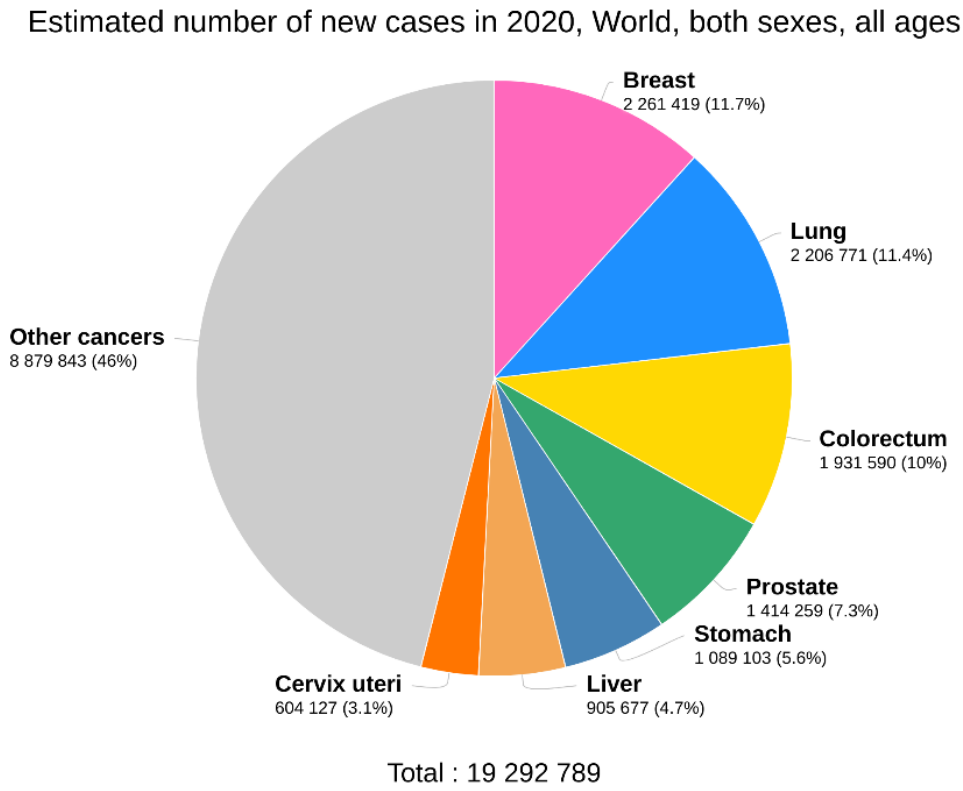

Figure 7. Estimated annual number of new cancer cases globally for the year 2020 [34]

Cancer is generally defined as the cell proliferation that has managed to elude central endogenous regulatory mechanisms. Uncontrollable cell proliferation and division results with invasion of healthy tissues and organs, and eventually can spread throughout the body. This behavior of cancer cells is explained by their lack of ability to adequately react to the signals that regulate normal cell behavior. As a result of accumulating aberrations in numerous cell regulatory systems, cancer cells demonstrate a broad loss of growth control that is reflected in a number of behaviors that set them apart from normal cells [35], [36].

There are more than a hundred different varieties of cancer, each with a unique behavior and response to treatment. Cancer can be caused by the abnormal multiplication of any type of cell in the body. The difference between benign and malignant tumors is the most crucial aspect of cancer pathology. Any aberrant cell proliferation, whether benign or malignant, is referred to as a tumor. A benign tumor, like a typical skin wart, stays in its original position and doesn't invade nearby healthy tissue or spread to other parts of the body. However, a malignant tumor has the capacity to both travel throughout the body through the circulatory or lymphatic systems and invade nearby normal tissue (metastasis). Only malignant tumors are appropriately referred to as cancers. While benign tumors may typically be surgically removed, malignant tumors are frequently resistant to such targeted treatment due to their tendency to metastasize to distant body regions [6].

The development of cancer is caused by a succession of gene changes that alter how cells operate. Evidently, chemical substances have a part in the development of cancerous cells and gene alterations. It's interesting to note that environmental chemicals with carcinogenic tendencies affect cells' cytoplasm and nuclei directly or indirectly, causing genetic diseases and gene alterations. Another 7% of all cancers are caused by carcinogenic agents such viruses, bacteria, and radiation. In general, cancer alters cellular interactions and causes critical genes to malfunction [35].

A set of functional characteristics that human cells acquire as they transition from normal growth stages to neoplastic growth states, i.e., abilities that are essential for the development of malignant tumors, is known under the concept of “Hallmarks of Cancer”. As shown in the Fig.8, the eight hallmarks currently comprise the acquired capabilities for sustaining proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing/accessing vasculature, activating invasion and metastasis, reprogramming cellular metabolism, and avoiding immune destruction. This evaluation also takes into account other suggested emerging hallmarks and enabling traits involving “unlocking phenotypic plasticity”, “nonmutational epigenetic reprogramming”, “polymorphic microbiomes”, and “senescent cells” [1].

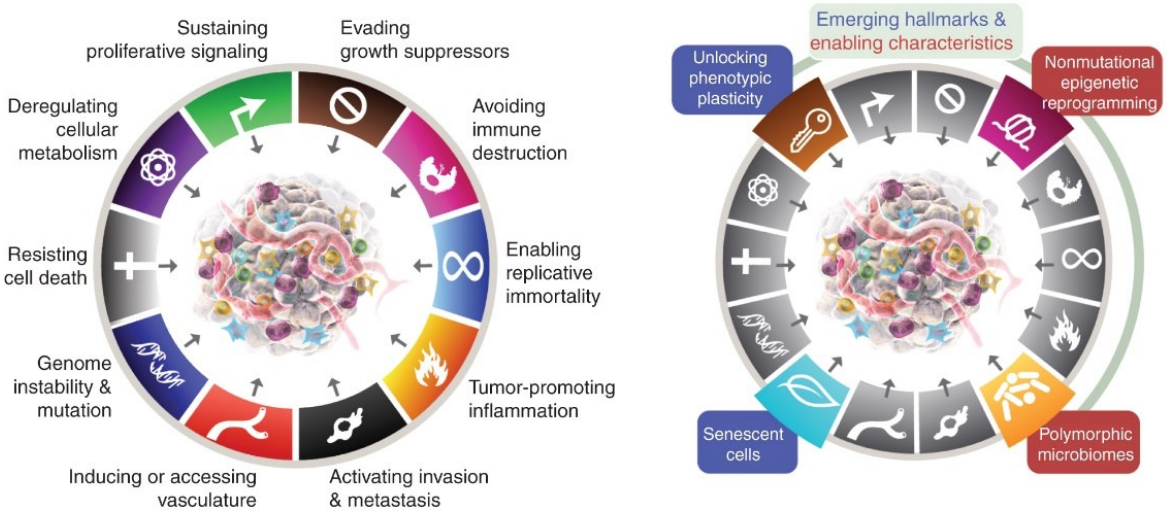

Figure 8. The hallmarks of cancer [1]

Even though the eight hallmarks of cancer and their two enabling characteristics have demonstrated their enduring heuristic value in the conceptualization of the disease, there may be new aspects of some generality that are relevant to better comprehending the complexities, mechanisms, and manifestations of the disease.

2.1. Cancer cells versus normal cells

Cancer cells can be differentiated from normal cells, based on the characterization of their growth patterns, chromosomal structure, regulation of cell death, presence of contact inhibition, ability to spread, cell specialization, maturation process and senescence, appearance, nuclear shape, and their ability to sustain organized arrangement in the body. (Fig.9) [37].

In the in vitro cell culture setting, growth factors are essential for normal cell division, whereas malignant cells can divide even when growth factors are absent. This ability to grow in the absence of growth factors is explained by cancer cells' capability to synthesize their own growth factors. Furthermore, cancer cells do not respond to the signals instructing them to stop further cell division. Immortality is another significant feature of cancer cells, which enables them to divide multiple times in contrast to normal cells. Normal cells' genomic integrity is maintained by the DNA telomere cap, and their progressive shortening occurs

during successive cell divisions. However, in cancer cells, telomere length is maintained by telomerase activity, which plays a critical role in cancer cell survival and development [38].

Cancer cells achieve replicative immortality by activating the silent telomerase encoding gene human TERT (hTERT), which possesses reverse transcriptase activity and aids in cancer cell senescence [39]. During cancer development, malignant tumor cells migrate to neighboring cells and tissues (a process known as metastasis) and promote the formation of new blood vessels (a process known as angiogenesis) as a source of oxygen and nutrients [40]. Cancer cells also avoid apoptosis by altering their metabolic strategy to allow for fast cell division [41], [42].

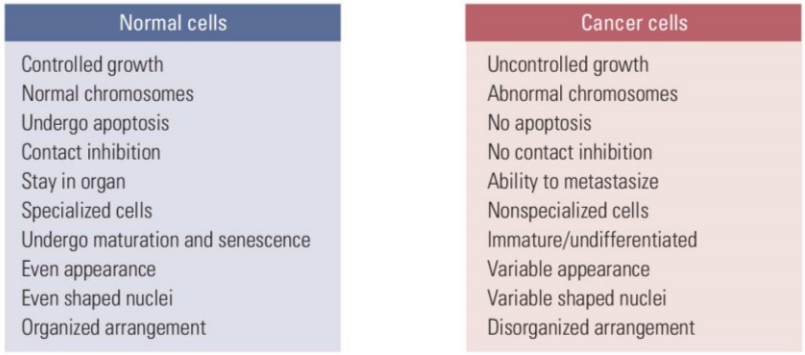

Figure 9. Differences between normal and cancer cells [37]

2.2. Energy metabolism in cancer cells

Changes in cancer cell metabolism are regarded as a key indicator of cancer progression. In contrast to normal cells, when cancer cells proliferate unchecked, they must rewire their metabolism to accommodate higher nutrient needs than healthy cells do. In order to survive the process of intravasation through vessels, where growth is essentially anchorage-independent, cancer cells try to grow in an oxygen and nutrient-deficient microenvironment at the primary site during tumor progression. They then colonize and grow in an entirely different microenvironment at the secondary site, which allows them to undergo metabolic reprogramming at each stage of cancer development. Cancer cells can obtain the nutrients they require from a nutrient-poor environment to both survive and produce new biomass thanks to this dysregulated metabolism [43].

