REVIEWS

MITOCHONDRIAL DNA AND CANCER

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ABSTRACT

Mitochondrial DNA has been proposed to be involved in carcinogenesis because of high susceptibility to mutations and limited repair mechanisms in comparison to nuclear DNA. In this paper, we review mitochondrial genome instability, relation of mitochondrial DNA mutations with apoptosis and mitochondrial genomic aberrations reported in solid tumors of the thyroid, colorectal, breast, and gastric cancers.

Keywords: Mitochondrial DNA, Genomic instability, Apoptosis, Mutation, Solid tumors

MİTOKONDRİYAL DNA VE KANSER

ÖZET

Mitokondriyal DNA' nın, nükleer DNA' ya kıyasla mutasyonlara daha duyarlı olması ve tamir mekanizmalarının sınırlılığı nedeniyle karsinogenezde rol aldığı öne sürülmüştür. Bu derlemede tiroid, kolorektal, meme ve gastrik kanserlerde, mitokondriyal genom kararsızlığı, mitokondriyal DNA mutasyonlarının apoptozis ile ilişkisi ve mitokondriyal genomdaki değişimler değerlendirilmiştir.

Anahtar Kelimeler: Mitokondriyal DNA, Genomik kararsızlık, Apoptozis, Mutasyon, Solid tümörler

The abbreviations used are: 8-oxodG, 8-oxodeoxyguanine; AIF, apoptosis-inducing factor; ATP, adenosine-3-phosphate; CD, common deletion; CRC, colorectal carcinoma; DAP3, death associated protein 3; DL, ductal lavage; D-loop, displacement loop; FA, follicular adenoma; FNA, fine needle aspirate; LOH, loss of heterozygosity; MMR, mismatch repair; MSI, microsatellite instability; MSNT, matched surrounding normal tissue; mt, mitochondrial; mtDNA, mitochondrial DNA; mtGI, mitochondrial genome instability; mtMSI, mitochondrial microsatellite instability; nDNA, nuclear DNA; NGI, nuclear genome instability; OXPHOS, oxidative phosphorylation system; POL γ, DNA polymerase γ; PTC, papillary thyroid carcinoma; RFLP, restriction fragments lenght polymorphism; ROS, reactive oxygen species; SSM, slipped-strand mispairing; TFAM, mitochondrial transcription factor A; TRAIL, TNF-related apoptosis-inducing ligand.

INTRODUCTION

Malignant cell transformation is a multistep process that usually involves a cascade of events in which the sequential malfunction of oncogenes, tumor suppressors, mismatch repair (MMR) genes and telomere replication regulators generate changes in the cell cycle. Recently, alterations of mitochondrial DNA (mtDNA), which were not found in normal tissues from the same individual, have been reported in many tumors. In this review, we will focus on the current knowledge on the role of these alterations in the carcinogenesis of human tissues.

1. Mitochondrial genome

Human cells have hundreds of mitochondria which are semi-autonomously functioning organelles producing cellular ATP as power plants of the cell. Each cell contains varying numbers of mitochondria depending on energetic requirements. The human oocyte is estimated to contain 100,000 mitochondria, while spermatozoa have relatively few. Each

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mitochondrium contains a number of copies of its own genome (10 to more than 1000) whose complete sequence was reported in 1981[1,2]. Human mitochondrial DNA is a double stranded, supercoiled, circular molecule, which consists of 16,568 base pairs. The compact mtDNA molecule encodes 37 genes; 13 of them encode polypeptides of the oxidative phophorylation system (OXPHOS), 22 tRNAs and two types of rRNAs. The OXPHOS consists of five large enzymatic complexes and is formed from the gene products of 74 nuclear genes and 13 mitochondrial genes (Fig 1) (Table I). The two complementary strands of mtDNA, based on their guanine (G) content, are named as heavy and light strands (H-strand and L-strand, respectively). Guanine-rich H-strand of mtDNA encodes 28 of the 37 genes while L-strand encodes the remaining genes[2,3]. A non-coding control region extending from 16,024 to 576 nucleotide positions contains three conserved sequence blocks and a displacement loop (D-loop). Moreover, promoters and enhancers for mitochondrial transcription, as well as the origin of replication for H-strand, reside in this region.

