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Mitochondrial DNA mutations in cancer - from bench to bedside

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1. ABSTRACT

Mitochondria are cell organelles mostly known for their production of ATP through oxidative phosphorylation. As suggested over 70 years ago by O. Warburg and recently confirmed with molecular techniques, alterations in respiratory activity and mitochondrial DNA appear to be a common feature of malignant cells. Somatic mtDNA mutations have been reported in many types of cancer cells. MtDNA mutation pattern may enhance the specificity of cancer diagnostics, detection and prediction of tumor growth rate and patients' outcome. Therefore it may be used as a molecular cancer bio-marker. Nevertheless recently published papers list a large number of mitochondrial DNA mutations in many different cancer types, but their role in cell patophysiology remains unsummarized. This review covers the consequences of mitochondrial genes mutations for human cell physiology and proliferation. We underline effects of mtDNA mutation-resulting amino acid changes in the respiratory chain proteins' structure, and propose changes in mitochondrial protein function. Mutations are critically evaluated and interpreted in the functional context and clinical utility of molecular mitochondrial research is summarized and new perspectives for 'mitochondrial oncology' suggested.

Table 1. The role of mtDNA mutations in cell transformation

Mutated mtDNA region	Role in cancer development and progression	Reference
D-loop	Disruption of mtDNA replication and copy number maintenance	(85).
	Depletion of mtDNA in the cell	(83)
	Disruption of mtDNA transcription	(84)
	Increase in the incidence of new deletions in mtDNA	(81)
	Complex I activity inhibition	(131)
	Elevated ROS production	(86).
tRNA	Impairment of mitochondrial protein synthesis	(101)
	Severe impairment of the oxidative phosphorylation system	(106) (105)
	Promotion of termination of mitochondrial genes transcription	(109) (107)
	Elevated ROS production	(32)
Complex I	Resistance to chemotherapeutics	(1).
	Metastasis promotion	(129)
	Overproduction of ROS accompanied by up-regulation of MCL-1, HIF-1 and VEGF genes	(129) (81)
	Increase in anchorage-dependent and independent	(130)
	Elevated ROS production	(130) (144) (129)
	Induction of aerobic glycolytic metabolic phenotype	(130) (144). (81)
Complex III	NF-κB signaling pathway up-regulation	(144)
	Rapid cell cycle progression and tumor growth	(144)
	Prevention of apoptosis	(31).
Complex V	Tumors growth promotion	(30)
	Elevated ROS production	(30)

2. INTRODUCTION

Over the past decades molecular cancer research has generated a rich and complex body of knowledge, revealing a multitude of parameters that either cause or favor cell transformation and cancer development and progression. Several features, however, emerge as key events in carcinogenesis: limitless cell proliferation (immortalization), apoptosis evasion, sustained angiogenesis and tissue invasion, as those are crucial properties of cancer cells. It is well established that certain nuclear DNA mutations are associated with tumor development and/or provide a selective advantage in cancer progression, thus, it is reasonable to postulate that similar principles may apply to mutations in mitochondrial DNA (mtDNA). At the present time, however there is still little information about which mtDNA mutations or polymorphisms are of functional importance in human tumors. Nonetheless, mtDNA mutations hold the promise of being useful clinical markers in the diagnosis and clinical management of cancer patients (1-3).

Mitochondria play essential and diverse roles in the physiology of eukaryotic cells. Not only do they provide ATP, but they also participate in numerous intermediate metabolic reactions and play central role in apoptosis. Impairments of mitochondrial functions have been implicated in a wide variety of human pathologies including cancer. It was over 30 years ago when cytoplasm from tumor cells was shown to transfer tumorigenic property when fused with caryoplasts from normal cells (4) and also until recently the 'aerobic glycolytic' metabolism of cancer cells had received little attention in molecular oncology despite being one of the first observations made in the cancer field (5). Recent progresses in cancer molecular genetics, biochemistry and proteomics have renewed interest in Warburg's views. It is being now widely understood that unlike most normal cells, cancer cells acquire a dependency on glycolysis for energy production and that this cannot be any longer considered as by-product of the oncogenic process, but this step is required for malignant progression (6). Under aerobic conditions, glycolysis is inhibited (the so-called *Pasteur effect*) and normal mammalian cells rely mainly on the mitochondrial OXPHOS for their energy supply, but aerobic glycolysis certainly confers a growth advantage to tumor cells (7). Above all, mechanisms decreasing oxidative metabolism during cell division are expected to direct substrates toward biosyntheses. Hence, disrupted OXPHOS is to provide a proliferating advantage to a transformed or tumor cell (8).

Nowadays, we are facing a renaissance of the role of mitochondria in cancer biology and attempt to analyze its significance in oncology and hence the important debate if mtDNA mutations are truly involved in tumor progression or if they are a consequence of it. Growing number of reports support the hypothesis that mitochondrial functions are profoundly altered in transformed cells and that these structures are prominent participants in the processes of clonal evolution. Several lines of evidence confirm that mitochondria contribute to neoplastic transformation by changing cellular energy capacities, increasing oxidative stress and modulating apoptosis and proliferation (Table 1, Figure 1). A shift in

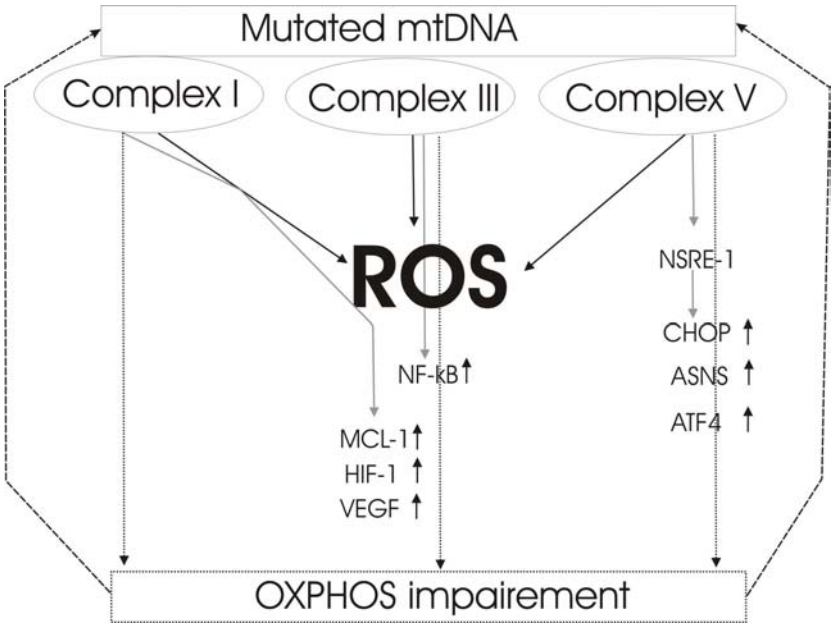

Figure 1. The role of mtDNA mutations in the viscous circle of cell transformation.The picture represents pato-physiological results of mutations in mitochondrial electron chain transport complexes (complex I, III) and ATPase (complex V) – in the subunits encoded by mtDNA. Excessive ROS are generated and in turn further damage mtDNA. ROS – dependent pro-angiogenic and survival signaling pathways are activated.mtDNA - mitochondrial genome; MCL1 - myeloid cell leukemia sequence 1 (BCL2-related); HIF-1 - hypoxia-inducible factor, basic helix-loop-helix transcription factor; VEGF - vascular endothelial growth factor, vascular permeability factor; AARE - amino acid regulatory element; NSRE-1 - nutrient-sensing response element-1, enhancer element; CHOP - C/EBP homologous protein; ASNS - asparagine synthase; ATF4 - activating transcription factor 4, tax-responsive enhancer element B67, NF-κB nuclear factor kappa-B.

the rate of mitochondrial respiration may provide biochemical explanation for the etiology of cancer, and moreover may offer additional strategies for therapeutic intervention (3, 9, 10).

Somatic mtDNA mutations are likely a general phenomenon occurring in the process of cell transformation. In the last few years mtDNA mutations have been found in solid tumors including carcinomas and sarcomas, and also leukemias and lymphomas. Comprehensive scanning of mitochondrial genomes has revealed that functionally relevant point mutations in mtDNA and polypeptide-encoding genes may be detected even in 50% of patients (11). Although there is this increasing evidence that mitochondrial mutations are associated with various neoplasms, it is yet not definitive evidence whether mitochondrial abnormalities are contributing factors in carcinogenesis or whether they simply arise as part of secondary effects in cancer development. In the biochemical aspect mutations in mtDNA are likely to cause alterations of the encoded protein and compromise the respiratory chain function. Mutations in mtDNA protein coding genes have been shown to affect assembly, stability or the functional structure of individual respiratory chain enzyme complexes, leading to OXPHOS deficiency (12, 13). Thus, the frequent mtDNA mutations observed in a variety of human cancers are thought to contribute to respiratory malfunction in cancer cells. Nevertheless until today numerous mtDNA mutations have been identified in various tumors, but the pathogenic implications (cell growth or transformation promotion? metastatic potential promotion?) of many of these mutations remain unclear (Table 1). It becomes even more complicated as little evidence confirm that mtDNA mutations and subsequent mitochondrial alterations (OXPHOS deficiency, ROS over-production) are directly involved in cellular metabolism and signaling pathways associated with cell transformation. Although the role of mitochondrial dysfunction in tumorigenesis has been investigated extensively using multiple approaches (13-16), direct link between the presence of mtDNA mutations and cell transformation in humans has not yet been described and published, but the codependence of cancer growth rate, metastatic potential and mtDNA mutation has recently been established in mice model (17). Moreover today there is little evidence of inherited mtDNA defects in tumors and development of cancers caused by germline mtDNA mutations – only breast, oral cancer and colorectal cancer have been shown to be influenced by such mutations (18-20). This obviously contrasts with the frequent occurrence of mtDNA somatic mutations in many different tumor types. For that reason some authors have even suggested that the majority of mtDNA mutations reported in tumors and hematological diseases are not significant factors in the process of carcinogenesis. Some reports underline that cancer progenitor cells achieve mtDNA mutation homoplasmy through stochastic redistribution of the mitochondrial mutation and hypothesize that no replicative or metabolic advantage of mutant mtDNA are required to promote