Healthy cells tend to have a slower rate of glycolysis followed by the reduction of pyruvate to form lactic acid in the mitochondria, whereas abnormal cells tend to produce

their energy primarily by a high rate of glycolysis followed by lactic acid fermentation in the cytosol. This phenomenon was characterized by Otto Warburg in his 1920s report [44]. However, the Warburg effect does not represent a fundamental difference between normal and cancer cells. Instead, it is considered to be the result of a metabolic shift in dividing cells. Cells in their dormant state maintain a constant rate of glycolysis, converting glucose to pyruvate, which is then oxidized to carbon dioxide in the mitochondria via the TCA cycle. The Warburg effect sparked the idea that cancer could be caused by defective mitochondrial metabolism. Several decades of research later, the concept that cancer cells switch to fermentation rather than oxidative respiration has become widely accepted, even though it is no longer regarded as the primary cause of cancer [45].

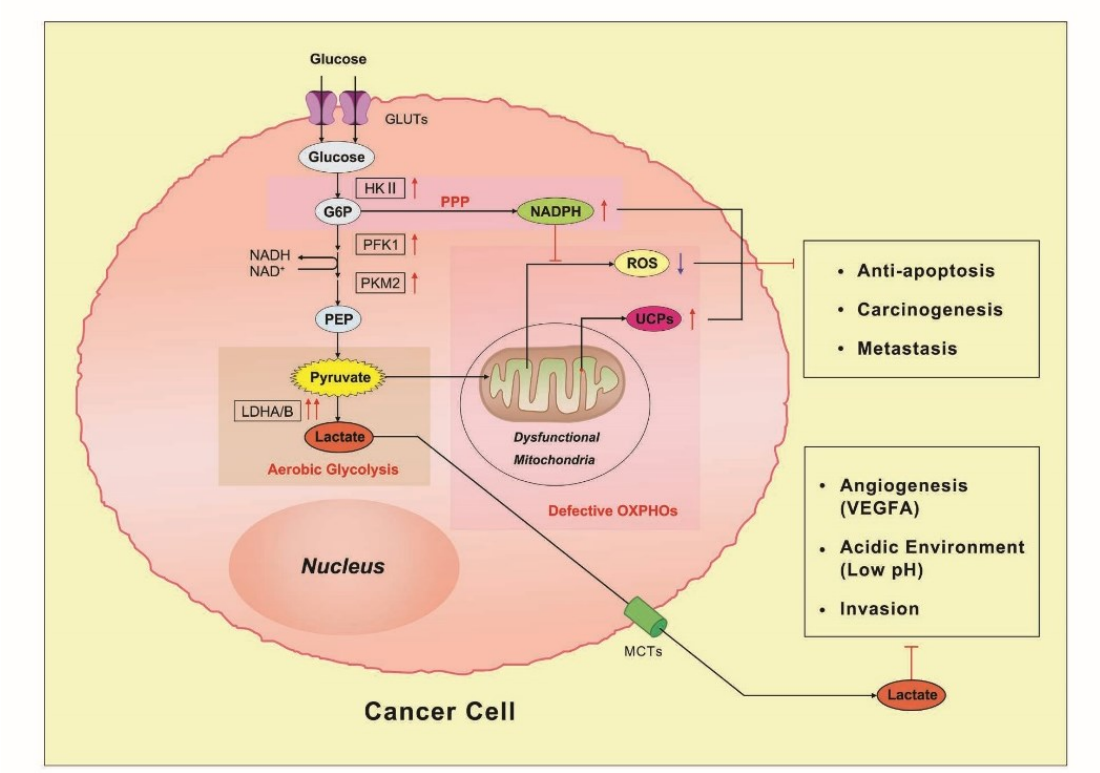

Figure 10. The Warburg effect in cancer cells. The Warburg effect is mainly driven by mitochondrial dysfunction. UCPs: uncoupling proteins; PEP: phospho-enolpyruvate; GLUTs: glucose transporters; HK: Hexokinase; G6P: glucose 6 phosphate; MCTs: monocarboxylate transporters; PPP: pentose phosphate pathway; PFK1: phosphofructokinase-1; LDHA/B: lactate dehydrogenase A/B [46]

2.3. Mitochondrial mechanisms in cell communication and cancer formation

To maintain cellular homeostasis, mitochondria have developed various ways for integrating external stimuli and communicating their condition to the rest of the cell. There are four major mechanisms, which enable mitochondrial communication with the rest of the cell: the release of cytochrome c to induce cell death, activation of AMP-activated protein kinase (AMPK) to control mitochondrial fission and fusion, production of ROS to activate transcription factors, and the release of mitochondrial DNA (mtDNA) to activate immune responses (Fig.11) [47], [48]. The fifth mechanism that is indicated in the recent studies is the release of TCA cycle metabolites by mitochondria to control cell fate and function [49].

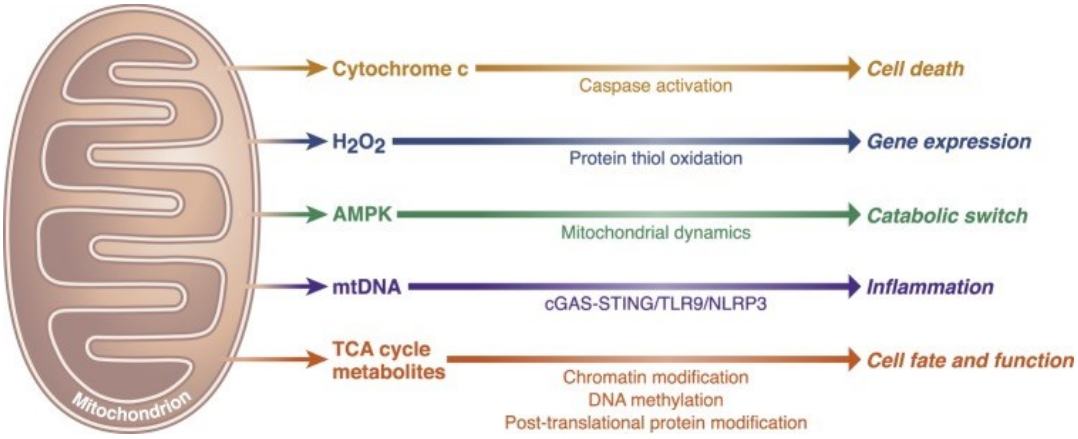

Figure 11. Crucial signaling functions of mitochondria**.** Three prominent mitochondrial-dependent signaling events are the release of cytochrome c to trigger caspase-dependent cell death, the release of reactive oxygen species to oxidize thiols within redox-regulated proteins and induce gene expression, and the stimulation of AMPK under energetic stress to control mitochondrial dynamics. Inflammasome activation and pro-inflammatory responses are also triggered by the release of mitochondrial DNA into the cytosol via the cGAS-STING cytosolic DNA-sensing pathway. Mediation of signaling activities of TCA cycle metabolites is enabled through influencing chromatin changes and DNA methylation, as well as post-translational protein modifications [49].

2.3.1. Cytochrome c

Cytochrome c (cyt c) is a tiny, globular nuclear-encoded metalloprotein with a covalently attached heme group. It is embedded in the IMS and functions as a mobile single electron carrier between Complexes III (bc_1 complex) and IV (cytochrome c oxidase, COX) of the ETC [50]. It has been found that cytochrome c plays an essential role in the critical

apoptotic processes of caspase-3 activation and DNA fragmentation. Its release from mitochondria is a key driving step in activation of cell death pathways. Upon permeabilization of the mitochondrial outer membrane, cyt c is released into the cytoplasm where it interacts with apoptotic protease-activating factor 1 (Apaf-1) to form the apoptosome. This step leads to the further activation of caspase-9 and the downstream caspase cascade (Fig.12). Under pathological conditions cyt c can also enter the blood circulation. Levels of circulating cyt c can be an indication of a novel in-vivo marker of mitochondrial damage after resuscitation from heart failure and chemotherapy. Additional to its role as an apoptosis biomarker, screening of cyt c can thus provide an understanding of certain abnormalities at cellular level [51].

Since apoptosis inhibition is one of the hallmarks of cancer, its activation in tumors can also be used as a treatment strategy. Apoptosis inhibition and induction are associated with lower and higher serum levels of cyt c, respectively. Therefore, the quantification of cyt c in serum is used in evaluating patient response to chemotherapy and may provide prognostic value [52].