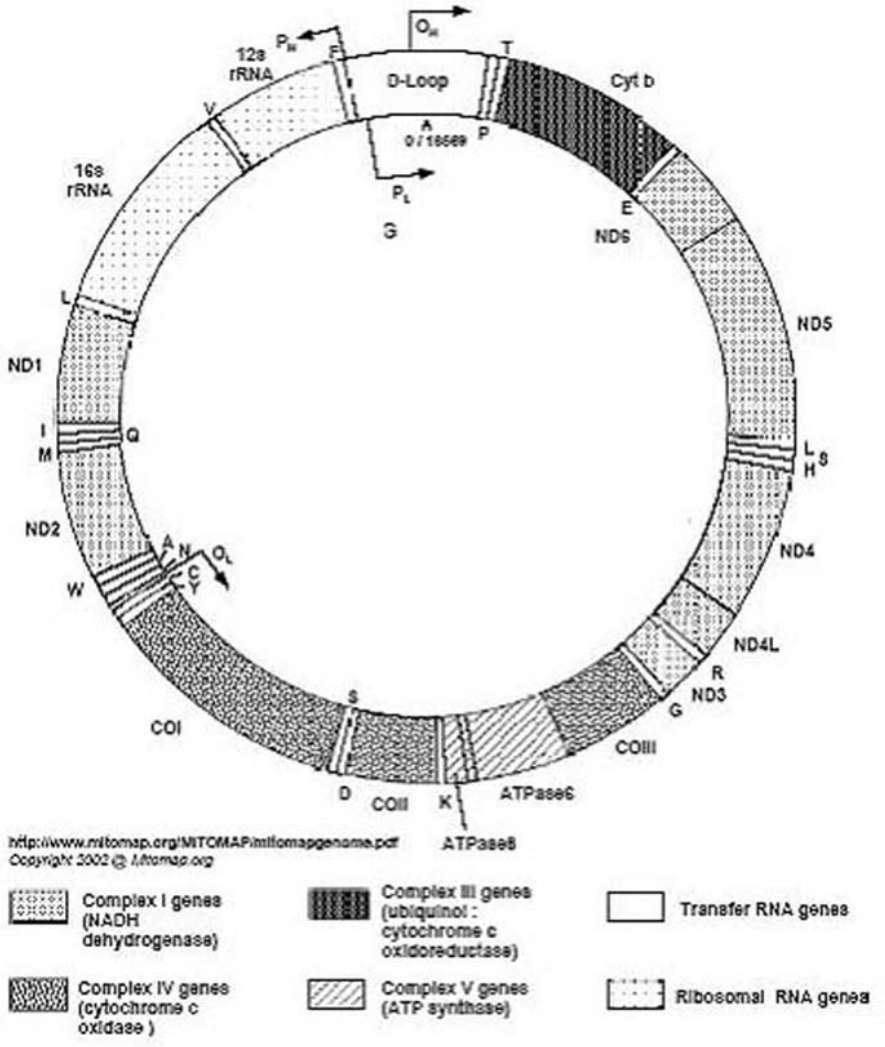

Figure 1: Human mitochondrial DNA3.

Mitochondria are the major intracellular producers of reactive oxygen species (ROS) as a side-product of OXPHOS. Deficiency in the OXPHOS reduces energy supply and enhances ROS production that may induce mutation and oxidative damage to mtDNA. This increase in the ROS concentration of mitochondrial microenvironment gives rise to a rate of mitochondrial genome mutations 10 to 100 fold higher than the nuclear DNA (nDNA) mutation rate. Moreover, in contrast to nDNA, mtDNA does not contain protective histon proteins and intronic sequences. However, it has been reported that mitochondrial transcription factor A (TFAM) is bound to mtDNA and forms histone-like structures[4,5]. In addition, mtDNA repair is poor and the replication process is error prone when compared to nDNA. Given the high copy number of mtDNA per cell, mutated mtDNA can be found together with wild-type mtDNA. This phenomenon is called as heteroplasmy. At the homoplasmic state, there is a single type of mtDNA existing in a cell. Clinical expression of a given mtDNA mutation mainly depends on the proportion of mutated and wild-type mtDNAs at the heteroplasmic state. Depending on the energetic demands of a particular cell, the level of mutated genomes is required to produce a phenotypic expression (threshold effect)6.