Table 2. Clinical significance and possible applications of mtDNA mutations screening

Mutated mtDNA region	Clinical application	Reference
D-loop	High number of mutations correlates with poor differentiation in ***HCC***	(87)
	High number of mutations correlates with late stage in ***gastric cancers, lung cancer, and colorectal cancers***	(84)
	High number of mutations correlates with the presence of metastasis correlates in ***ovarian carcinoma***	(88)
	Mutations are characteristic for nodular and metastatic ***melanoma***	(89)
	T allele at 16519 in ***pancreactic cancer*** in patients with locally advanced disease correlates with diabetes mellitus development and shorter life expectancy	(90)
	Positions 146, 152 and 186 are hotspots for ***oral squamous cell carcinoma***	(91)
	Mutations are marker of hypopharyngeal ***head and neck cancer*** in tobacco users	(92)
	Mutations are factor of resistance to fluorouracil-based adjuvant chemotherapy in stage III ***colon cancers***	(93).
	Mutations in stage III ***colon cancers*** correlate with the relative risk of death	(93).
	Mutations are typical for older onset age (>or=50) as well as a higher histological grade in ***breast cancer***	(85)
	Mutations are prognostic factor of poorer disease-free and overall survival in ***breast cancer***	(85)
	Mutations are prognostic factor of lack of estrogen receptor and progesterone receptor expression in ***breast cancer***	(95)
Mt rRNA	Variant frequencies of 12S rRNA-tRNA(phe) correlate with differentiation degree in ***gastric cancer***	(119).
	Mutations correlate with gastric (vs. intestinal) type carcinoma of ***gastric cancer***	(120).
	High mutation number differentiate cancer from dysplasia in ***gastric carcinoma***	(120).
Complex IV	Nearly 100% of ***Hürthle cell tumors*** display the mitochondrial common deletion	(143)
	A clear-cut difference between the benign oncocytomas and the clear-cell/chromophilic carcinomas can be assessed based on complex IV activity in ***clear cell renal cell carcinomas*** (CCRCCs)	(8)
	Mitochondrial impairment increase from the less aggressive chromophilic carcinomas to the high-grade ***CCRCCs***	(8)
Complex V	In ***CCRCs*** correlation exists between complex V activity and tumor aggressiveness	(8)

cancer development, and therefore selective expansion of cancer cells with specific mutant mtDNA is not required to explain homoplasmy of mutant mtDNA in cancer (21). Others even claim that mitochondrial oncology research needs to be revised as to methodological, methodical and/or technical errors because these have been common in many published papers on mtDNA mutations in cancer (14, 22, 23).

Nevertheless mtDNA mutations might provide important and significant markers in the detection of tumor, tumor classification, tumor recurrence and possibly also prognostic markers used also in individual readjustment of chemotherapy (Table 2) (9, 16, 24) as unfortunately, prognostic markers currently accepted for clinical use, such as nodal status, tumor size, histological grade, steroid receptor status and others, frequently do not adequately identify patients at higher risk of recurrence. For that reason correlations of the mitochondrial markers with the clinicopathological classification of the tumors must be further established. Recently it has been proven that the inheritance of mitochondrial haplotype U is associated with approximately 2.0-fold increased risk of prostate cancer and 2.5-fold increased risk of renal cancer in white North America individuals (25), which again provides first strong evidence for the importance of mtDNA sequence for cancer development. The next step towards explanation of mitochondrial-dependent cell transformation has been made *in vivo* model. Growth advantage of the cancer cells with specific mutant mtDNA was demonstrated in mouse model system using cybrid (trans-mitochondrial hybrid) cells (26, 27). The apparent conundrum of whether mtDNA mutations found in tumors are either a cause or a consequence of the carcinogenic process has also been partially solved after the demonstration that pathogenic mtDNA mutations do influence excessive reactive oxygen species signaling (28, 29), diminish cellular apoptotic potential (27), initiate mitochondria - nucleus signaling that promotes cellular invasive phenotype (30-32), and regulates epigenetic modifications in nucleus (33) and nucleotide pools as well as chromosomal stability (34).

As the mechanisms that generate mtDNA mutations and the importance of mtDNA mutations in tumor development and progression are getting more complex, it would be pivotal step towards understanding cancer etiology, prediction of the course of disease and designing novel therapeutic and/or preventative strategies and drugs. Metabolic phenotype of the cell, more specifically its bioenergetic-mitochondrial phenotype, could provide a marker for the analysis of cancer progression (16, 35-37).

2.1. MtDNA mutation mechanism

MtDNA mutation rate is known to be 100 times higher than nuclear genome mutation rate. The evolution rate of mtDNA is much faster than the one of the nuclear genome and several reasons are likely to explain this fact: mtDNA is poorly protected by proteins, it is physically associated with the mitochondrial inner membrane where damaging reactive oxygen species (ROS) are generated, and mitochondria appear to have less-efficient repair mechanisms than the nucleus (38-40). Also malfunction of mismatch repair (MMR) and slipped strand misspairing (SSM) should be considered as mitochondrial genome instability (mtGI) factors (38, 41). Population studies in the UK have shown that at least one in 8000 adults harbours a pathogenic mtDNA mutation (42), and it is believed that respiratory chain disorder prevalence may be as high as 1 in 5000 newborns (43). The frequency of mitochondrial genome instability and mitochondrial mutation rate is even higher in cancer cells, with example of breast cancer cells with 216-fold increase over the spontaneous rate in the female germline and 16-fold increase over the rate of spontaneous somatic mutations (44). The mtGI is expected to be even more pronounced in cancer cells with impaired p53 signalling pathway/mutated p53 as it governs base excision repair (BER) in mitochondria (45).

Point mutations or other alterations (large deletions, inversions or insertions) in mtDNA that have been reported in a variety of human cancers may arise due to different mechanisms. The mutagenic agents are represented by chemical and physical factors including nitrosamines in drinking water, IFN therapy, UV or X radiation, reactive oxygen species (ROS) along with reactive nitrogen species (RNS), but most often arise in a consequence of poly errors, as in the case of repetitive sequences of mononucleotides within the mitochondrial genome that are unstable and subjected to deletions (46-48). Until today, only one *in vivo* mitochondria-mutation cancer model has been established. In the case of colorectal cancer it has been proven that mutations in the mtDNA occur in one of normal crypt stem cells, so they are maintained in the replication process and the daughter cells (with mutated mtDNA) finally occupy the whole crypt; later on mtDNA-mutated crypts clonally expand by crypt fission and crypt that divides into two daughter crypts, give rise to two new crypts, both mutated (49). In the case of prostate tumors it has been indicated that the mitochondrial mutations are locally confined, and that the multiple mutations exist on the same molecules and more than one mtDNA mutant genomes may co-exists in the same neoplastic focus. Based on those findings the authors have suggested rapid mtDNA mutagenesis during tumor progression - the process of mitochondrial hyper-mutagenesis, and suggested it arises as a consequence of cellular oxidative stress (50).

2.2. The role of reactive oxygen species in mitochondrial carcinogenesis

Recent evidence indicates that oxidative stress is central to the pathogenesis of a wide variety of diseases including cancer and a special role in the mitochondria-related carcinogenesis has been attributed to reactive oxygen species (ROS) (47). The mitochondrial respiratory chain is the major source of ROS, which in excess destroy mtDNA, eventually contributing to the promotion of vicious circle of cancer development (Figure 1). The constant generation of reactive oxygen species within the mitochondria and the increased free radical stress in cancer cells may cause further damage to both mtDNA and the electron transport chain, thus amplifying respiratory malfunctions and dependency on glycolysis; ROS production increases when electron transport is reduced or inhibited, which occurs at low aerobic respiration rate resulting from mtDNA mutations and aberrant OXPHOS protein structure (51). The main sources of mitochondrial ROS are complex I and complex III of the respiratory chain, as mitochondria generate ATP through oxidative phosphorylation with formation of O_2^- and derived reactive oxygen species such as hydrogen peroxide and ·OH occurs, because complex I flavin active site, complex I and complex III iron-sulfur centers or ubisemiquinone can transfer electrons to oxygen and thou give rise to superoxide anions. At the same time if the respiratory chain is inhibited downstream of complex III (as in the case of some mtDNA mutations), electrons coming from succinate oxidation could also lead to superoxide anion generation by reverse electron transport from complex II to complex I (52). Subsequently, oxidative stress develops as the delicate balance between production and detoxification of reactive oxygen species gets disturbed and cells respond to this condition in several ways, among which is also a change in mitochondrial morphology (53).

The scenario that emerges from mtDNA mutations and cancer studies is rather complex, since different mtDNA mutations do not result in the same profile in terms of ROS production. For example it has been proven that mutations that affect the function of complex I and III, but not isolated COX mutations without I and III complex involvement, are able to produce an increase in ROS (28) and that specific mutations in complex I and subsequent ROS overproduction may promote tumor growth (26) and cancer metastasis (17). Low levels of ROS formed as a consequence of mtDNA mutation - OXPHOS deficiencies provide a mitotic stimulus (54). Mitochondrial genome mutations observed in cancer cells are in power to be potent OXPHOS inhibitors, to increase ROS production and to promote tumor cell proliferation, but also it cannot be excluded that some mtDNA variants and their physiological consequences permit tumors to better adapt to their new environment (52).