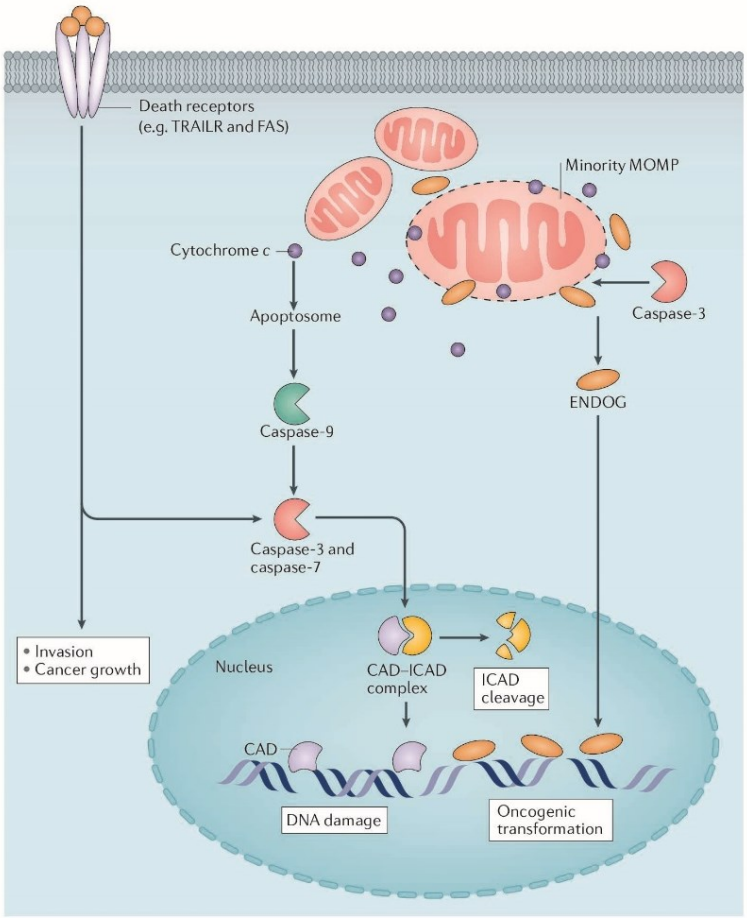

Figure 12. Role of cytochrome c in apoptotic processes of caspase-3 activation and DNA fragmentation [53]

Other essential cyt c functions include oxidization of cardiolipin, the mitochondrial membrane lipid, at earlier stages of apoptosis, which leads to the cyt c release from the IMS into the cytosol. Cyt c can also play a role in the destruction of ROS and at the same time in their production, which takes place via reduction of $p66^{shc}$, a protein involved in ROS generation and apoptosis [54]. Furthermore, cyt c serves as the electron acceptor in the Erv1-Mia40 redox relay system involved in mitochondrial IMS protein import [55].

2.3.2. Reactive oxygen species (ROS)

ROS are highly reactive oxygen-containing molecules, which include hydroxyl (HO•) and superoxide (O_2•) free radicals as well as nonradical molecules such as hydrogen peroxide (H_2O_2). In eukaryotic cells ROS are generated via aerobic metabolism and have emerged as key regulators of crucial signaling pathways. Production of ROS takes place in mitochondria (mostly via the electron transport chain, where approximately 1-2% of O_2 is reduced to create superoxide anions), peroxisomes (by β-oxidation of fatty acids), and the endoplasmic reticulum (via protein oxidation) [56].

Early studies on the role of ROS in tumor initiation discovered that ROS can cause DNA-damage, significantly raising the mutation rate inside cells and stimulating oncogenic transformation. The majority of ROS-induced DNA damage is caused by the non-specific, destructive behavior of hydroxyl radicals. Recent research has shown that, in addition to inducing such non-specific actions, ROS (except hydroxyl radical) can specifically activate certain intracellular signaling cascades, thereby contributing to tumor development and metastasis via the regulation of cellular phenotypes such as proliferation, death, and motility [57].

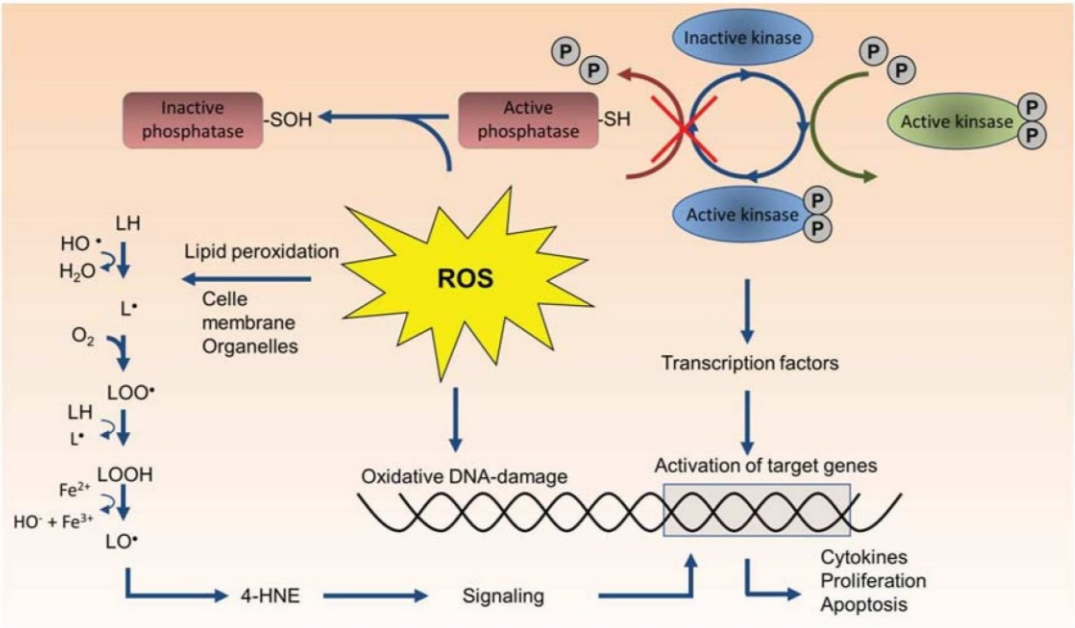

Figure 13. Impact of ROS on cellular structures. A large group of intracellular signaling proteins called kinases are activated via phosphorylation from up-stream kinases and exert their effects by phosphorylation of down-stream targets. Protein phosphatases act as kinase suppressor. SH-groups on phosphatases are being oxidized by ROS (H2O2), enabling reversible inactivation of the phosphatase, which in its turn leads to increased kinase activation and increased signaling. H2O2 may further oxidize thiolate anions into sulfinic (SO2H) or sulfonic (SO3H) species under higher concentrations, which can lead to the irreversible protein damage. High oxidative stress levels (especially from •OH) can damage proteins, DNA, and membrane lipids (lipid peroxidation). Lipid peroxidation can cause cell membrane and organelle damage, as well as the generation of reactive oxygenated, α,β-unsaturated aldehydes as 4-hydroxy nonenal (4-HNE) [58].

2.3.3. AMP-activated protein kinase (AMPK)

AMPK is a stress response kinase and an important energy sensor that is activated to control diverse signals and metabolic pathways in response to various stimuli such as caloric restriction, exercise, aging and obesity. It is considered as a metabolic guardian stimulating mitochondrial biogenesis, which generates ATP to meet the energy demands of cells in response to metabolic stress. This process is accomplished through the ability of AMPK to regulate the mitochondrial protein transcription. By phosphorylating mitochondrial fission factor (MFF), a protein of the mitochondrial outer membrane, AMPK acts as a sensor to monitor the cell's energy state. MFF then stimulates mitochondrial fission by recruiting a cytoplasmic guanosine triphosphatase [59], [60].

AMPK contributes to inhibition or promotion of cancer progression, through the phosphorylation of several downstream substrates and ubiquitination or phosphorylation of AMPK by upstream enzymes [59].

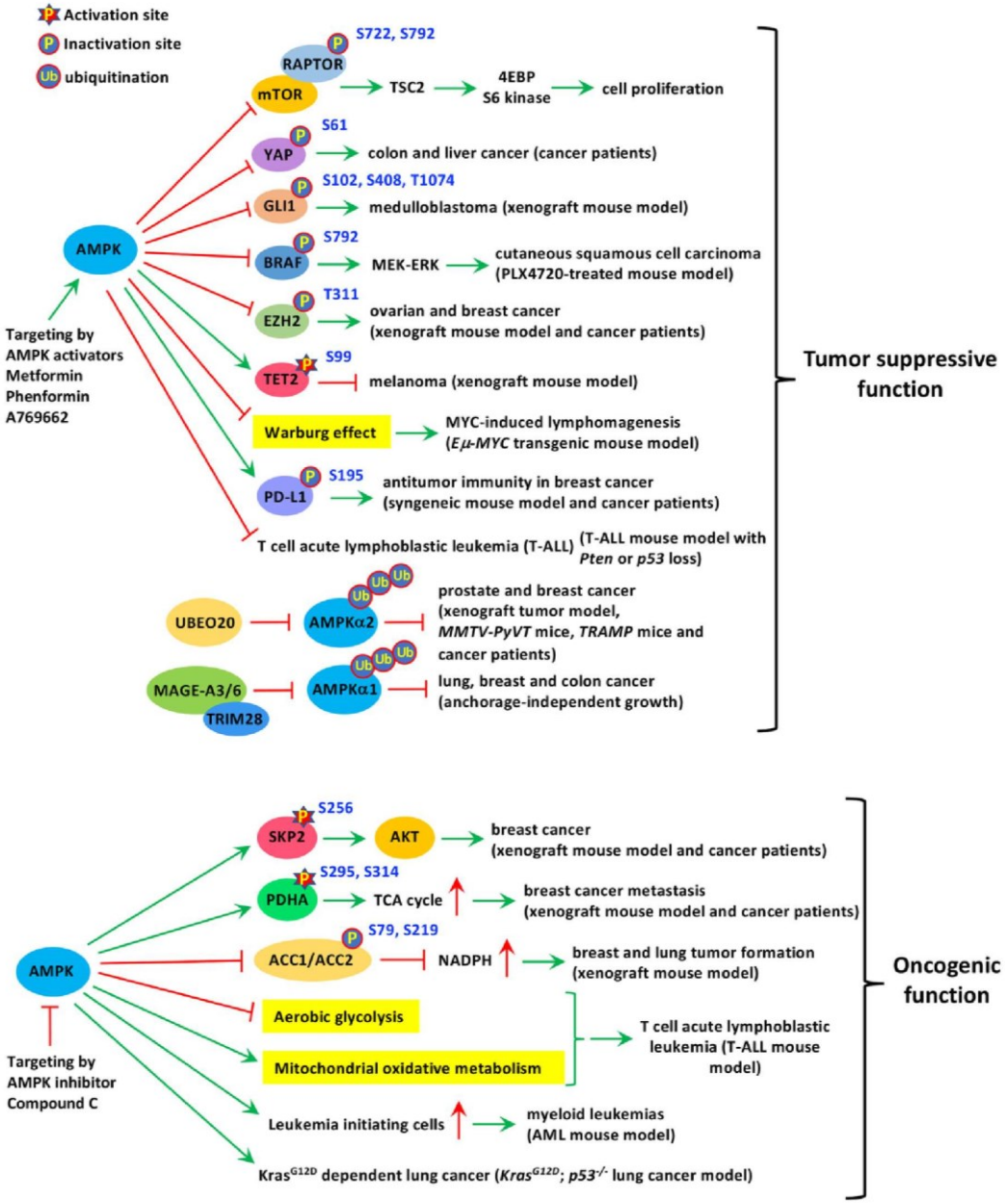

Figure 14. Role of AMPK as a tumor suppressor and an oncogene, regulated by a wide range of signaling cascades [59].