1.1. Reactive oxygen species (ROS) and mtDNA

As noted above, mitochondria are the main targets for ROS, which are produced by themselves. Oxidative damage to DNA results in the base or sugar adducts, single and double strand breaks, as well as cross-links to other molecules. Many of these mtDNA modifications are mutagenic, and thought to contribute to cancer, aging and neurodegenerative diseases[7]. More than 20 base lesions have been identified as a result of hydroxyl radical attack. Thymine glycol, the most common pyrimidine lesion, has been determined to block replication and transcription rather than to cause mutagenesis[8]. Generally, hydroxyl radicals produce purine modifications. The most common and extensively studied purine modification is 8-oxodeoxyguanine (8-oxodG) and it has been found to cause GC → TA transversions[9]. The mtDNA polymerase γ (POL γ) also introduces this signature mutation at high frequency when replication pasts 8-oxodG[10]. Since increased formation of ROS-related DNA modifications has been reported in various studies with human cancers, it is not clear whether over-production of ROS is a result or consequence of neoplastic transformation[11]. On the other hand, the effect of ROS on mitochondria may result in deficiency in the apoptotic behaviour of cells and mitochondrial genome instability (mtGI) as well as nuclear genome instability (NGI), which are the main molecular events frequently observed in malignant cells[11], as will be discussed below.

Mitochondrial DNA undergoes fragmentation and degradation in the mt-environment or in the cytoplasm followed by severe damage. In some cases, transposition of mtDNA fragments within the nuclear genome is detected, which may be responsible for malign transformation in certain cases of cell transformation[12].

1.2. Mitochondrial genome instability

High rates of spontaneous mutations, epigenetic factors such as abnormal methylation and loss of heterozygosity (LOH) in MMR gene/s explain nuclear genome instability in several types of hereditary and sporadic human tumors. So far, different rates and types of mitochondrial genome instability have been analyzed in almost all human tumors, but the information is still insufficient to explain the role of the mtGI in the origin and evolution of human cancers. Since DNA damage, slipped-strand mispairing (SSM) and defective DNA repair generate the genomic changes detected for the nDNA of tumors, it has been suggested that these three mechanisms are also involved in the formation of mtDNA mutations.

Mitochondrial DNA contains several mono- and dinucleotide repeats. $(CA)_n$ microsatellite starts from 514 bp position of the D-loop and it shows different alleles varying in size by one repeat in human populations.

Table I: Number of mitochondrial and nuclear DNA-encoded gene products of electron respiratory chain[67].

Protein complex	mtDNA gene product	mtDNA gene product (N)	nDNA gene product (N)
I	ND1, ND2, ND3, ND4, ND4L, ND5, ND6	7	36
II	-	0	4
III	Cytochrome b	1	10
IV	Cytochrome c oxidase I, II and III	3	10
V	ATPase 6 and 8	2	14
Total		13	74

Homopolymeric C tract extends from 16,184 to 16,193 bp of the D-loop, which is interrupted by a T at 16,189 bp position.

It has been proposed that the enhanced sensitivity to ROS and lipid peroxides due to the triple stranded DNA structure and attachment to the mt-membrane of the D-loop region may explain the increased rate of the D-loop point mutation in breast cancer[13].

The first step leading to allelic changes in homopolymeric tracts and microsatellites is the misalignment of repeats in complementary DNA strands with the formation of single strand loops of one or more bases[14,15]. SSM is an intra-helical event involving the misalignment of repeats in complementary DNA strands. The mechanism producing the allelic changes in breast cancer (CA)n microsatellite can be explained by SSM[13].