3. D-LOOP MUTATIONS IN HUMAN CANCERS

The D-loop region (the region between proline and phenylalanine 16024-576) was found to be a "hot spot" for mutation in mtDNA of the tumors. The control region of mtDNA is highly polymorphic and contains two hypervariable regions: HV1 (nucleotides 16024–16383) and HV2 (nucleotides 57–372). Thus somatic mutations occurred preferentially at hypervariable polymorphic sites in the control region (55). Other alterations of the noncoding displacement (D) loop of mitochondrial DNA

present in many cancers include changes in a homopolymeric C tract at positions 303-309 and are represented by single base substitutions. All the D-loop mtDNA mutations including small deletions and insertions occur primarily at mononucleotide sequence repeats and dinucleotide microsatellites (55, 56). Although many reports claim mtDNA mutation rate is significant in cancer tissues, however effects of the D-loop mutations on the copy number or transcription of mtDNA in tumor tissues are poorly understood. D-loop mutations have been reported till date in: gastric tumors (57), breast cancer (44), esophageal squamous cell carcinoma (58), glioblastoma multiforme (59), ovarian carcinomas (60), prostatic preneoplastic lesions and cancer (56, 61), lung and head and neck tumors (55), hepatocellular, carcinoma (62), melanoma (63), endometrial carcinomas, including uterine serous carcinoma (64), invasive gestational trophoblastic disease (choriocarcinoma) (65), thyroid cancer, including papillary carcinomas, medullary carcinomas, anaplastic carcinomas, and follicular carcinomas (66), renal cell carcinomas (67), and bladder primary tumors (2, 68). Therefore although the mechanisms of generation and functional impact of mtMSI (mitochondrial microsatellite instability) are still not clear, the high incidence of mtMSI in the D-loop and its broad distribution in human cancers render it a potential marker for cancer detection (69).

The finding of mutations in the mitochondrial genome raises the question what is their functional importance for the evolution of cancer cells. It has been proposed that mitochondrial mutations may themselves affect mitochondrial function (68). Little data is available to explain published correlations. For example, in colorectal carcinomas D310 instability was not associated with nuclear microsatellite instability, indicating different mechanisms of occurrence (70) and no correlation between mtMSI and nuclear microsatellite instability (nMSI) was found in colorectal, breast, and renal cancers (71). Moreover correlation between mtMSI and nMSI or between *de novo* occurrence of the delta4977 mtDNA deletion and nMSI could not be detected in head and neck squamous cell carcinomas (72), or tumors of the central nervous system (73). In Hürthle cell tumors the percentage of deleted mtDNA molecules was significantly higher in tumors with D-loop mutations than in mtDNA stable tumors (74).

3.1. Consequences for cell physiology

In colorectal carcinogenesis model it has been shown that mitochondrial DNA D-loop transferred from colorectal cancer cell (SW480) into mouse embryonic fibroblast cell line (NIH3T3) on expression vector, promotes the malignant phenotype of NIH3T3 cells. Such manipulation is in power to significantly increase growth rate and colony formation rate of NIH3T3 cells and also decrease its apoptotic rate (75). The mutations in D-loop have been associated with depletion of mtDNA in the cell. In hepatocellular carcinoma mtDNA copy number was significantly decreased in the patients with somatic mutations in the D-loop of mtDNA. Moreover this decrease in mtDNA copy number was highly associated with the occurrence of point mutations near the replication origin of the heavy-strand of mtDNA. At the same time some cases without D-loop mutations had a reduced copy number of mtDNA, therefore the authors suggested that also other unidentified factors are involved in mitochondrial biogenesis and are defective in the tumor (76). This trend was further confirmed in gastric, lung, and colorectal (77) and breast cancer. In breast cancer patients, those harboring mutations at the polycytidine stretch or close to the replication origins of the heavy-strand a significantly lower copy number of mtDNA is detected (78). Moreover in hepatocellular carcinoma with mutations in the D-loop region of mitochondrial DNA, the ROS level in the tissue was higher than that hepatocarcinoma tissue with normal mtDNA D-loop (79).

3.2. Clinical implications

Until today already clinical correlations have been described for D-loop mutations (Table 2). Correlation between cancer characteristics and mitochondrial D-loop DNA mutations in hepatocellular carcinoma (HCC) has been reported. The mean number of mtDNA mutations was 1.7 in well-differentiated HCC, as compared with 4.5 in moderately differentiated HCC and 4.6 in poorly differentiated HCC. The frequency of mtDNA mutations was thus higher in less differentiated HCC (80). Also the frequency of D-loop mutations in gastric, lung, and colorectal cancers of later stages was higher than that of the early-stage cases (77). Not only differentiation stage but also metastasis correlates with D-loop mutations. In cases with bilateral metastatic ovarian cancer it has been shown that metastatic foci showed identical D-loop variants to those found in at least one of the ovarian tumors (81). At the same time D-loop point mutations are typical for nodular and metastatic samples of melanoma (82). Moreover in pancreatic cancer in patients with locally advanced disease the T allele of mtDNA 16519 correlates with shorter life expectancy and interestingly 16519 variant seems to be a predisposing genetic factor for diabetes mellitus in this population. It is known that diabetes mellitus developing with pancreatic cancer is a negative prognostic factor (83). D-loop mutations may also be associated with cigarette smoke exposure. In oral squamous cell carcinoma three D-Loop mutation hotspots were observed at positions 146, 152 and 186. Those mutations occur predominantly in male smokers and female non-smokers and that this association with gender is statistically significant (84). In head and neck carcinoma D-loop mutations seem to be a marker of hypopharyngeal cancer location especially in tobacco users, but presence of D-Loop in this group of patients is not associated with prognosis or with the response to neoadjuvant chemotherapy (85). This region of mtDNA has also emerged as a mutational hotspot recently found to associate with prognosis and response to 5-fluorouracil (5FU) in colon cancer. Those mutations are a factor of resistance to fluorouracil-based adjuvant chemotherapy in stage III colon cancers. After adjustment for age, stage, and microsatellite instability status, the relative risk of death in patients with D-loop mutation was 1.40 as compared to those without (86).

Not only sporadic, but also familial cancer cases have been correlated with D-loop mutations. Sequence

variants within the mtDNA D-loop region of mtDNA have been reported to be involved in familial breast cancer development. Some polymorphism particularly those in D310 segment (mononucleotide repeat (poly-C) between 303 and 315 nucleotides), and position 263, 489, 522, and 527 were highly frequent in breast cancer families and might be responsible for inherited cancer susceptibility. Moreover D310 have been suggested as molecular biomarker for cancer susceptibility early-detection strategy (87). At the same time in sporadic breast tumor cases harboring D-loop mutations reduced mtDNA copy number was associated with an older onset age (>or=50) as well as a higher histological grade. Also survival analysis measured by the Kaplan-Meier curves and the log-rank test indicated that patients with reduced mtDNA content have significantly poorer disease-free and overall survival (78). In other study of sporadic breast cancer the occurrence of D-loop mutations was also associated with an older onset age (>or=50 years old), lack of estrogen and progesterone receptor expression. In these study patients with mtDNA D-loop mutations have significantly poorer disease-free survival in Kaplan-Meier curves and log-rank test. Also multivariate regression analysis indicated that D-loop mutation may be used in clinic as significant independent marker to assess the prognosis (88).

4. tRNA GENES MUTATIONS IN HUMAN CANCERS

Mitochondrial genome encodes 22 tRNA, but the tRNA genes spreaded among protein coding genes phenylalanine tRNA (577-647), valine tRNA (1602-1670), leucine tRNA (3230-3304 and 12266-12336), isoleucine tRNA (4263-4331), glutamine tRNA (4329-4400), methionine tRNA (4402-4469), tryptophan tRNA (5512-5579), alanine tRNA (5587-5655), asparagine tRNA (5657-5729), cysteine tRNA (5761-5826), tyrosine tRNA (5826-5891), serine tRNA (7446-7516 and 12207-12265), aspartic acid tRNA (7518-7585), lysine tRNA (8295-8364), glycine tRNA (9991-10058), arginine tRNA (10405-10469), histidine tRNA (12138-12206), glutamic amid tRNA (14674-14742), proline tRNA (15955-16023) and are often found in larger deletions reported in cancer cells (89, 90).

Until today only few somatic mtDNA mutations have been reported to localize in the tRNA genes. The homoplasmic A3243G mutation in colon cancer sample was reported (91). Another mitochondrial tRNA mutation has been described in the mtDNA methionine-tRNA gene - a G-to-A transition at position 4450, in a patient with a splenic lymphoma with villous lymphocytes. It was associated with morphological alterations of the mitochondria, with defects of respiratory chain complexes activities and with a decrease in the mitochondrially encoded cytochrome c oxidase subunit II (92). A8344G or G8363A tRNA lysine gene mutations have been described in lipomas (93). Multiple symmetric lipomas with high levels of mtDNA with the tRNA(Lys) A/G(8344) mutation was the only manifestation of disease in a carrier of myoclonus epilepsy and ragged-red fibers (MERRF) syndrome, and the tRNA(Lys) mutation might be either the direct or the indirect cause of perturbation of the maturation process of the adipocytes, leading to an increased risk of lipoma formation, but it has not been proven (94). Also cervical lipoma hereditary syndrome of cerebellar ataxia, photomyoclonus, skeletal deformities and lipoma, originally described by Ekbom, lipoma showed a high level of the tRNA-Lys A8344G mutation (95). Also lung cancer cases have been described to harbor tRNA mutations including G5521A lung tumor (68) and G3244A found in lung tumor and gastric carcinoma (91). Thyroid tumors seem to be the best analyzed cancer and mutations that have been reported include C4312T, C5633T, G7521A, and G12236A (74). Interesting mutations have been found in endometrial tumor - C12258G (91), as this mutation is characteristic for Diabetes-Deafness Syndrome (Midd-Ballinger-Wallace Syndrome), a noninsulin-dependent diabetes mellitus with deafness. The other mutation found in this type of cancer is T10463C (91). Finally pancreatic cancer cell line was shown to harbor T15983C mutation (96) A3243G transition in the gene encoding leucine tRNA was detected in oncocytoma case (97) and interesting G4450A mutation has been reported in transfer RNA methionine gene, in a patient with a splenic lymphoma with villous lymphocytes (92).