2.3.4. Mitochondrial DNA (mt-DNA)

mtDNA is a special type of deoxyribonucleic acid, which constitutes a genetic material outside the nucleus that is replicated, transcribed, and translated independently [61]. Human

mtDNA has a circular-shape and a double-stranded DNA measuring about 16,569 bp [62]. It encodes for 13 proteins that take part in the ETC and OXPHOS, which is crucial for ATP production. Mitochondrial DNA is a main target of ROS and absence of histone protection, makes it particularly prone to oxidative damage [63].

It has been established, that mtDNA can serve as a significant genetic biomarker in disease, cancer, and evolution. Mutations in mtDNA, single-nucleotide polymorphisms (SNPs) in mtDNA, mtDNA-encoded microRNAs (mitomiRs), mitochondria-derived long noncoding RNAs (lncRNAs), and mitochondrial proteins have all been linked to cancer development in functional and clinical investigations (Fig.15). Nuclear-encoded genes, on the other hand, have been identified to be coding for thousands of mitochondrial proteins and affecting mitochondria-encoded gene expression, which in turn regulates mitochondrial homeostasis. These findings highlight the significance of interaction between the nuclear and mitochondrial genomes for cellular activity [64].

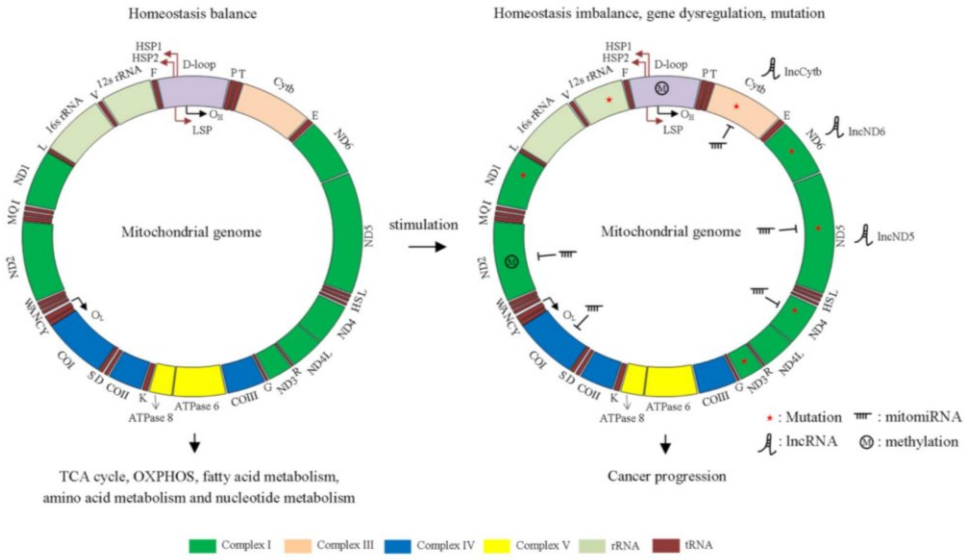

Figure 15. A graphical illustration of the human mitochondrial genome, comprising protein-coding genes, noncoding RNAs and control regions. The mtDNA encodes 2 rRNAs, 22 tRNAs and 13 mitochondrial protein subunits. Teal colored part represents the rRNA genes. Complex I genes are represented in green. Complex III genes are in peach buff. Complex IV genes are in blue and Complex V genes are in yellow. Amethyst colored part illustrates the D-loop, the regulatory region. Heavy strand promoters (HSP) and light strand promoters (LSP) are two mtDNA promoters. Left panel represents normal mitochondrial functions as regulators of cellular homeostasis, such as the TCA cycle, OXPHOS and fatty acid metabolism. Right panel represents mitochondrial DNA mutations (red star), mitochondrial genes dysfunction and methylation, which lead to homeostasis imbalance and cancer progression [64].

According to current evidence, mtDNA mutations are more common in cancer cells than in normal cells, which can possibly be explained by the increased production of ROS in cancer cell mitochondria [65]. An overview of mtDNA mutations in the coding regions of respiratory chain components found in various kinds of cancer tissues are summarized in Fig.16. Mutations may arise in any part of the mtDNA, while there may be particular “hot spots” where mutations are more abundant. Many mutations have also been discovered in the non-coding region (D-loop), which contains the replication origin and transcription promoter regions. D-loop mutations do not directly impact the structure and function of any specific protein encoded by mtDNA, but they may impair mtDNA replication and transcription [66].

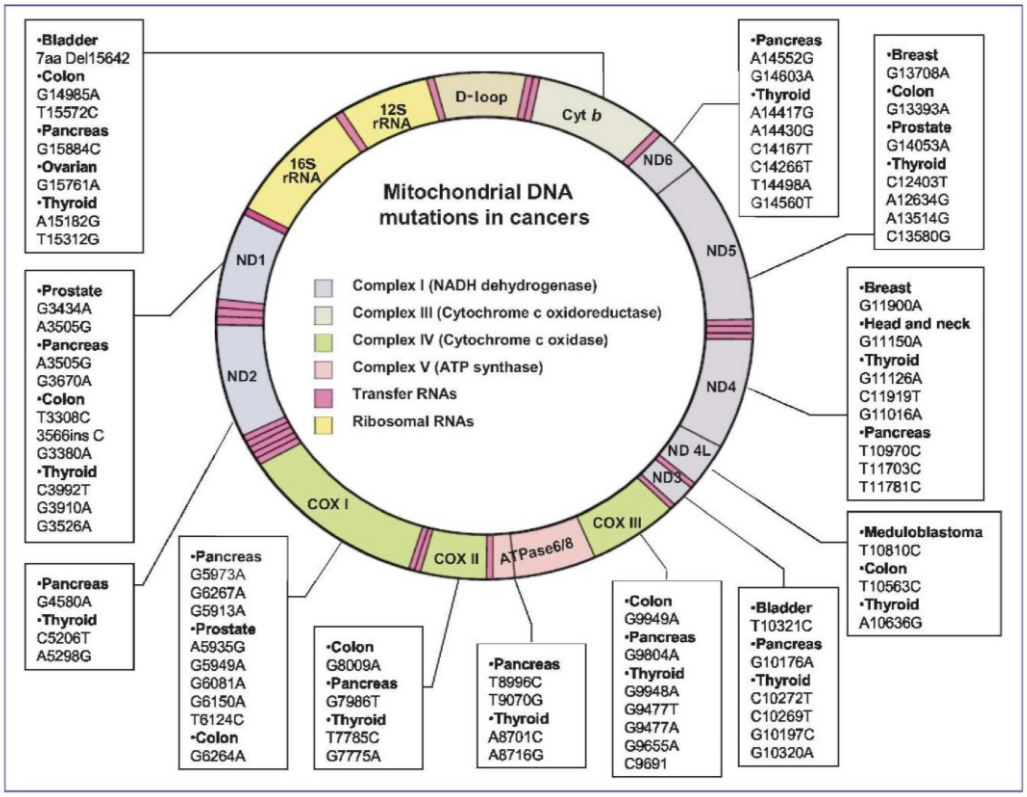

Figure 16. Map of human mtDNA and cancer types related to mutations in mtDNA metabolism. Mutations at the 13 ETC-encoding genes may cause alterations of the respiratory chain activity and thus affect metabolism. Boxes represent a partial list of mutations that have been identified in mtDNA of human cancers referring to particular mitochondrial gene region and the specific cancer types are indicated. The locations of the mutated bases are represented by numbers. (C, cytosine; G, guanine; T, thymine; A, Adenine). Note that the two adjacent genes for ATPase6 and ATPase8 are shown in a single box [66].

2.3.5. TCA cycle metabolites

As mentioned earlier, additional to their role in regulation of metabolic homeostasis and ATP production, mitochondria are responsible for other cell activities such as cell death decisions and immune signaling. Wide range of metabolites, urea (in hepatocellular mitochondria), heme molecules, steroids etc. are synthesized by mitochondria, for further utilization in mitochondria itself or as signaling molecules to other regions of the cell. The communication of mitochondrial metabolites with the nucleus provides the cells with a dynamic regulatory mechanism capable of responding to changing metabolic conditions. Dysregulation of the interaction between mitochondrial metabolites and the nucleus has been linked to cause of number of diseases, including cancer and type II diabetes [67].