The chance of alignment shifting is higher for homopolymeric or dinucleotide repeats than for repeats of three or more base pairs and higher for continuous homopolymers than for homopolymers interrupted by a base different from the repeated one[16]. Moreover, the cause of the homopolymeric tract instabilities have been detected in colorectal, gastric and breast cancers[13,17-19].

The pairing between two direct repeats separated by an intervening DNA fragment is one of the mechanisms producing mt-deletions. During this SSM, the intervening segment forms single strand loops and becomes thereafter deleted, including in the deletion of one of the repeats[20-24]. Accordingly, this is the mechanism for the 4997 bp deletion in breast cancer[20] and the 50 bp deletion in gastric carcinomas[25].

It is very well known that defective DNA repair is always a component of the mechanisms leading to DNA changes. A homozygous defective function of one or more MMR genes plays an important role in malignant transformation. Since MMR gene defect shows a marked positive correlation with high-frequency of microsatellite instability (MSI)[26,27,] there is no correlation between mtMSI and mtGI in colorectal[17,18] and mammary cancers[13]. These results indicate that NGI and mtGI in these tumors respond to different mechanisms of defective repair.

POL γ is encoded by nuclear genes as all other proteins required for mt-genome replication[28]. It is responsible for mtDNA replication and error prone due to a ineffective proofreading efficiency[29,30]. Thus, probably most forms of mt-genome mutations are explained by increased DNA damage plus normal POL γ DNA synthesis. It has been proposed that the rate of mtDNA mutations is increased when POL γ is defected due to

mutations, epigenetic factors or inhibition by certain antiviral drugs[31-33]. Zeviani et al.[34] proposed that mitochondrial topoisomerases I and II genes are additional causes of mtGI in tumors.

1.3. Mitochondrial DNA mutations and apoptosis

Mitochondria play an important role in the apoptotic processes. At the intrinsic pathway of apoptosis (e.g. apoptosis due to internal signals such as DNA damage), the first step is the induction of pro-apoptotic (BAX, BID, BAD, NOXA, PUMA) and the down-regulation of antiapoptotic (Bcl-2, Bcl-xL, Bfl-1) Bcl family proteins. The second step is the formation of pores in the mitochondrial membrane and release of several mitochondrial proteins such as cytochrome c, SMAC/Diablo and apoptosis-inducing factor (AIF) to cytoplasm. These factors then form large apoptosome complexes, which lead to caspase activation and then apoptosis.The contribution of mtDNA mutations to the apoptotic behaviour of cells is a great interest of research.

Recently, Lee et al.[35] have reported that the resistance against apoptosis, when induced by TRAIL (TNF-related apoptosis-inducing ligand), was increased in the cells with diminished mitochondrial function Thus, it has been suggested that mutations in mitochondrial genes might serve as a selection advantage over normal cells with intact mitochondrial function. In contrast, Liu et al.[36] have reported that mutation or depletion of mtDNA increased the susceptibility of the cell to apoptosis, induced with strausporin. Moreover, Shidara et al.,[37] investigated the contribution of mtDNA mutations in tumor development and progression. In this study, cybrids containing a single nucleotide mutation at mitochondrial ATPase6 gene were inoculated to nude mice, which demonstrated higher rates of tumor development and progression, compared to wild-type mice. Furthermore, they reported a lower frequency of apoptotis at cybrids both in vivo and in vitro and concluded that mutations at the mitochondrial genome offer an advantage to cells for promoting tumorigenesis, especially at the early stages. On the other hand, Schoeler et al.,[38] reported a relatively higher sensitivity to TRAIL induced apoptotic stimuli at cybrid cells with low levels of mtDNA 4977 bp common deletion (CD). Controversial results obtained by various laboratories may be due to experimental differences such as cell lines used and types of apoptotic induction.