4.1 Consequences for cell physiology

Lipomatosis due to tRNA(Lys) mutations is associated with a pattern of altered expression of master regulators of adipogenesis consistent with enhanced proliferation but maintenance of adipocyte features, and with a distorted pattern of brown versus white adipocyte differentiations (93). In case of 8344 (A8344G) of the tRNA(Lys) gene of mtDNA, mutation impairs mitochondrial protein synthesis and causes a respiratory chain dysfunction (94). Two other mutations (G8363A and A8296G) in the mtDNA tRNA(Lys) gene have been associated with severe mitochondrial diseases in a number of reports. Mutation in the tRNA(Lys) gene (G8363A) have defective respiratory-chain enzyme activities and low oxygen consumption, indicating a severe impairment of the oxidative phosphorylation system. G8363A resulted in an important alteration in the conformation of the tRNA(Lys), but did not affect tRNA steady-state levels. Mutants have an important decrease in the proportion of amino-acylated tRNA(Lys) and, consequently, mitochondrial protein synthesis. The pathogenicity of G8363A arises as a consequence of a change in the conformation of the tRNA that severely impairs aminoacylation (98). The 3243 - the MELAS mutation occurs within the mtDNA binding site for a protein factor (mTERF) that promotes termination of transcription at the 16S rRNA/tRNA(LeuUUR) gene boundary. A marked decrease in affinity of purified mTERF for the mutant sequence is observed. The COII, COIII, and ND2 polypeptides were more severely affected and a marked decrease in 0_2 consumption is reported (99). Cytochemical evidence show that cytochrome c oxidase deficiency is associated with the MELAS mutation, as affected nucleotide is conserved not only in many mitochondrial tRNAs but in most cytosolic tRNA molecules (100) and thus single point mutation causes the functional abnormality in the respiratory chain of mitochondria (101). In 3243 cells mitotic segregation goes

always toward increasing levels of mutant mitochondrial DNA. Rapid segregation is frequently followed by the loss of mtDNA (102). In cell lines carrying A3243G mutations in their mitochondrial tRNA genes elevated ROS production was also found (by 83 %). Mutants showed also 1.5-fold increased of catalase activity, a significant increase in the activity of Se-GSH-Px (glutathione peroxidase) and in total- superoxide dismutase activity (SOD) both- CuZn-SOD and Mn-SOD (28). The G4450A methionine tRNA is associated with morphological alterations of the mitochondria, with defects of respiratory chain complexes activities and with a decrease in the mitochondrially encoded cytochrome c oxidase subunit II associated with a severe respiratory chain dysfunction (92).

4.2. Clinical implications

Until now no reports are available to prove the correlation of mitochondrial tRNA genes mutations with patient's prognosis or with tumor stage and/or grade. It is not established if tRNA mutations reported above arise as early or rather late events in the process of carcinogenesis.

5. rRNA GENES MUTATIONS

Mitochondrial genome encodes two rRNA genes - 12S ribosomal RNA (648-1601) and RNA (1671-3228) (90) and its mutations have also been reported in a variety of human cancers, as endometrial carcinomas (64) or cell tumors arising in end-stage renal disease (103). 16S rRNA gene mutations were reported in gestational trophoblastic disease (65). C2998CT (91) and T2664C are described in lung cancer (68), A1811G in head and neck cancer, T2445C, G3054A in bladder cancer (68), and G2923A in prostate cancer (61). Many rRNA mutations have been reported in pancreatic cancer cell lines: G687A, T1243C, T1406C, A1676G, G2015A, T2222C, A2805T, and A2905G (96). It needs to be pointed out that T at nucleotide 1243 in conserved positions, and the prediction of RNA secondary structure shows change in this 12S rRNA variant's structure and this mutation is present in sensoneuronal hearing loss (104).

Interesting mutations are found in endometrial carcinomas. Above all 12S rRNA gene is considered as instability hot spot region in endometrial carcinomas and it has been suggested that mtDNA replication errors may account for high frequency of mtDNA mutations in endometrial carcinomas (64). In other report 12S rRNA gene at positions 772, 773, and 780 in stage IIIC endometrial tumors occurred with a frequency of 100%. Furthermore, two mutations were observed in serous tumors only at position 1657 in stage IV (100%), and at position 8221delA in benign cystadenomas (100%) and borderline tumors (again 100%), which suggest that certain mtDNA mutations can reliably distinguish the different histological subtypes of epithelial ovarian tumors and that rRNA mutations are one of these mutations (105). Other female specific cancers are also reported to harbor mitochondrial rRNA mutations. In ovarian carcinomas 16S and 12S rRNA genes are often mutated with G to A transitions most common, and G664A, G1401A, T1952C and they may represent the zone of preferred mtDNA mutation in ovarian cancer (60). In a recent paper where stringent data quality control has been used to evaluate spectrum of mtDNA somatic mutations in breast cancer T2275C transition was found in the primary cancerous tissue but not in normal tissues and what's more important this is the transition that has been previously reported in the colonic crypts and it is located at a highly conserved site in the 16S rRNA gene (106).

The first report on colorectal tumor mtDNA mutations revealed T710C, T1738C, T1967C, and T2299A mutations (107) and further homoplasmic mutations distributed in 16S and 12S rRNA genes of colorectal cancers have been suggested to be involved in tumor progression (18). At the same time in gastric cancer there is a significant correlation between differentiation degree of gastric cancer and variant frequencies of 12S rRNA-tRNA(Phe). The 12S rRNA-tRNA(Phe) variations are more likely to be poorly found in differentiated cancers (108). To further evaluate rRNA mutation effects on the development of gastric carcinomas, the distribution and RNA secondary structure of 12S rRNA mutants has been analyzed. Statistically significant difference existed in variant frequency of 12S rRNA between intestinal type (29.4%) and diffuse type of gastric carcinoma (70.6%), also variant frequency of 12S rRNA in cancer was higher than that in dysplasia. During the process of progression from normal cell through dysplasia to cancer, 12S rRNA tended to transit from wild type homoplasmy and heteroplasmy to mutant type homoplasmy with 12S rRNA 652G insertion having more adverse effect on secondary structure stability of 12S rRNA (109).

6. OXPHOS COMPLEX I GENES AND HUMAN CANCER

Complex I (NADH:ubiquinone oxidoreductase, EC 1.6.5.3) is the largest multisubunit assembly of the OXPHOS system, comprising 39 nuclear encoded and 7 mitochondrially encoded subunits: NADH dehydrogenase subunit 1 (3307-4262, ND1), NADH dehydrogenase subunit 2 (4470-5511, ND2), NADH dehydrogenase subunit 3 (10059-10404, ND3), NADH dehydrogenase subunit 4L (10470-10766, ND4L), NADH dehydrogenase subunit 4 (10760-12137, ND4), NADH dehydrogenase subunit 5 (12337-14148, ND5) and NADH dehydrogenase subunit 6 (14149-14673, ND6). Malfunction of this complex and mtDNA mutations in ND1-6 genes are associated with a wide variety of clinical syndromes. In human tumor both the non-coding D-loop region and Complex I seem to be mutational hotspots (55) and it has been hypothesized that the resultant somatic mutations in mtDNA play a causal role in oncogenic transformation. This hypothesis has been supported by report of ND2 mutations in chemical carcinogen-induced rat bladder and human bladder cancers (46). In clinical oncology research other pivotal role of complex I has also been proven. It was shown to be involved in activation of adriamycin - a member of the anthracycline class of anticancer drugs. This drug is activated in by complex I redox cycling between the quinone and the semiquinone. Cancer cells with mtDNA mutations and lacking complex I activity as a consequence,

are resistant to adriamycin. Thus, complex I mutations that frequently occur in human tumors have the potential to contribute to resistance to chemotherapeutic agents that require redox cycling for their activation (1).

Until today complex I mutations have been found in a variety of human cancer with colon cancer as the first one reported to bear ND genes mutation. Colorectal cancer was show to have both synonymic G3357A (61), missense T10563C (C-R), T3308C (M-T), and frameshift A12418AA (107) mutations. Other digestive tract cancer with well characterized mtDNA genome in pancreatic cancer with C3654T, G4580A, A4811G, C10715T, T11009C, T11152C, C11674T, A11974G, T12414C, G12561A, C12717T, T12414C, G12561A, C12717T, T12954C, T13500C, C14650T, T12954C, T13500C - synonymic mutations and G3421A (V-M), A3505G (T-A), G3670A (A-T), T10970C (F-L), T11703C (L-P), T11781C (I-T), A14552G (V-A), G14603A (S-F), G10176A (G-T) - missense mutations (96). Extensively characterized by mitochondrial groups are head and neck cancers. Oral cancer samples had A4986C (T-P), A5026G (H-R), T11794C mutations (110). In the population of smoking male patients with oral squamous cell carcinoma (SCC) mutation hot-spot is the ND2 gene (84). At the same time in oral cancer samples from betel quid chewers three were missense mutations (C14F, H186R, T173P) also in NADH dehydrogenase subunit 2 (111) have been described. In the head and neck group thyroid tumors have been analyzed in details. In thyroid tumors respiration rate, mitochondrial ATP synthesis driven by complex I substrates and complexes I and III was described as dramatically reduced and production of reactive oxygen species enhanced; mtDNA sequencing identified a frameshift mutations in ND1 (112), and later on more of mutations including C10793T (68, 74), C3594T, A4613G, C4940T, C10181T, A4985G, C11840T, C12918T, G14560T, C11332T, C12918T, C10691G, G3526A (A-T), G3910A (E-K), C3992T (T-M), A10639G (N-S), G10197C (A-P) , C10269T (L-F), C10272T (L-F), G10320A (V-I), A12967C (T-P), T14498A (Y-F), C13943T (T-M), A12967C (T-P), C13943T (T-M), G11016A (S-N) (74) and A10398G (T-A) (113) and A5298G (I-V), A13514G (D-G), A14417G (V-A), G11126A (E-K), A13514G (D-G), 5408A-del (frameshift) in papillary thyroid carcinoma (114) have been described. This suggests that mtDNA variants and mtDNA somatic mutations of complex I (and also complex IV) genes are involved in thyroid tumorigenesis and follicular and papillary carcinomas carry a significantly high prevalence of non-silent point mutations of complex I genes (74).