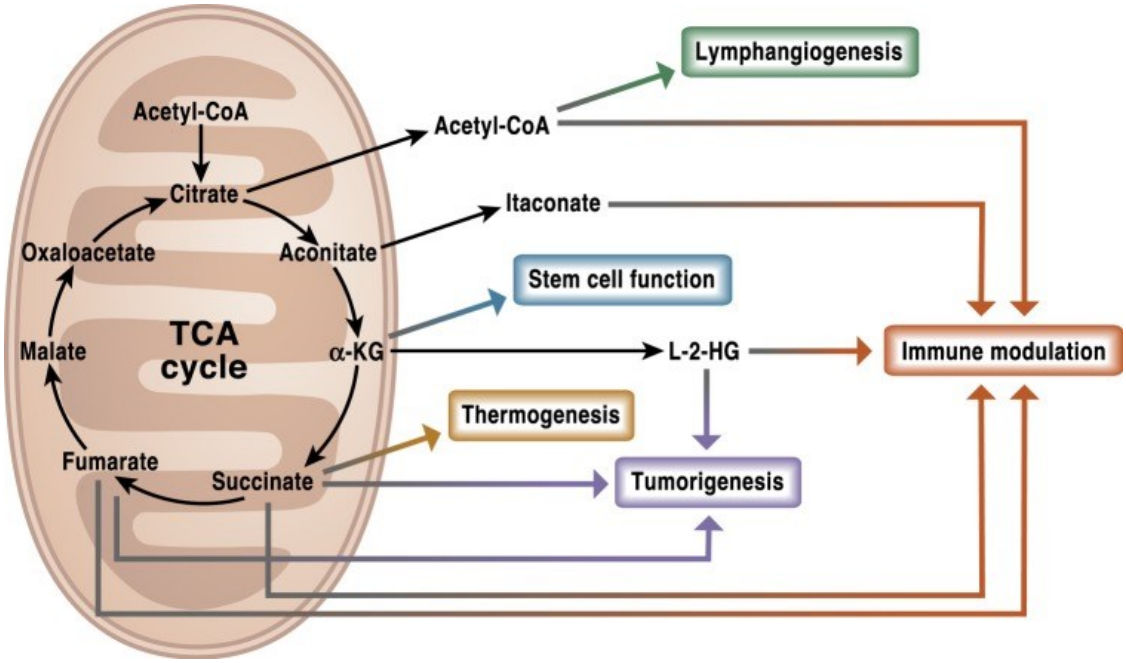

Figure 17. The TCA cycle as a signaling hub. Metabolites of TCA cycle play a wide range of non-metabolic signaling roles in physiology and disease. Acetyl-CoA, itaconate, succinate, fumarate, and L-2-hydroxyglutarate (L-2-HG) are all metabolites that can affect the innate and adaptive immune systems. Other processes, including lymphangiogenesis and stem cell pluripotency maintenance, have been linked to acetyl-CoA and α-ketoglutarate (α-KG), respectively. Succinate, L-2HG, and fumarate are well-known tumor-promoting oncometabolites. Succinate, in addition to its intracellular roles, can operate as a systemic signal to modulate thermogenesis when exposed to cold temperatures [49].

TCA cycle metabolites were primarily considered as byproducts of cellular metabolism important for the biosynthesis of macromolecules such as nucleotides, lipids,

and proteins. Although this is an essential function for the maintenance of cellular homeostasis, it is rapidly appreciated that metabolites in the TCA cycle are also involved in controlling chromatin modifications, DNA methylation, and post-translational modifications of proteins to alter their function [68].

3. ABNORMALITIES IN TCA CYCLE ENZYMES AND TUMOR FORMATION

A crucial pathway for oxidative phosphorylation in cells, the TCA cycle satisfies the needs of cells for bioenergetic, biosynthetic, and redox equilibrium. The TCA cycle has a series of metabolic processes in the mitochondria that result in CO_2, H_2O, and the bioenergetic products GTP, NADH, and $FADH_2$ are catalyzed by specific enzymes including citrate synthase, aconitase, isocitrate dehydrogenase (IDH), α-KG dehydrogenase complex (α-KGDHC), SDH, fumarate hydratase (FH), and malate dehydrogenase [69]. Even though it was once believed that cancer cells mainly used aerobic glycolysis and skipped the TCA cycle, new research shows that some cancer cells, particularly those with aberrant oncogene and tumor suppressor expression, heavily depend on the TCA cycle for energy synthesis and macromolecule synthesis [70]. TCA cycle enzymes are found in the mitochondrial matrix except for succinate dehydrogenase, which faces the matrix in the inner mitochondrial membrane. These enzymes are encoded by nuclear DNA (nDNA) and a number of them have been reported mutated in both sporadic and hereditary cancers throughout the last decade [71].

The TCA cycle is involved in many different disease disorders. Rare but severe genetic illnesses, such as FH deficiency, cause the TCA cycle to malfunction due to hereditary cycle enzyme abnormalities. Citrate synthase, which has decreased activity in obese mice, is one of the TCA cycle enzymes that is deregulated in obesity. Reduced activity of α-KGDHC has been linked to a number of neurodegenerative diseases, including Alzheimer's disease [70].

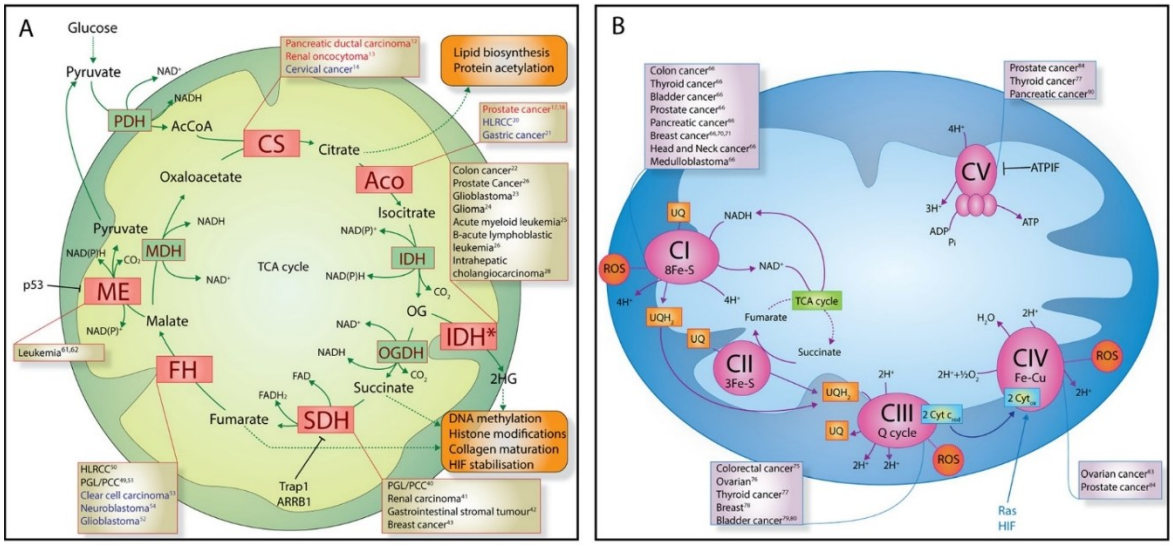

Figure 18. Cancer-related mitochondrial dysfunction. A schematic illustration of mitochondrial enzymes involved in cancer, with a focus on TCA cycle enzymes (A) and respiratory chain and ATP synthase (B). Boxes describe the type of cancer related with each specific enzyme. The text color shows whether the enzyme was discovered to be elevated (red), decreased (blue), or mutated (black) in the particular tumor type. CS: citrate synthase; Aco: aconitase; IDH*: mutant IDH; OGDH: oxoglutarate dehydrogenase; ME: malic enzyme; MDH: malate dehydrogenase; PDH: pyruvate dehydrogenase; OG: 2-oxoglutarate; 2-HG: 2-hydroxyglutarate; HLRCC: hereditary leiomyomatosis and renal cell cancer; PGL/PCC: hereditary paraganglioma and pheochromocytoma; CI–CV: complex I–V; UQ: ubiquinone; UQH_2: ubiquinol; ATPIF: ATP synthase inhibitory factor. Dashed lines indicate a series of reaction in a complex pathway, whereas solid lines indicate a single step reaction [71].

3.1. Mechanisms of tumor formation

Genes that encode the SDH, SDHB, SDHC, and SDHD, and the FH enzymes are described as tumor suppressors and have been found mutated in many forms of cancer. Deficiency of these TCA cycle enzymes leads to the accumulation of succinate and fumarate, which was observed to inhibit hypoxia-inducible factor (HIF-1α) prolyl hydroxylases (PHDs) in the cytosol. The similar case is observed in the mutation of FH, or IDH, leading in the Krebs cycle to an excess of fumarate, or 2-hydroxyglutarate, which exert the same role of oncometabolite [72]. High levels of these metabolites promote tumor development and progression by inhibiting several dioxygenases dependent on 2-oxoglutarate (2-OGDD) [73]. The inhibition of PHDs leads to the stabilization and activation of HIF-1α, which enables the transcription of several genes involved in enhanced glycolysis and angiogenesis [74]. Therefore, the downregulation of mitochondrial respiration in tumor cells leads to

buildup of the tricarboxylic cycle substrates, which may act as a signal for increased glycolysis, hence enhancing the Warburg effect [45], [75].

The dysfunction of SDH and FH (through SDH mutations) results in the accumulation of succinate or fumarate in the mitochondria. The accumulated metabolites then make their way into the cytosol, where they inhibit PHD. PHD inhibition in its turn causes the buildup of HIF, which is reliant on the von Hippel-Lindau (VHL) tumor suppressor protein. Inhibition of the PHD enzymes (and HIF induction) can promote cancer by increasing tolerance to apoptotic signals [76] and/or by a pseudohypoxic signaling that enhances glycolysis in tumor cells [77]. The rise in HIF caused by a lack of degradation can also stimulate angiogenic signaling and the new blood vessel formation, which will further feed and nourish the tumor [78].