Since a lot of studies revealed these findings, molecular mechanisms underlying the effect of mtDNA mutations on apoptosis are still a mystery. It has been found that the mtDNA mutations induce protective expression of Bcl-2 and Bfl-1, prosurvival proteins in cardiac myocytes[39]. Park et al.,[40] reported an increase in the expression of antioxidant enzymes at mitochondria depleted cells. They suggested that this adaptive response in the gene expression renders cancer or aged cells with aberrant mtDNA mutations, resists against oxidative stress, hosts anti-cancer surveillance against chemotherapeutic agents, conferring survival advantage.

Jacques et al.,[41] showed that expression of DAP3 (Death associated protein 3) is strongly dependent on mtDNA maintenance. DAP3 is a component of the mitochondrial ribosome small subunit and a major positive mediator of apoptosis. According to their data, DAP3 was not expressed at mtDNA-less cells, which exhibited a relative resistance to apoptosis induced with drugs such as strausporin. In the mtDNA-less cells 12S and 16S rRNAs expression is low and this may result in a failure to assembly mitochondrial ribosomes. The authors concluded that mutations of mtDNA, resulting in lower rRNA expression, might be responsible for apoptotic resistance in diseased cells.

2. Mitochondrial DNA mutations and cancer

2.1. Thyroid cancer

Using two-dimensional gene scanning technique, Yeh et al.,[42] examined 21 cases of thyroid tumors [1 Hurtle cell carcinoma, 4 follicular adenomas (FA), 13 papillary thyroid carcinomas (PTC), 1 follicular carcinoma, 1 insular carcinoma and 1 medullary carcinoma], 6 cases with non-neoplastic thyroid pathology, 30 control tissues, 9 foetal

thyroid tissue and 9 non-thyroid tissues for mtDNA mutation. They identified 3 somatic missense mutations in 23% of the papillary thyroid cancer cases. These three mutations were found in $tRNA^{asp}$, ND3 and CYT B genes. Moreover, sequence variations in both neoplastic and non-neoplastic thyroid tissues were identified, and the authors concluded that these non-somatic variations may be related to tumour progression in the thyroid.

Maximo et al.,[43] investigated the existence of 4977 bp common deletion in 79 benign and malignant thyroid tumors (Hurtle and non-Hurtle cell neoplasms). They found mtDNA CD in all of the Hurtle cell tumors (100%), in 33.3% of adenomas and 18.8% of papillary carcinomas without Hurtle cell features. The authors also found 57 somatic mutations, mostly transitions, in coding genes including tRNA genes of tumor samples. Follicular and papillary carcinomas displayed a significantly higher prevalance of mutations of complex I genes than follicular adenomas. On the other hand, the prevalance of OXPHOS mutations in Hurtle cell tumors did not statistically differ from non-Hurtle cell tumors.

Rogounovitch et al.,[44] investigated mutations in mtDNA samples of PTC and FA patients from regions contaminated with radioisotopes after the Chernobyl accident. Common deletion (4977 bp) was quite prevalent in PTC and FA, therefore it was unlikely to be representative of thyroid tumors. The authors also reported that large scale deletions other than CD were higher in radiation associated tumors as well as CD when compared to the non-radiation associated group (spontaneous PTC or FA).

Sequence alterations in the D-loop region of mtDNA were also investigated in thyroid cancer, as well as other types of human cancers. Tong et al.,45 investigated D310 polymorphisms of the D-loop region. This region consists of two cytosine stretches interrupted by a thymidine nucleotide (C_7TC_5). Alterations of the cytosine number at the first stretch were found in 5.7% PTC, 5.6% medullary carcinomas, 11.1% anaplastic carcinomas and 11.1% follicular thyroid carcinomas. Another study including thyroid cancer patients from Belarus after the Chernobyl accident and 40 sporadic thyroid cancer patients from Germany showed that alterations of D310 region are related to the age of patients at the time of surgery, but not to the degree of radioactive contamination[46]. Recently, Maximo et al.,[47] examined sequence alterations in three repetitive regions (D310, D514, D568) in malignant and benign thyroid tumors. They found that these alterations were common in both malignant and benign tumors and cannot be considered as a marker of malignancy.