ND1, 4 and 5 mutation are found already in early stage prostate cancer; not only synonymic as A11947G, A3480G, G12372A, C12705T, G12372A, C12705T, but also missense mutations A14053G (A-T), A3434G (Y-C) (61), T12414C (96) and 11032:11038 A(7)-A(6) frameshift mutations correlated with loss of complex I activity and assembly (61) (97). In renal oncocytomas, the mitochondrial NADH-coenzyme Q oxidoreductase activity (complex I) is strongly decreased and important reduction in the amount of assembled complex I was shown by two-dimensional blue native-PAGE and/or immunological titration of some complex I subunits (115). Mitochondrial genome scan revealed variety of frameshift mutations correlated with loss of complex I activity and assembly T13493C (L-P), 12384T-TT, 3566:3571 C(6)-C(7) and 3571 C-del, 11032:11038 A(7)-A(6), 10952:19052 C-CC (97), and 264 bp deletion (116). Other types of cancers also have reported complex I mutations. This includes breast tumor with G3918A, G11900A (V-M), T12344A (M-K) (117), bladder tumor - T10071C, A10792G, G11518A, A10978G, C10822T, A11065G, C12049T, T12519C, T10321C (V-A) (68), endometrial adenocarcinoma - T10551C and T10640C (91) and lung tumor G12345A (68).

6.1. Consequences for cell physiology

Recently a paper by Ishikawa *et al.* points out complex I mutations as metastasis promoting factors. The authors have shown down-regulation of complex I activity in highly metastatic cells and were able to transfer the feature of high metastatic potential to cybrids with mtDNA of donor cells. The mutated mtDNA has been shown to harbor metastatic potential. Mutations responsible for metastasis formation are located in ND6 gene - G13997A and 13885insC. Those two mutations cause a deficiency in complex I activity and also overproduction of ROS accompanied by up-regulation of MCL-1, HIF-1, and VEGF genes. In mice metastasis assays it was shown that recipient tumor cells acquired the metastatic potential of the transferred mtDNA. Pretreatment of metastatic cells with ROS scavengers suppresses their potential to give rise to metastatic, which demonstrates that ND6 mutation related ROS are responsible for metastatic potential enhancement (17).

Even before the recent paper of Ishikawa (17) large body of evidence supported the hypothesis that complex I disruption is of pivotal role in cancer patho-physiology. In human squamous cell cancers of the head and neck the function of the mtDNA mutations was assessed with molecular genetics techniques. Cancer ND2 mutants ware cloned and expressed in cancer cell lines resulted in increased anchorage-dependent and independent growth, accompanied by increased reactive oxygen species production and an aerobic glycolytic metabolic phenotype with hypoxia-inducible factor (HIF)-1-alpha induction. All this data suggests cancer-specific mitochondrial mutations may contribute to development of a malignant phenotype by direct genotoxic effects from increased reactive oxygen species production as well as induction of aerobic glycolysis and growth promotion (118).

The best studied cancer in mitochondrial oncology is oncocytoma. Oncocytic tumors represent a distinctive set of lesions with specific granular cytoplasmic eosinophilia and cells are called oncocytes because of striking accumulation of mitochondria. Although generally uncommon, oncocytic tumors have been in the focus of mitochondrial research due to their apparent morphological disruption of mitochondrial structure. A great variety of biochemical and molecular changes have been identified, and the aberrant biogenesis of mitochondria in oncocytic

cells seem to bear similarities to that reported in mitochondrial encephalomyopathies and oncocytoma cell lines are all addicted to glucose for their survival and growth (119). Recently the relationship between the accumulation of mitochondrial disturbances and the occurrence of tumors is being revealed (120). In renal oncocytoma enzymatic activity of complex I is often undetectable or greatly reduced as proven by in-gel activity assay of complex I. This is accompanied by lack of assembled complex I and numerous frame-shift mutations in the genes of either subunit ND1, ND4, or ND5 of complex I. Remarkably, tissues that originate from the same patient with two independent oncocytomas harbor different mutations, but both localize in ND genes. In the reported case one tumor had an insertion of a cytosine residue into a stretch of six cytosines at mtDNA positions 3566 to 3571 in the ND1 gene and second tumor had insertion in the stretch of six cytosine residues at positions 12385-12390 of the ND5 gene with >95% mutational load (97). In other report mtDNA mutations again resulted in complex I deficiency. Of 7 tumors with complex I defects, all had ND genes mutations and one had a defect in the mitochondrial gene of complex III (CIII) that is necessary for complex assembly. All mtDNA mutations reported are expected to result in defective protein. Complex I was also inhibited in tumors harboring mutations in the D-Loop (119). It is important to notice that in cells with mutated cytochrome b gene of complex III may in fact have secondary induced complex I deficiency, because respiratory complexes are bound into super-complexes while assembled and the absence of the cytochrome b protein may cause the dissociation of such super-complex and promote the degradation of free subunits (121)

Oxyphilic thyroid tumor specimen tissues removed from tumor with the normal part of the thyroid gland serving as a control was studied. Fresh tumor samples were analyzed by paleography to investigate the mitochondrial respiratory chain activity, and the rate of oxidative phosphorylation (ADP/oxidation ratio) and to determine the mitochondrial ATP synthesis. In tumors samples the ATP synthesis, adjusted to the mitochondrial protein level was significantly increased from 5.8+/-1.4 □mol/mg per 10 min, to 12.1+/-3.1 □mol/mg in matched controls. In those tumors expression of ND2 and ND5 mitochondrial genes was also 12 times higher and when adjusted to the mtDNA/nDNA ratio, the relative mitochondrial transcript ratio was 3.8 times higher in the tumor samples. However, the ADP/O ratio was only 75% of the normal value (122). In oncocytic phenotype thyroid tumors sequenced the entire mtDNA and 57.8% samples analyzed harbored 30 mtDNA mutations, 25 of which were in complex I genes. 26% cases presented nonsense or frameshift mutations. All those disruptive mutations were found in complex I subunit genes, and the association between these mutations and the oncocytic phenotype was statistically significant. In three samples, the disruptive mutations coexisted with potentially damaging missense mutations and 31.1% of the mutations were potentially damaging missense changes, but patient's age, sex, and size of the lesion were not associated with mtDNA mutations (123). In primary papillary thyroid carcinomas sequencing of the entire mtDNA coding regions, revealed somatic mutations in 36.8% cases. This was followed by flow cytometry analysis of mitochondria respiratory function which revealed a severe defect in mitochondrial complex I activity. The majority of the mutations detected were involved in genes located in the complex I of the mitochondrial genome with majority of A-G or C-T transitions, and most often resulting in a change of a moderately or highly conserved amino acid in protein. The majority of the mutations were located in the complex I of the mitochondrial genome. Somatic mtDNA mutations were also detected in one of four multinodular hyperplasias examined, which might suggest that mtDNA mutations are involved in the early stage of tumor development. Two interesting mutations were described - 7476 reported previously in patients with Alzheimer's disease and mutation at 13514 known to be atypical mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes (MELAS) mutation (114). Yeh *et al.* have shown that the distribution and spectra of nonsomatic mitochondrial gene variants seem to differ between patients with thyroid cancer and normal individuals, with complex I variants favored among cancer patients. Significant differential distributions of mtDNA sequence variants between thyroid carcinomas and controls have been found. Interestingly, these variants appear to be more frequent in the genes which encode complex I of the mitochondrial electron transport chain compared to normal population controls. These findings suggest first, that somatic mtDNA mutations may be involved in thyroid tumorigenesis and second, that the accumulation of certain non-somatic variants may be related to tumor progression in the thyroid (113). These results were confirmed by Maximo *et al.* (2002). A detailed research on metastatic oncocytic thyroid follicular carcinoma was performed on XTC.UC1 cell line. This cell line is unable to rely solely on mitochondria for energy production and survive in galactose supplemented medium. In this cancer the rate of respiration and mitochondrial ATP synthesis driven by complex I substrates was severely reduced in XTC.UC1 cells. The enzymatic activity of complexes I and III was dramatically decreased while ROS production was strongly enhanced. Sequencing of mtDNA revealed a frameshift mutation in ND1 and a non-conservative substitution in cytochrome b. This proves that mitochondrial dysfunction in XTC.UC1 cell is a result of a combined complex I and III mtDNA mutations. This biochemical phenotype was cotransfered along with CYTB G15557(E271K) and A15326G(T194A), substitutions and ND1 (C) insertion at 3571 resulting in frameshift premature stop codon (G101X) mutations giving rise to early degraded truncated polypeptide. This research has proven that occurrence of a severe energetic dysfunction in a thyroid oncocytic cells can be attributed to two pathogenic mtDNA mutations. Moreover remarkably reduced level of ND6 subunit was also found in those cells, which suggests possible derangements in complex I assembly, for example sub-complex formation may be impaired by the absence of ND1 protein, as similar co-dependence of ND protein presence-complex I assembly was shown in Leigh's syndrome, wherein mutations in ND6 cause a defective complex I assembly (112). Since several of these subunits are necessary for proper assembly of the complex, the lack

of only single subunit, such as one encoded by MT-ND4 or MT-ND6 or GRIM19/NDUFA13 (nuclear DNA), or of an assembly factor may lead to degradation of all the remaining subunits (124, 125). Recently, somatic missense mutations in the complex I assembly protein GRIM-19 were identified in 15% of sporadic oxyphilic Hürthle cell tumors of the thyroid (126). It has been reported that about 60% of ND5 mRNA is required to support a normal rate of ND5 subunit synthesis for mutants which create a stop codon downstream, resulting in truncated polypeptide (for example 13885 frameshift mutation). Below this threshold ND5 protein content in the cell declines progressively, as no upregulation at the translational level takes place. With the decreasing percentage of functional mRNA - the decreased protein synthesis and respiration rate, which indicate that there is very little excess of ND5 protein synthesis capacity over required to maintain assembly of functional complex I, and it cannot be excluded that this is truth for other ND subunits. Overall respiration might be tightly regulated by ND genes expression (127). Moreover it was recently shown that the nuclear encoded subunit NDUFS1, which is part of the iron-sulfur clusters of complex I, is proteolytically cleaved by caspase-3 and it seems interesting that mtDNA encoded subunits are possibly involved in apoptosis. Taking into account all data on complex I it seems to have a dual function—creating a proton gradient under normal conditions and participating in apoptosis after caspase-3 cleavage. Loss of this intermediate step of apoptosis due to a deficiency of complex I might lead to a selective advantage for tumor cells (128).