3.1.1. Succinate dehydrogenase

SDH (also known as succinate-ubiquinone oxidoreductase, or Complex II) is an enzyme complex attached to the inner mitochondrial membrane that transforms succinate to fumarate in a mechanism linked to the reduction of FAD to $FADH_2$ [79]. It is a highly conserved heterotetrameric protein complex, which acts as a bridge between the tricarboxylic acid cycle and the electron transport chain. The absence of subunits encoded by the mitochondrial genome and the lack of proton pumping function makes SDH differ from the other respiratory chain enzymes [80], [81].

SDH is comprised of four subunits, which are encoded by the SDHA, SDHB, SDHC, SDHD genes. Each of these subunits are independently transported to the mitochondria and together they form a mature protein complex placed in the inner mitochondrial membrane. SDHA and SDHB are the catalytic subunits of SDH, which protrude into the mitochondrial matrix and are anchored to the inner membrane by SDHC and SDHD (Fig.19). These latter subunits can also serve as the ubiquinone binding site [82].

It has been reported that the mutations responsible for the inactivation of SDH subunits and assembly factors are associated with a variety of hereditary and sporadic cancers. Particularly SDHB, -C,-D, and SDH5 mutations have been linked to PGLs, including head and neck paragangliomas (HNPGLs) and PCCs. Other tumors associated with mutated SDH genes include GIST (gastrointestinal stromal tumor), thyroid cancer, renal tumors, and even neuroblastoma [81], [83].

SDH has ability to function as a typical tumor suppressor gene due to the mutant allele, which is transmitted in a heterozygous form, whereas the rest of the wild type allele is lost in tumor samples. The majority of the carcinogenic activity of SDH mutations has been linked to a metabolite, succinate, which accumulates in SDH-deficient cells [84].

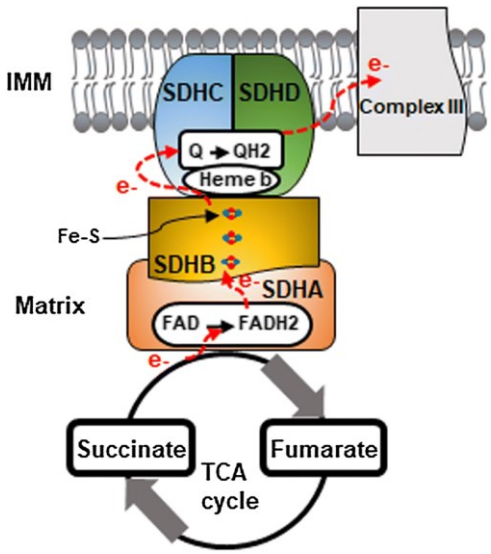

Figure 19. The structure of complex II (succinate dehydrogenase). This enzyme functions as a bridge between oxidative phosphorylation and TCA cycle. SDHA-B oxidizes succinate to fumarate in the TCA cycle and the derived electrons are further transported to coenzyme Q and then to complex III. Along this path, electrons reduce FAD of SDHA subunit, later passing through Fe-S clusters in SDHB subunit, and finally reducing the ubiquinone before transferring to complex III [85].

3.1.2. Succinate as a cancer metabolite

Succinate is one of the TCA cycle metabolites that plays a crucial role in variety of intracellular functions, as well as organismal functions. It is regarded as an oncometabolite that accumulates as a result of SDH inactivating mutations. Through two key metabolic pathways (TCA cycle and OXPHOS), succinate buildup influences gene expression regulation and promotes cancer [86]. Because succinate is a byproduct of the processes of 2-OGDD enzymes, its buildup inhibits these enzymes. As a result, variations in succinate have a significant impact on histones and DNA methylation, altering the epigenetic landscape of cells and gene expression [49].

Few other studies highlight the significance of succinate in the regulation of innate immunity. It was shown that a high abundance of succinate, that was detected in the metabolic profile of LPS treated macrophages, leads to HIF-1α stabilization as well as the

transcriptional activation of the pro-inflammatory cytokine IL-1β [87]. A number of signaling cascades have been described in response to succinate binding to its specific receptor SUNCR1 in various cell types, including dendritic cells and macrophages where it appears to contribute to their functionality in driving endo- and paracrine modulation and inflammation [88]–[90].

Succinate was also described as a systemic molecule that promotes thermogenesis in brown adipocytes in response to cold exposure [91]. Brown adipocytes were highly receptive to increased levels of circulating succinate, which was later oxidized by SDH. This further leads to the increase in the ROS levels and uncoupling protein 1 (UCP1) activity, a proton transporter/channel located on the inner mitochondrial membrane, which is involved in heat production of cell [68], [92].

Recent reports describe succinate as an archetypal "epigenetic hacker", which may function as inhibitor of both DNA [93], [94] and histone demethylases [95], resulting in epigenetic changes similar to those found in mutant IDH cancers [84].

3.1.3. The relationship between succinate and HIF-1a in cancer

HIF-1α has been described as a critical succinate target that acts as a transcriptional regulator in the transition to glycolysis [96]. Many key enzymes in the glycolytic pathway are specifically targeted by HIF-1α, which enables the stimulation of most glycolytic enzymes expression by binding to HIF response elements (HREs) in target genes. The ability of HIF-1α to promote cancer development is linked to the increase in the glucose metabolism, generation of proliferative intermediates, and stimulation of angiogenesis.

Under extreme hypoxia, HIF-1α blocks pyruvate entrance into the tricarboxylic acid cycle and increased mitochondrial ROS by activating the 3-phosphoinositide-dependent protein kinase 1 (PDK1) protein, preventing a huge rise in ROS that would trigger apoptosis. It has been established that the metabolic alterations are attributable to tumor cells adapting to a hypoxic environment [97].

Another recent study discovered that up-regulation of HIF-1α in tumor cells makes them resistant to apoptosis and proliferation hindrance caused by hypoxia and hypoglycemia, whereas inhibition of HIF-1α expression makes cancer cells more susceptible to apoptosis and proliferation hindrance caused by hypoxia and hypoglycemia [98].

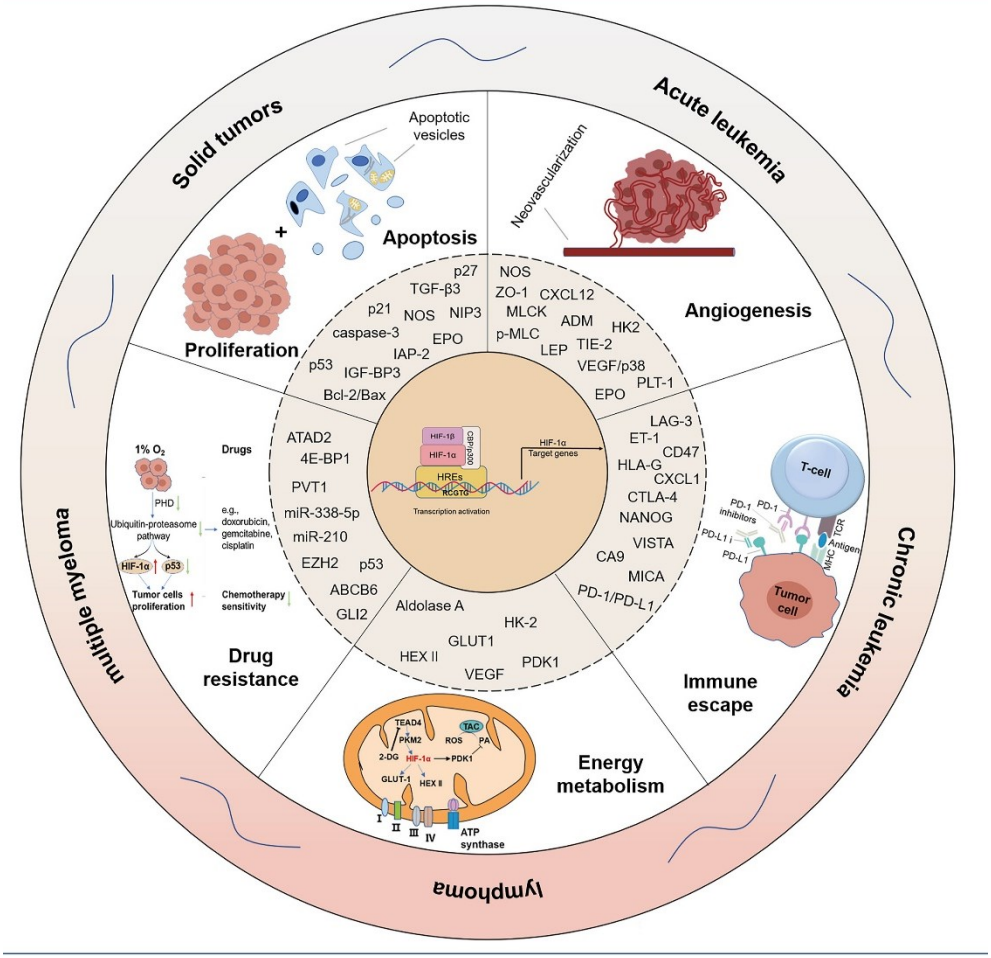

Figure 20. Behaviors of malignant HIF-1α in tumorigenesis under hypoxic conditions. Tumor cells reside in a complex environment called the tumor microenvironment (TME). The TME is essential for carcinogenesis, growth, and metastasis. Oxygen consumption is enhanced as malignant tumor cells proliferate rapidly, whereas metastasis leads to decreased oxygen supply, therefore resulting in a hypoxic microenvironment (HME) for tumor growth. In a hypoxic environment, HIF-1α accumulation can regulate the expression of downstream genes through a variety of mechanisms, promoting tumor cell proliferation, angiogenesis, energy metabolism, epithelial-mesenchymal transition (EMT), and immune escape, among other things, thereby making tumor cells more tolerant to HME and acquiring a greater capacity for proliferation, metastasis, and invasion [98].