In an ongoing research performed in our laboratory, we are examining D310 alterations using restriction fragments length polymorphism (RFLP) assay in thyroid cancer patients. In this way, enzymatic digestion of PCR products of D310 by using BsaXI, it is possible to distinguish 7-C carriers at the first stretch. Thus BsaXI positive case is identified as 7-C carrier whereas BsaXI negative case contains more or less than 7-C in this region. According to our preliminary data, 16 of 37 thyroid cancer patients were BsaXI positive (eg. 7-C at D310 region) and 20 of 37 patients were BsaXI negative. Only one patient showed heteroplasmy and there was no difference between tumoral and non-tumoral tissues.

2.2 Colorectal Cancer

Aikhionbare et al.,[48] examined the relationship between mtDNA alterations and colorectal carcinogenesis in 25 adenomas, 27 colorectal carcinomas (CRC) and their matched surrounding normal tissues (MSNT). A total of 38 nucleotide variants were identified; however, none of them appeared to be a marker for a particular adenoma of CRC. Moreover, the numbers of these variants were less in precancerous tissues than in CRC. They suggested that this may be a useful approach for distinguishing the progressive stages of colorectal adenomas.

Nishikawa et al.,[49] found a higher mtDNA mutation rate in the colonic tissues of patients with ulcerative colitis than in those of inflammatory disease-free patients. They suggested that accumulation of mtDNA

mutations, as well as nDNA mutations after inflammation, may be an indicator of carcinogenesis. On the other hand, the authors reported the existence of homoplasmic mutations in both inflammatory and non-inflammatory regions of the colonic mucosa. Greaves et al.,[50] showed that a mitochondrial mutation in a single cryptic cell could be dominated in a patch of cryptic tissue of the colon by clonal expansion by cryptic fission.

Lee et al.,[51] analyzed the D-loop region of mtDNA in a variety of human tumors including CRC and found sequence alterations in 40% of the patients, mostly in the D310 region (90%). They also reported a significant decrease in the mtDNA copy number in 28% of the cases when compared to MSNT, especially in the later stages of CRC (stage III and IV). The authors suggested that D-loop mutations were important markers of carcinogenesis, but the molecular mechanism by which the mtDNA copy number is decreased by cancer-associated D-loop mutations is not clear. However, in another study, D-loop alterations were found in only 8% of colorectal cancers without a significant relation between the stage of the disease and the occurrence of mutation[52].

Lievre et al.,[53] analyzed entire mitochondrial genome in 11 CRC patients and found 10 somatic mutations in seven patients. Most of the nucleotide changes (80%) were found in the D-loop region, while the remaining were in the coding regions (cytochrome c oxidase 1 and 3). The authors further analyzed D-loop region (nt 190-590) in 365 CRC patients and found 142 D-loop alterations. Most of these alterations (132) were found in the D310 region. They also reported that 3-year survival rate and the efficacy of adjuvant chemotherapy were significantly lower in patients with D-loop mutations.

Recently, using PCR-based restriction fragments length polymorphism assay with BsaXI restriction enzyme, we analyzed the D310 region in the CRC and MSNT samples, as well as in the samples from healthy individuals[54]. Thirty-six percent of the studied samples were homoplasmic BsaXI (+) and 24% were heteroplasmic (BsaXI +/-). No significant differences were found between CRCs and MSNTs. When we compared the BsaXI status of CRC patients with that of the healthy controls, a significant difference was found.

2.3. Breast cancer

The D-loop region of 40 breast cancer samples and their MSNT were analyzed using RFLP analysis[13]. It was found that 47.5% of the cases were mutated in MnlI restriction sites representing a 216-fold increase over the spontaneous rate in the female germline.

In another study that involved entire mtDNA mutation scanning by temporal temparature gel electrophoresis (TTGE), 74% of the cases (14/19) were found to be mutated[55].The percentages of these mutations were 3.7%, 14.8% and 81.5% in rRNA genes, protein encoding genes and D-loop region, respectively.