6.2. Clinical implications

In renal carcinomas (conventional, papillary, unclassified) reduction of all mitochondrial enzyme activities including complex V due to mitochondrial DNA content depletion is significant. Mitochondrial enzyme activities and mitochondrial DNA levels were not statistically different between the conventional, papillary and unclassified sarcomatoid type of renal carcinoma and do not correlate with tumor grade, metastasis, ploidy and proliferative activity. Moreover a co-ordinated down-regulation of all components necessary for mitochondrial energy metabolism occurs in most renal carcinomas as an early event in carcinoma formation, and does not change with progression of the disease (129).

7. OXPHOS COMPLEX III GENES

Cytochrome b (CytB) of mitochondrial electron transport complex III (QH2:cytochrome c oxidoreductase, ubiquinol-cytochrome-c reductase) is encoded in mtDNA - 14747-15887 and has also been reported mutated in a large variety of human tumors as pancreatic cancer - C14866T, C15646T, T15784C, G15884C (A-P) (96), breast cancer - G14869A (117), colon cancer - C14920T (130), G14963A (V-M), G15084A, C15540CC, G14985A (R-H), T15572C (F-L), G15756A (107, 130), thyroid tumor - G15179A (V-M), A15182G (I-V), T15312G (I-S) (131) and ovarian cancer - G15761A (60). Cytochrome b gene has been suggested as the zone of preferred mtDNA mutation in ovarian cancer (60). COIII mitochondrial DNA mutations have also been reported to arise in chemical carcinogen-induced rat bladder and human bladder cancer (46)

7.1. Consequences for cell physiology

The thyroid cancer cell line - XTC.UC1 was shown to harbor nonconservative substitution in cytochrome b and its mitochondrial dysfunction was proven to be of combined complex I/III defect associated with mtDNA mutations origin (112). In a murine xenograft and human model of bladder cancer, the functional effect of overexpression of a 21-bp deletion mutation of cytB has revealed that overexpression of mtCYTB is accompanied by increased ROS production, increased oxygen consumption and lactate production. In cells with CYTB mutations NF-κB signaling pathway is up-regulated, giving rapid cell cycle progression and tumor growth *in vitro* and *in vivo* providing physiologic and functional evidence for the role of a *bona fide* mitochondrial gene mutation in cancer (Figure 1, 2) (132).

8. OXPHOS COMPLEX IV GENES AND HUMAN CANCER

Complex IV of mitochondrial electron transport, (NADH cytochrome c oxidase, Warburg's respiratory enzyme, EC 1.9.3.1) being the last enzyme in the respiratory electron transport chain receives an electron from each of four cytochrome c molecules, and transfers them to one oxygen molecule, converting molecular oxygen to two molecules of water. Three subunits of complex IV are encoded by mitochondrial genome - cytochrome c oxidase subunit I (5904-7445, COI), cytochrome c oxidase subunit II (7586-8269, COII) and cytochrome c oxidase subunit III (9207-9990, COIII) and these have also been found mutated in human cancers, especially frequently in oral cancer of betel quid chewers (111). MtDNA variants and mtDNA somatic mutations of complex I and complex IV genes seem to be involved in thyroid tumorigenesis (74) with G8697A, A8701G (T-A), A8706G, A8716G (K-E), C9030T, T9137C (I-T) mutations reported (131). Also colon cancer and pancreatic cancer harbor complex IV mutations - G8557A (A-T) and G8572A have been reported (130) and T8696C (M-T), T9070G (S-A) and T9078C mutations (96), respectively. In the case of prostate cancer COI was found to be mutated in 12% of patients and reported mutations altered conserved amino acids (26).

8.1. Consequences for cell physiology

A high percentage (nearly 100%) of Hürthle cell tumors display the mitochondrial common deletion and/or somatic mitochondrial point mutations (131), but only a partial deficiency of cytochrome-c oxidase has been found in oncocytic nodules of hyperplastic or adenomatous parathyroid glands and Hürthle cell lesions (131), while CO activity is reduced in pulmonary alveolar cell carcinoma cell (133). At the same time cytochrome c oxidase (COX) mutant cells analyzed till today show neither an increase in ROS production nor elevation of antioxidant enzyme activities or oxidative damage (28).

8.2. Clinical implications

In clear cell renal cell carcinomas (CCRCC) a clear-cut difference between the benign oncocytomas and

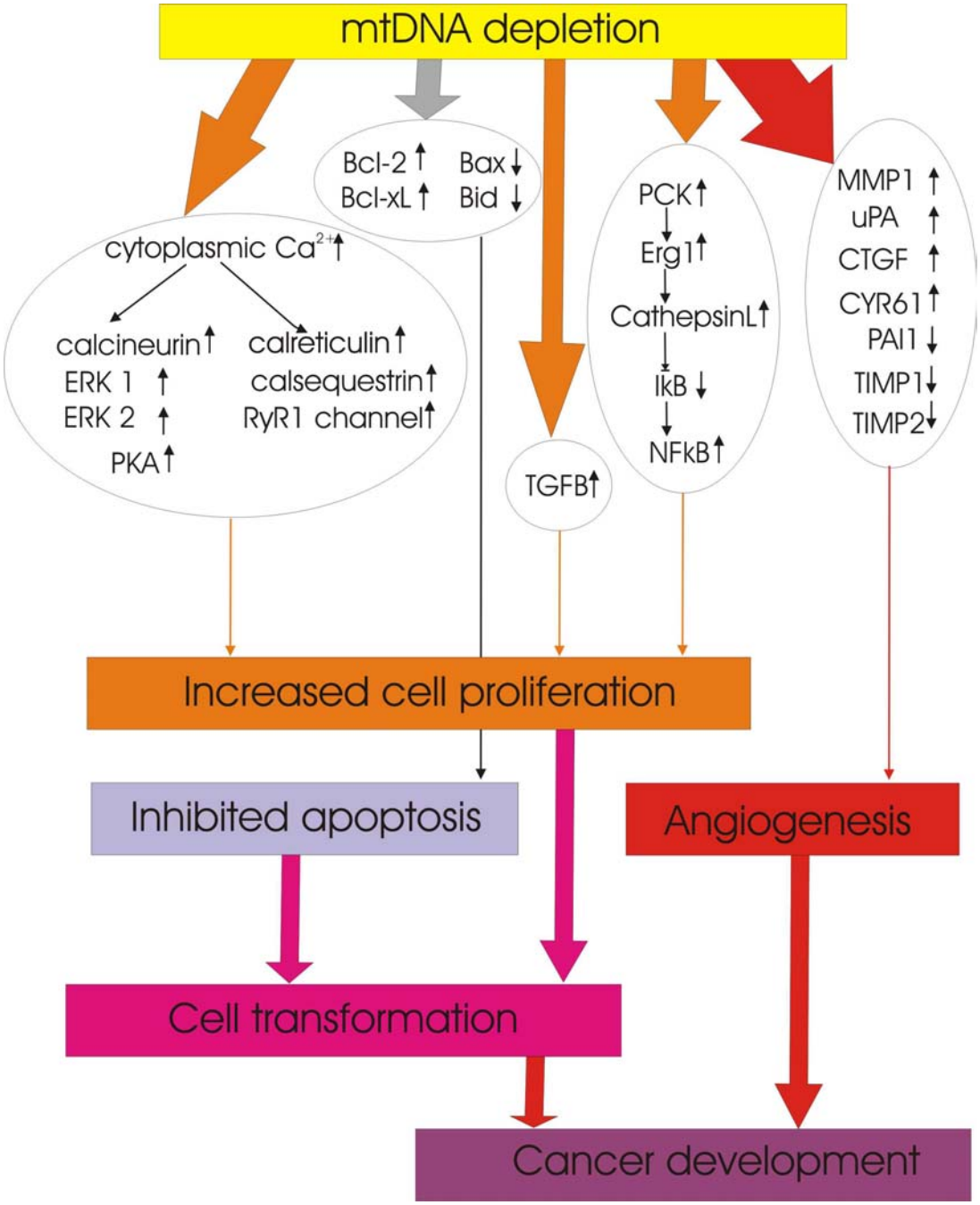

Figure 2. The role of mtDNA depletion in cancer development. Pato-physiological consequences of mtDNA depletion in cell transformation: 1) increased cell proliferation due to activation of Ca^{2+} signaling (calreticulin mediated pathways of MAP/ERK kinase activation and calsequestrin releted pathway) , TGFβ signaling and PCK signaling pathway; 2) apoptosis inhibition (upregulation of anti-apoptotic Bcl-2, Bcl-xL; downregulation of pro-apoptotic Bax and Bid); 3) angiogenesis activation (upregulation of matrix metaoproteinases eg. MMP-1 and down regulation of its inhibitors). ERK1 = MAPK3 mitogen-activated protein kinase 3; ERK-2 = MAPK1 mitogen-activated protein kinase 1; RyR1 - ryanodine receptor 1; PKA - cAMP-dependent protein kinase; PCK - protein kinase C; ERG-1 - early growth response 1; IκB - inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta; NF-κB nuclear factor kappa-B; MMP-1 - matrix metallopeptidase 1 (interstitial collagenase); uPA - plasminogen activator, urokinase; CTGF - connective tissue growth factor; CYR61 - cysteine-rich, angiogenic inducer, 61; PAI1 - plasminogen activator inhibitor 1; TIMP - TIMP metallopeptidase inhibitor 1; TIMP2 metallopeptidase inhibitor 2

the clear-cell or the chromophilic carcinomas can be assessed based on mitochondrial – CO marker (Table 2). In oncocytomas all mitochondrial markers are increased but to a variable degree, complex IV activity is increased by factors of about 7, while complexes V, III, and II are slightly increased or close to normal. On the contrary, in other tumor types such as chromophilic and low-grade CCRCC tumors, all parameters are decreased with the exception of complex V. In the most aggressive high-grade CCRCCs, complex V activity is also significantly decreased (8). Nevertheless no significant difference between carcinomas with renal vein invasion, distant metastases and without metastases can be defined based on mitochondrial activities. In addition, no differences of OXPHOS activities and mitochondrial DNA levels is seen in tumors smaller or larger than 7 cm in diameter (129). Another important result is the gradual worsening of the mitochondrial impairment from the less aggressive chromophilic carcinomas to the high-grade CCRCCs. In chromophilic tumors the OXPHOS activities are less reduced than in CCRCCs of all grades. The ATPase activity is higher than expected from the activities of complexes II, III, and IV which is similar to low grade CCRCCs. The differences in CO activity are clearly associated with the tumor type, not to their stage of progression. In this study enzymatic activities in various zones of the tumor taken from one patient shown no differences (8).