PHD enzymes play a role of strict HIF regulators and are considered to be a type of dioxygenases that utilize oxygen generated from the conversion of α-KG to succinate to hydroxylate HIF-1α. This prepares it for ubiquitination by E3 ubiquitin ligase and, later allowing its proteasomal destruction [99]. Succinate has been found in tumor cells to limit PHD activity via product inhibition and to promote HIF-1α stability [100]. This significant discovery was the first evidence that succinate can serve as an intracellular messenger, altering gene expression in tumors by targeting HIF-1α [101].

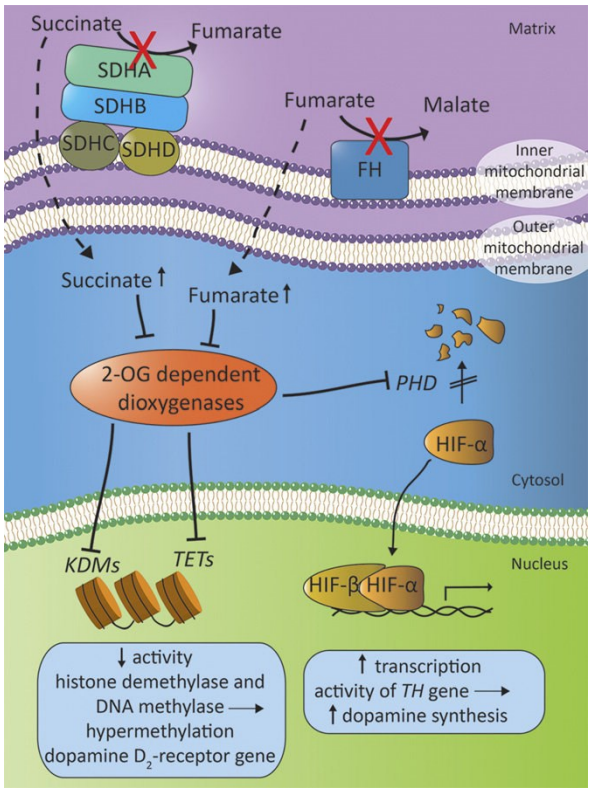

Figure 21. The proposed mechanism of the interaction between mitochondrial complex II and FH in the HIF-α signaling pathway stability. Mitochondrial complex II, positioned on the inner mitochondrial membrane, is made up of SDH-proteins and acts as a catalysator in the succinate to fumarate conversion. Another mitochondrial enzyme that converts succinate to malate is FH. In the case of a mutation in one of the SDH subunits (SDHA, SDHB, SDHC, or SDHD gene mutations) or FH genes, the intracellular concentration of succinate and fumarate rise, resulting in inhibition of 2-oxyglutarate-dependent dioxygenases, including PHD, histone lysine demethylases (KDMs), and the Ten-eleven translocation (TET) enzymes, and leading to HIF-stimulated increase of transcription of the gene encoding TH, and to hypermethylation of the dopamine D2-receptor (DAD2-receptor) gene [102].

3.1.4. Prolyl hydroxylase domain (PHD) enzymes

The PHD enzymes function as the regulator of the HIF stability, based on the response to oxygen availability. The inhibition of PHD enables the stabilization of HIF, during the oxygen limitation, which leads to the cellular adaptation to hypoxia. This adaptability is particularly important for solid tumors, which are frequently exposed to a hypoxic environment [103].

PHD enzymes are considered to be a part of 2-oxoglutarate (αKG)-dependent, non-haem iron-binding dioxygenase family. As previously stated, the major target of PHD enzymes is the transcription factor HIF (three main subunits reported, HIF-1, HIF-2, and

HIF-3), which governs cell response to hypoxic circumstances. In the presence of oxygen, PHD hydroxylates HIFα in its oxygen-dependent degradation (ODD) domain at two proline residues (P402 and P564). This hydroxylation process causes HIFα to bind to the von Hippel-Lindau (pVHL) tumor suppressor protein, which leads to ubiquitination and subsequent proteolytic destruction by the E3 ubiquitin ligase complex [104]. Whereas in the hypoxic environment, PHD are inactivated, and HIFα is stabilized, allowing target genes to be expressed [105], [106].

Despite their original purpose as oxygen sensors, PHD are now recognized to have HIF-independent and hydroxylase-independent activities in the modulation of several physiological processes, including the mammalian target of rapamycin (mTOR) system, the nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB) pathway, apoptosis, and cellular metabolism [103].

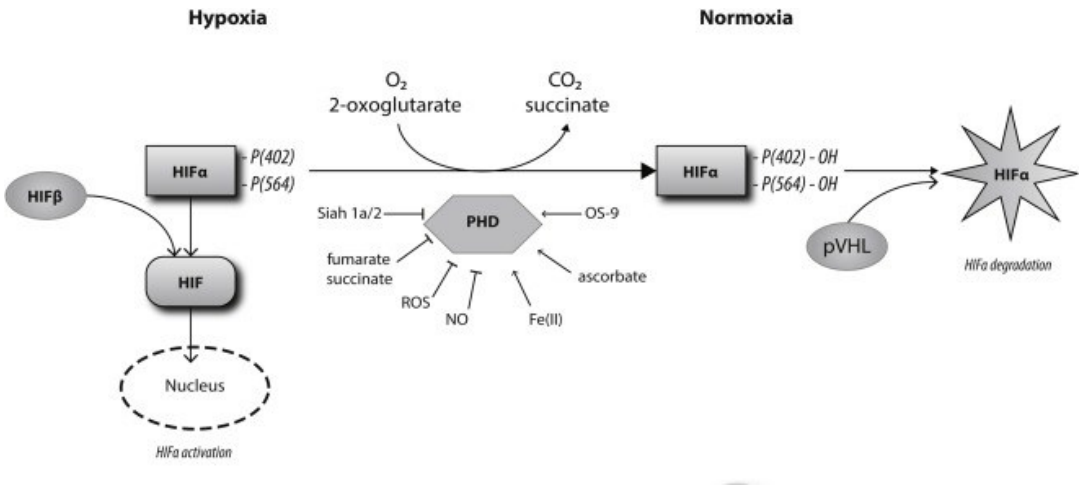

Figure 22. Metabolic components controlling PHD activity. The hydroxylation of HIFα by PHD proteins takes place depending on the oxygen level. Under normoxic conditions, HIFα is hydroxylated in its ODD domain at two proline residues (P402 and P564 in HIF-1α). This hydroxylation causes the HIF subunit to bind to the pVHL protein, causing ubiquitination and subsequent proteolytic destruction by the E3 ubiquitin ligase complex. PHD activity is suppressed and HIFα accumulates as oxygen levels fall under hypoxic conditions. Stable HIFα further interacts with HIFβ to stimulate the expression of various target genes. Several upstream inputs make PHD susceptible to the modulation of their activity in response to changes in external factors. Activity of PHD depends on various factors, such as oxygen and αKG as co-substrates, or iron and ascorbate as co-factors [106].

4. MITOCHONDRIAL BIOMARKERS

A biomarker is defined as a trait that may be tested and assessed objectively as an indicator of normal biological processes, pathogenic processes, or pharmacological responses to a therapeutic intervention [107]. Based on their definition and application, biomarkers can be categorized as diagnostic, monitoring, pharmacodynamic/response, predictive, prognostic, safety, and susceptibility/risk biomarkers [108]. Detection and analysis of biomarkers can be conducted in tissue, in blood or lymph circulation, and in body fluids (urine, stool, sputum, breast nipple aspiration, etc.). Furthermore, biomarkers may be detected in cells of concern (premalignant or even existing cancer cells) or in tissues around the region of interest (for example, indications of neo-vascularization or inflammation surrounding a tumour). Biomarkers may also be found in exfoliated cells, soluble or suspended molecules, such as proteins, DNA, microRNA, in circulation or in secretions. Lastly, inherited germline biomarkers can be assessed from circulating leukocytes or exfoliated cells from easily accessible tissues, such as from a cheek swab [109].

According to the research conducted by Hubens, W.H.G., et al. [110], mitochondrial biomarkers can be divided into three main classes: 1) functional markers measured in blood cells, 2) biochemical markers of serum/plasma and 3) DNA markers. It's important to highlight the fact, that none of the above-mentioned single biomarkers can fully explain the whole underlying mitochondrial dysfunction. However, the biomarker panel, that has been brought together by various aspects of mitochondrial impairment, may help with the diagnosis of primary mitochondrial patients. Additionally, the panel may serve to assess mitochondrial dysfunction in complex multifactorial diseases, such as Alzheimer's disease and glaucoma, as and enable selection of patients who could benefit from therapies targeting mitochondria.

4.1. Functional markers measured in blood cells

One of the functional markers measured in blood cells is the diagnosis of defective OXPHOS, which is considered by the Mitochondrial Medicine Society to be the main criterium for MD [111]. The research conducted on 101 primary mitochondrial disease (MD) patients, showed the results of 69 patients' blood cell respiration levels to be lower when compared to the average reference values of control subjects.

Another important blood cell functional marker is the mitochondrial membrane potential (ΔΨm), which is formed as the result of active pumping of protons transferred by the mitochondrial complexes. In healthy cells its potential is maintained at approximately −180 mV [112], [113]. It is still not clear how mitochondrial dysfunction increases or decreases ΔΨm. However, it has been demonstrated that the altered ΔΨm over a longer period of time is damaging for the cell and can trigger apoptosis [114], [115]. The staining technique, which is based on the intensity of staining the negatively charged ΔΨm by positively charged dyes (tertramethylrhodamine (TMRM), JC-1), was used to quantify of the ΔΨm. As a result, ΔΨm in blood cells of primary MD patients reported a significant difference, either elevated or decreased levels of ΔΨm, when comparing with the average ΔΨm of healthy controls [116].

4.2. Biochemical markers of serum/plasma

Lactate

As a product of anaerobic ATP production, lactate can be used as an effective biomarker for MDs [117]. In fact, lactate was the first circulating biomarker used to identify patients with suspected mitochondrial dysfunction. Studies show the elevated lactate levels in 172 of 234 MD patients.

Pyruvic acid

Another suggested biomarker for primary MD is pyruvic acid. As a diagnostic tool for MDs, blood pyruvic acid levels tend to be assessed along with lactate levels. Similar to lactate, blood pyruvate levels are also elevated in many MD patients. It's important to mention that both lactate and pyruvic acid levels tend to vary depending on the physical activity of the studied individual. Higher levels are detected during exercise with further gradual post exercise recovery [118], [119].

Creatine and creatine kinase

Tissues with higher energy demand (skeletal muscle, brain, or the liver) store their energy in the form of phosphocreatine (PCr). When cells need high energy supply and ATP production through OXPHOS is not sufficient, PCr can be converted into creatine by cytosolic creatine kinase (CK) enzyme and later release ATP. Dysregulation of this

alternative energy metabolism may cause an OXPHOS related mitochondrial dysfunction [120]–[122].

Only 26.5% of primary MD patients were presenting enhanced creatine levels in a study of a large cohort as well as an untargeted liquid chromatography with tandem mass spectrometry [122], [123].

Some other energy-balance-associated metabolites include acylcarnitines and amino acids. Acylcarnitines are metabolic compounds for transportation of activated long-chain fatty-acids into the mitochondria, for further beta oxidation [124]. Based on literature, the mechanism underlying the higher levels of acylcarnitines in these disorders remains unknown and there is no clear rationale to quantify acylcarnitines in order to diagnose general mitochondrial dysfunction [125]–[127]. However, quantification of the amino acid profile (in particular alanine, guanine, proline, threonine) is common for diagnosing mitochondrial dysfunction [128].

Markers of mitochondrial integrated stress response

Studies conducted on gene expression of muscle tissues with MD, revealed two crucial markers that showed enhanced expression when compared to average control values. These two markers are known as fibroblast growth factor-21 (FGF-21), a growth factor that regulates lipid and glucose metabolism and growth differentiation factor (GDF-15), a member of the transforming growth factor beta family.

4.3. DNA markers

The use of mtDNA as a biomarker is gaining more interest in many different research fields, such as cancer, uncommon metabolic diseases, aging, tracing ancient human migration patterns, demographic characterization via maternal markers, and human identity. mtDNA use as a cancer biomarker is considered advantageous due to its absence of introns (mutations are detected in coding regions), relatively small size, simplicity of extraction, lack of genetic rearrangements and rapid mutation rates [129]. Furthermore, a large numbers of mtDNA copies (up to hundreds of copies per cell) allows to conduct the rare disease studies with minimal amount of tissue samples. Mitochondrial genome mutations can be detected in primary tumor tissues and non-invasively obtained bodily fluids. Therefore, further studies on mtDNA mutations may provide a significant molecular tool for cancer identification and prognosis [130].

Damage in mitochondrial function can be caused by mtDNA variations, such as deletions, depletion, and point mutations, which may result in clinically heterogeneous primary mitochondrial or, also known as, OXPHOS disorders [131]. Mitochondrial dysfunctions that are caused as a result of mtDNA alterations can be measured by several assays conducted on DNA that is isolated from white blood cells (for example quantifiying the mtDNA amount and detection of inherited (pathogenic) variants).

According to Nashwa Mohd Khair, Siti, et al. [132] the main mtDNA biomarkers can be classified as small- and large-scale deletions, mtDNA copy number, cell-free mtDNA, mitochondrial microsatellite instability (mtMSI), somatic mtDNA alterations, and mtDNA and mitochondrial RNA (mtRNA) methylation.

4.3.1. Small- and large-scale deletions

During mtDNA studies it was observed that diseases are mainly related to the deletions of mtDNA [133]. The term "multiple mitochondrial DNA deletions" describes missing portions of mitochondrial DNA with different size ranges. Functional cellular mtDNA would be reduced in prostate cancer due to small- and large-scale deletion. Specifically, quantification of the 3.4 kb (3379 bp) large-scale deletion enables the detection of cancer and is particularly used for the identification of breast and prostate cancer. The detection of deletions in neighboring benign tissue, also known as field-effect or cancerization, which occurs prior to carcinogenesis was detected using quantitative polymerase chain reaction (qPCR) to identify the presence of small tumor foci. This has raised the possibility of detecting the early carcinogenesis or cancer cell transition, additionally allowing to distinguish proximal benign prostate tumor biopsies from malignant ones [134]. Higher rate of $mtDNA^{4977}$ was detected in patients with breast carcinoma and hepatocellular carcinoma, which makes $mtDNA^{4977}$ a useful non-invasive biomarker for the early cancer diagnosis. Another major large-scale deletion, which serves as an indication for the breast cancer is the 4576 bp loss [135].

4.3.2. mtDNA copy number

Another important biomarker in cancer detection is known as mtDNA copy number. mtDNA copy number refers to the number of copies of the mitochondrial genome, or mtDNA, in each cell, which ranges from 10^3 to 10^4, depending on the type of cell and the

stage of development [136]. Decrease in the mtDNA copy number can be an indication of alterations in nuclear encoded genes related to mtDNA biosynthesis and maintenance, which leads to mitochondrial impairment. Other factors that can cause the alteration of mtDNA copy number are aging, disease or drugs like antiviral therapy in human immunodeficiency virus infection [137].

A lower mtDNA copy number was observed in patients with a mtDNA depletion syndrome (MDDs) [138], [139]. However, in some other MDs, such as Leber's hereditary optic neuropathy (LHON), mitochondrial encephalomyopathy lactic acidosis and stroke-like episodes (MELAS) and myoclonus epilepsy with ragged-red fibers (MERRF), a higher mtDNA copy number could be observed, and patients with a higher mtDNA copy number were less severely affected [140]–[145].

4.3.3. Cell-free mtDNA

The third significant biomarker of mtDNA is the cell-free mtDNA, which is defined as the cellular mtDNA that has leaked from within mitochondria into the cytosol or peripheral blood circulation as a result of disturbance of the regular mitochondrial life cycle (mtDNA replication and replacement), with further disruption of the damaged mtDNA mitophagy [146], [147].

Studies show that the analysis mtDNA content from peripheral blood of patients with hepatitis C, lung cancer, and neck cancer may serve as a noninvasive biomarker and may predict their further risk of developing into hepatocellular carcinoma [148]–[152]. Additionally, the levels of cf-mtDNA were enhanced in the developing of cancer linked to lymph node metastasis [152]. However, it is important to mention the conflicting findings in certain research, which demonstrates cf-mtDNA content in cancer patients to be lower value than in control samples. These findings may imply that the association between mtDNA copy number and breast carcinogenesis is governed by an underlying mechanism that is still not fully understood.

4.3.4. Mitochondrial microsatellite instability (mtMSI)

Next possible mtDNA biomarkers are the microsatellites, which are short tandem repeats (mononucleotide or dinucleotide) ranging in size from 1 to 6 bp that are dispersed

throughout the mitochondrial and nuclear genomes [153]. The D-loop region is home to the most often reported mtMSI overall [154]. The D310 region, which is defined as a highly polymorphic homopolymeric C stretch, is a mutational hotspot in primary cancers [155]. According to some reports, the D310 mutation is the first sign of malignancy and has the potential to be a precancerous tumor marker [156].

4.3.5. Alterations in somatic mtDNA

Studies conducted on neoplasms, suggest 25 to 80 percent of somatic mtDNA mutations are thought to be responsible for neoplastic transformation by altering energy sources of cells, regulating apoptosis, and raising oxidative stress [157]. Numerous studies have been done on the mtDNA A12308G change, and they demonstrate that it may be used as a diagnostic tool for colorectal cancer, lung cancer, prostate cancer and breast cancer when combined with other mtDNA alterations [158].

4.3.6. MtDNA and mitochondrial RNA (mtRNA) methylation

In recent years, a number of studies have found a link between cancer and mtDNA methylation deficiencies. In their cellular models, the mechanism linking mtDNA methylation and cancer is similar to decreasing 5-methylcytosine (5mC) levels of glioblastoma and osteosarcoma cells during tumor progression were found at mtDNA-specific locations, which may be consistent with the growth of the mtDNA copy number. Later, the 5mC levels would also rise to prevent further mtDNA replication, suggesting that enough mtDNA had been recovered to start carcinogenesis. When mtDNA is translated into RNA utilizing continuous polycistrons, this mechanism is known as posttranscriptional modulation, and it is critical for RNA processing [159]. Alterations in mitochondrial N1-methyladenosine (m1A) and N1-methylguanosine (m1G) tRNA methylation levels are seen after identifying the varied increase of mtRNA transcripts in human malignancies [160], showing that they have a substantial impact on mitochondrion-mediated metabolism.

CONCLUSION

In this review, we explored the relationship between mitochondrial metabolic abnormalities, particularly alterations of mitochondrial enzymes induced by mtDNA mutations or altered oncogenes/tumor suppressors. Additional to energy production, mitochondria play a crucial role in cancer initiation, growth, survival, and metastasis. We have briefly explained the tumorigenic potential of mitochondria, associated with its mass, dynamics, regulation of metabolism and cell death, redox homeostasis, and cell signaling. It is now clear that the interaction of various components of mitochondrial biology leads to coordinated programing of cellular physiology, displaying mitochondria's multifaceted roles in cancer. Mitochondrial alterations and malfunction have been related to a wide range of diseases in all fields of medicine. Many mtDNA features, such as increased sensitivity for mutations, simplicity of extraction and minimal need for the tumor tissue sample makes mtDNA particularly advantageous for cancer studies. Recent discoveries in mitochondrial intervention and gene therapy for mitochondrial diseases may serve as a blueprint for future research towards high specificity and sensitivity mtDNA biomarkers, which may provide a promising strategy for the development of selective anticancer treatment.

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