9. OXPHOS COMPLEX V GENES AND HUMAN CANCERS

In mitochondrial genome ATPase8 ATP synthase F0 subunit 8 (8366-8572) and ATPase6 ATP synthase F0 subunit 6 (8527-9207) are encoded. ATPase8 mutations have been shown to arise in chemically induced carcinogenesis in rat bladder and human bladder cancer (46) and germline polymorphisms of the ATPase 6 gene along with somatic mutations G8697A, A8706G, C9030T, A8701G (T-A), A8716G (K-E), T9137C (I-T) in the thyroid are to be associated with Hürthle cell tumors (74). In esophageal cancer progression from heteroplasmic to homoplasmic of A9182G (N219S) in ATPase 6 was reported (58) and additional somatic mutations are reported in pancreatic cancer T9078C, T8696C (M-T), T9070G (S-A) (96).

9.1. Consequences for cell physiology

Complex V genes seem of high importance not only to their function in ATP generation, but also other cell processes as apoptosis and therefore it is not surprising that if ATP6/8 are mutated a cell transformation is favored. Sequence variants of the ATPase 6 gene, of the complex V genes thought to play a role in mtDNA maintenance and integrity (74) and moreover efficient execution of apoptotic cell death requires the molecular components of the ATP synthase (134).

If cybrids with T8993G or T9176 mutations in the MTATP6 gene, derived from patients with mitochondrial encephalomyopathy are transplanted into nude mice, the MTATP6 mutations conferred an advantage in the early stage of tumor growth did contribute to promotion of tumor development by prevention of apoptosis (27). Interestingly T8993G/T8993C point mutations in the mitochondrial ATP6 gene destabilize the human F1F0-ATP synthase without preventing enzyme assembly and oligomerization (135). PC3 prostate cancer cell line with ATP6 T8993G mutation was also tested for tumor growth in nude mice. The mutant cells (cybrids) generate 7 times larger tumors than wild-type cells, and generated significantly more ROS (26). DNA microarray analysis performed using cells harboring 3243A>G (tRNA-Leu (UUR)) and 8993T>G (ATPase6 Leu156Arg) mtDNA mutations revealed that mRNA and protein levels of ATF4, CHOP (C/EBP homologous protein) and ASNS (asparagine synthase) are up-regulated in with mtDNA mutations. The transcription of CHOP and ASNS genes is up-regulated by specific transcription factors through the AARE (amino acid regulatory element) and NSRE-1 (nutrient-sensing response element-1) enhancer elements (136).

9.2. Clinical implications

Renal cell carcinomas (RCCs) of clear cell type (CCRCCs) have been used to study ATPase activity as clinical marker. Most renal carcinomas lose ATP synthase activity, and low content of complex V protein is found in all CCRCC. However F1-ATPase activity impairment is not associated with increased aggressiveness. Nevertheless disturbed assembly of complex V may be detected in CCRCC and chromophilic tumors with immunohistochemistry methods staining for free F1-sector of complex V (8). CCRCCs, that are more aggressive renal cancers than chromophilic cancers, exhibited the lowest respiratory chain activities and oncocytomas which are the most benign contain increased amounts of complex III, IV and V, suggesting a correlation between mitochondrial OXPHOS disruption and tumor aggressiveness (8). Aurovertin-sensitive ATPase activity is always decreased in high-grade CCRCCs, it is sometimes increased in low-grade CCRCCs and in chromophilic tumors, but unlike ATPase activities, complex V protein is uniformly decreased in clear cell and chromophilic type carcinomas and increased in benign oncocytomas (8). Furthermore, no significant difference between ATPase activity in carcinomas with renal vein invasion, distant metastases and without metastases can be detected. In addition, no differences of OXPHOS activities and mitochondrial DNA levels were seen in tumors smaller or larger than 7 cm in diameter (129). The described discrepancy between high ATP hydrolysis activity and low complex V protein amount again suggested instability of complex V. As the F1-ATPase activity is necessary to maintain the mitochondrial membrane potential, so mtDNA depleted cells or ATPase mutant cells use glycolysis for ATP supply, but may maintain the mitochondrial membrane potential, required for mitochondrial matrix activities (8).

Although ß-F1 is encoded in the *nuclear genome* it is an interesting mitochondrial protein, as it has been recently established as relevant cancer marker in human kidney tumors, colon carcinomas, primary breast ductal invasive adenocarcinomas, gastric adenocarcinomas,

squamous esophageal carcinomas and lung adenocarcinomas. Highly significant down-regulation of the ß-F1-ATPase protein was found when compared with the normal tissues and decrease of the ß-F1-ATPase:Hsp 60 ratio was seen in the process of carcinogenesis that involves a selective down-regulation of the expression of the ß-F1-ATPase. Subsequently ATPase:Hsp 60 ratio was suggested as bioenergetic index of the cell that could be used for classification and prognostic purposes in certain types of cancers (137). Reductions in ß-F1-ATPase is more pronounced in tumors derived from patients with progressive disease and the Kaplan-Meier survival curve showed the association of the expression level of ß-F1-ATPase in the tumors with overall survival and disease free survival (DFS) and the time of recurrence of the disease and in assessing the clinical outcome of patients with Dukes' stage B2 (T3N0M0) colorectal carcinomas (137) (138). Cross-validation analysis using the ß-F1-ATPase/Hsp 60 ratio as predictor revealed a classification sensitivity of 97.3%. It was also observed that the overall correct classification of the biopsies was 91.4%, with a sensitivity of 97.3% for the tumor biopsies. The same analysis using the BEC index as a predictor variable showed an overall correct classification of the biopsies of 92.2% with a sensitivity of 92.6% (139). This marker allows the identification of a subgroup of cancer patients with significantly worse prognosis and multivariate Cox regression analysis indicated that tumor expression of ß-F1-ATPase is a significant marker independent from clinical variables to assess the prognosis of the patients (140). All this suggests that more effort is needed to establish usefulness of mtDNA encoded genes role in cancer prognostics and diagnostics.

10. LARGE mtDNA DELETIONS AND mtDNA DEPLETION IN HUMAN CANCERS

Large deletion of mitochondrial DNA – the 4977 bp (at position 8,482-13,460 of mtDNA) accumulates in patients with heteroplasmic mtDNA mutations and in normal individuals during aging. The common deletion affects several transfer RNA and respiratory chain genes. It is known to be involved in myopathies, Alzheimer's disease, as well as chronological aging (141), in lung carcinoma (142), nonmelanoma skin cancer (NMSC) and in cutaneous photoaging (143), skin basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) (141), esophageal squamous cell carcinoma (144), hepatocellular carcinoma and hepatocellular nodular hyperplasia (145, 146), oncocytoma of the parotid gland (147), nasopharyngeal carcinoma (148), gastric cancer (149), Hürthle cell tumors (74). The poor bioenergetic state in mitochondria containing mtDNA with the 4977-bp deletion has been well documented. In those cells self-accelerating vicious cycle of mitochondrial ROS production is initiated by the deletion and should be proposed that this mechanism may play an important role in the pathophysiology of the disease process caused by mitochondria containing mtDNA with the 4977-bp deletion (150). Another large deletion (4981 bp) was detected frequently in nasopharyngeal carcinoma (NPC) and evidence was provided that mtDNA mutation is involved in the development and progression of NPC (148).

In many human cancers including HCCs and renal tumors a significant reduction in mtDNA levels was observed when compared with normal corresponding tissues (137) (146) (129). Partial depletion of mitochondrial DNA induced a stress signaling associated with increased cytoplasmic-free Ca2+ with overexpression of tumor-specific markers like cathepsin-L and transforming growth factor beta (TGFbeta) and activation of Ca2+-dependent protein kinase C (PKC). MtDNA depleted cell lines showed also 2.5 to 18-fold increased levels of mRNA for RyR1 (ryanodine receptor 1) Ca2+ channel, calreticulin, calsequestrin, MMA, cathepsin L, TGF-beta and phosphoenol pyruvate (PEP) carboxykinase, Insulin receptor substrate 1 and mouse melanoma antigen (151). Along with mRNA levels a 3 to 5-fold increased in cathepsin L protein level is found and the level of TGF-beta protein increased by 4.5-fold. Moreover elevated secretion of matrix protease cathepsin L is 10-fold (151). Calcium-dependent MAP kinases (ERK1 and ERK2) and calcineurin are activated, while the levels of anti-apoptotic proteins Bcl2 and Bcl-XL were increased, and the cellular levels of pro-apoptotic proteins Bid and Bax were reduced. Ca2+ specific PKC effects are mediated through Egr-1 factor (member of immediate early Jun/Fos family genes) binding to the cathepsin L promoter, and activation of NF-κβ/Rel factors through selective inactivation of inhibitory factor - I-κβ (151). This mitochondria-nucleus stress signaling is in power to induce invasive phenotypes in non-invasive C2C12 myoblasts and human pulmonary carcinoma A549 cells. These cells exhibit 4- to 6-fold higher invasion in matrigel membrane mice model as well as rat tracheal xenotransplants in SCID mice (30). Also cells injected subcutaneously into SCID mice show the highest level of invasion. These mtDNA-depleted cells show highly invasive behavior and are resistant to apoptosis in response to varied agents including staurosporine and etoposide (151) (152). The effect of the mitochondrial DNA reduction on the expression of mitochondrial and nuclear-encoded COX subunits was also investigated in renal carcinoma tissues and a significant reduction of nuclear and mitochondrial-encoded subunits was found and median reduction of mitochondrial-encoded subunit I of COX was in the same range as the reduction of the nuclear-encoded subunit IV of COX (129).

At the same time in large scale study OXPHOS-deficient – mtDNA depleted – cells had 338 genes expression changed, of which several are involved in ECM remodeling and few genes known to participate in neoplastic transformation of particular MMP1, TIMPs 1 and 2, CTGF, uPA, PAI 1 and CYR61. This correlation suggests a common mechanism in which cells undergoing OXPHOS dysfunction stimulate ECM remodeling, which *in vivo* could lead to neovascularization or invasion (Figure 1) (153).

Inhibition of complex I or complex IV of OXPHOS leads to changes in the expression of cytoskeletal and cytoskeleton-associated proteins and those involved in apoptosis, glycolysis, the tricarboxylic acid cycle, oxidative stress responses and other OXPHOS proteins. The inhibition of complex IV results in increased expression of

the translation elongation factor - EEF1D, the pyruvate kinase (PKM2), and down-expression of the phosphorylated form of the cytoskeletal protein KRT8, sterol biosynthesis protein HMGCS1 and also downregulation of NDUFV2 (NADH dehydrogenase flavoprotein 2) and SDHA - mitochondrial respiratory chain proteins downregulation (154). Recently disorganization of the vimentin network in cultured OXPHOS deficient cells has been show in osteosarcoma cells upon complex I and complex IV inhibition. With the inhibition of complex IV the vimentin network collapsed around the nucleus and forms thick bundles and mitochondria form a perinuclear crescent (154).

11. OXPHOS GENES EXPRESSION IN HUMAN CANCERS

Neoplastic transformation was found to have a marked effect on the expression of mitochondrial DNA encoded oxidative phosphorylation genes. The mRNA levels for the mtDNA-encoded 12 S rRNA, ND2, ATPase6/8, COIII, ND5/6, and Cytb genes were reported increased in SV 40-transformed fibroblasts (155), while ATP6, COXII, COX III, ND1, ND4, and 12S rRNA transcripts were decreased in glioblastomas and prostate cancer (156, 157). In gestational trophoblastic diseases including complete hydatidiform mole (CHM), persistent gestational trophoblastic disease (PGTD) and the human choriocarcinoma the expression of COX I, ATP6, 12S rRNA and the transfer RNA for phenylalanine (tRNA(Phe)) were decreased. In addition, mitochondrial transcription factor A (mtTFA) mRNA level was observed to correlate with the decrease (158). On contrary in the papillary thyroid carcinomas ND5, ATP6, cytochrome b, and COX I were down-regulated, but mitochondrial transcription factor A showed similar mRNA expression levels in tumor and non-tumor tissue (159). In the model of polyp formation in familial polyposis coli a change in the rate of synthesis and/or degradation of the mtRNA was even suggested (160).

The mechanism responsible for mtDNA transcription depletion is unknown at present moment. Reactive oxygen species production induced by tumor necrosis factor alpha (TNF-alpha) was associated with a decrease in the steady-state mRNA levels of ATP 6/8 and this was associated with decreased protein levels of the ATPase subunits and also cytochrome c oxidase subunit II. Since TNF-alpha has no effect on the amount of mitochondrial DNA, it must act at the transcriptional and/or post-transcriptional level (161). The down-regulation of mtDNA transcription may overcome with the overexpression of PGC-1α and PGC-1β, at least in osteosarcoma cybrids, where this overexpression stimulates mitochondrial respiration and partially rescue OXPHOS defect. Transcriptional coactivators of the PGC-1 (peroxisome proliferator-activated receptor g coactivator 1) gene family are master regulators of mitochondrial biogenesis and oxidative metabolism and are potent regulators of mitochondrial function and biogenesis may improve mitochondrial respiration and OXPHOS function in cells with mtDNA mutations (54).

12. SUMMARY AND PERSPECTIVES

All presented data clearly support the hypothesis that genetic instability of mtDNA might play a significant role in tumorigenesis and these findings are important in the search for novel therapeutic approaches to cancer treatment (Table 2). The high incidence of mtDNA mutations in cancer suggests that mtDNA and mitochondria play pivotal role in the process of carcinogenesis ant that mtDNA instability plays a significant role in cell transformation. Although it needs to be undermined that if stringent data quality and methodology control is applied to the experiments the overall rate of somatic mutations has been shown much lower than previously reported in many cancer cells. Recent results by Wang *et al.* (106) gave support to the claims of Bandelt *et al.* (162, 163) that the high frequency of mtDNA somatic mutations in cancer studies is overestimated. Nevertheless based on the mtDNA mutation pattern in early stage cancer samples the enthusiasm and efforts to look for somatic mutations of diagnostic value in cancer early detection should be kept (106).

Although the mechanisms of generation and functional impact of mtDNA mutations are still not clear, its high incidence and its broad distribution in human cancers render it a potential marker for cancer detection. The accumulation of mtDNA mutations is a useful predictor of carcinogenesis. MtDNA mutations are endogenous factors that induce subsequent somatic nuclear and mitochondrial mutations in nuclear genome and etiologically contribute to cell transformation. The role of mtDNA mutations in the maintenance of tumor cell phenotype or in tumorigenesis remains to be elucidated.

Until today analysis of molecular and other clinical implications and pathological findings does reveal significant correlation between somatic mtDNA mutations and/or mtDNA content, or between mtDNA content and metastatic status. Certain mtDNA mutations can reliably distinguish the different histologic subtypes of tumors. In addition, these data raise the possibility that certain mtDNA mutations may be useful biomarkers for predicting tumor aggressiveness and may play a potential role in tumorigenesis. At the same time the identification of mtDNA mutations may complement other early detection approaches for cancer. A quantitative mitochondrial bio-markers may aid in the histopathological analysis of tumor biopsies for the diagnosis, classification and characterization of the differentiation state of the tumors. Now there is a general consensus that other prognostic factors, different from those included in the TNM-stages system, are required to improve the accuracy in the management of cancer patients, therefore the development of mitochondrial oncology is to benefit clinical practice. Also carriers of some germ-line mtDNA polymorphism could be more susceptible to cancer development and therefore selected as candidate population for intensive prevention and early detection programs (17).

More extensive biochemical and molecular studies will be necessary to determine the pathological

implications of these somatic mutations. The detection of increased somatic mtDNA mutations in cancer tissues is clearly intriguing and raises many questions that have yet to be analyzed. It remains unclear if these mutations are homoplasmic or heteroplasmic, do those mutations actually affect mitochondrial function and do mtDNA mutations initiate the ROS production or are they a consequence of damage to the cell. In future, in addition to further analysis of mitochondrial mutations, it would be worth looking at the functionality (*in silico* OXPHOS structure modeling, OXPHOS measurements etc.) and the gene expression pattern of mitochondria (RTReal time -PCR, RNA chips etc.) to obtain a more complete picture of the role of mitochondria in health and disease. Although it is generally recognized that somatic mutations suggest pathogenicity we still need to establish if these mutations contribute to neoplastic transformation by changing cellular energy capacities, increasing mitochondrial oxidative stress, or modulating apoptosis. The significance of each individual mutation on the mitochondrial function and tumorigenesis is recently unknown and only functional analysis of mutants in relation to cell behavior, proliferation, and apoptosis is warranted to determine the significance of these mutations and their association with tumorigenesis. Enhanced understanding of the pathophysiology of mitochondria, with the final aim of developing new cancer treatment strategies to stabilize or even cure them is now just emerging.

Until today only few chemotherapeutic interventions in oncology target mitochondria or aerobic glycolysis and therefore work against Warburg effect. Respiration deficient leukemia cells are substantially insensitive to 1-beta-D-arabinofuranosylcytosine (ara-C), doxorubicin, taxol, and vincristine. If these cells are treated with 50 3-BrPA (3-bromopyruvate) - hexokinase II inhibitor it is possible to substantially increase the cytotoxic effect of doxorubicin, vincristine, or ara-C (51). The inhibition of glycolysis in cancer cells with mitochondrial respiration defects leads to rapid dephosphorylation of BAD (Bcl2-antagonist of cell death) and relocalization of BAX (Bcl2-associated X protein) to mitochondria, and massive cell death also in multidrug-resistant cells (51). Other intriguing codependence emerged from molecular analysis of new class of cancer drugs. It seems that cells treated with hsp90 inhibitors exhibit pleiotropic changes, including an expansion of the mitochondrial compartment, accompanied by mitochondrial fragmentation and apoptosis. Several mitochondrial oxidative phosphorylation complex subunits, including several encoded by mtDNA are up-regulated by hsp90 inhibitors - without corresponding changes in mRNA abundance. Post-transcriptional accumulation of mitochondrial proteins observed with hsp90 inhibitors is also seen in cells treated with proteasome inhibitors. Analogous to defective protein folding in the endoplasmic reticulum, a mitochondrial unfolded protein response may play a role in the apoptotic effects of hsp90 and proteasome inhibitors (164). Although new term 'mitocans' has been suggested for drugs targeting mitochondrial metabolism and signaling pathways, sill the number and effectiveness of these drugs are not satisfactory. Nevertheless it has been proposed that mitocans should include drugs affecting: hexokinase inhibitors; electron transport/respiratory chain blockers; activators of the mitochondrial membrane permeability transition pore targeting constituent protein subunits, either the voltage dependent anion-selective channel (VDAC) or adenine nucleotide transporter (ANT); inhibitors of Bcl-2 anti-apoptotic family proteins and Bax/Bid pro-apoptotic mimetics (165); and overcoming the Warburg effect which is promising approach in cancer therapy (166).

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