The D310 region in the D-loop region of mtDNA was also examined by various researchers in breast cancer patients. Parrella et al.,[56] analyzed D310 mononucleotide repeat changes in 64 breast tumors and found D310 alterations in 12 cases (19%). The majority of these alterations were 1 or 2 bp insersions or deletions of the first stretch of cytosines, except the two cases that showed either an 8 bp or a 9 bp deletion. The same deletion or insersions were also found in the corresponding fine needle aspirate (FNA) specimens when available. The entire mitochondrial genome was sequenced in 18 primary breast tumors and 7 mutations were detected in the coding (ND1, ND4, ND5 and cytochrome b genes) and non-coding D-loop region. Similarly, several studies revealed the possibility of the examination of D310 alterations in ductal lavage (DL) and FNA specimens[57,58].

Zhu et al.,[59] scanned mtDNA for large deletions in breast cancer tissues, their MSNT and in normal breast tissues from women without breast cancer. Common deletion of mtDNA had similar frequency in both cancerous and in normal tissues, whereas novel 3,938 bp and 4,388 bp deletions were

more frequent in the cancerous tissue than observed in MSNT. Therefore, it was suggested that these novel deletions may be a marker for breast malignancy. Consistent with these data, Dani et al.,[60] also noticed a lower frequency of CD in tumoral tissues including breast cancer.

In our recent study[54,] we examined 25 breast cancer specimens for D310 polymorphism with RFLP. BsaXI negative cases had lower frequency in the breast cancer samples (11/25, 44%) when compared to the control group (27/41, 65.9%), but this difference was not statistically significant.

2.4. Gastric cancer

Tamura et al.,[61] screened control region of mtDNA in 45 Japanese gastric carcinoma patients with PCR-single stranded conformation polymorphism assay (SSCP) and found only two heteroplasmic mutations in nucleotide positions 248 and 16,129. In a different study, Maximo et al.,[62] found a high percentage of CD in gastric cancer (53.1%) patients and suggested that gastric cancer is more prone to have gross genetic alterations of mtDNA than to exhibit signs of fine genetic instability.

The D-loop and downstream genes 12s rRNA-tRNAphe were studied for sequence variations in 22 gastric cancer samples and their MSNT by direct sequencing. In this study, PolyC and (CA)n instabilities were demonstrated in the D-loop including the D310 region without a significant difference between cancerous tissue versus MNST[63]. Moreover, sequence variations in 12s rRNA-$tRNA^{phe}$ genes and a significant correlation between the frequency of these variations and the differentiation degree of gastric carcinoma were detected. Recently, D310 alterations were found in 14 of 94 gastric cancer samples without a significant correlation with clinicopathological features, such as patient age or sex, tumor location, depth of tumor invasion and lymph node metastasis[52]. Lee et al.,[51] identified sequence alterations in D-loop region (mostly in D310) of mtDNA in 51.6% of gastric cancer samples. According to their data, 54.8% of the gastric cancers had significantly decreased mtDNA copy number (below 90%) when compared to MSNT. By contrast, 22.6% of the samples had significantly higher mtDNA copy numbers (above 110%). In contrast to colorectal carcinomas indicated above, decrease or increase of mtDNA copy number appeared to be independent of the stage of the disease.

Eigtheen D-loop mutations were reported by Zhao et al.,[64] in 20 gastric cancer patients. In this study, the level of ROS, rate of cell apoptosis and proliferation were found to be higher in patients with mutations than those in controls. It was concluded that D-loop mutations were important in the development and progression of gastric cancer through the effect of increased ROS.

Wu et al.,[65] examined D-loop mutations and the presence of CD in 31 gastric cancer samples. In this study, 10 of the patients showed sequence alterations in the D310 region and a total of 17 mutations were reported, including a case with 3 mutations. Approximately half of the mutations were reported as homoplasmic. On the other hand, CD was found in 10% of the cancerous tissues of the patients, whereas it was found in 55% of the MSNT. It was also shown that 55% of the patients had significantly decreased mtDNA copy number, which was not associated with D-loop mutations.

3. Conclusion

Mutations in the mtDNA have been reported in almost all forms of primary tumors examined. As recently classified by Carew and Huang[66,] (1) the majority of the mutations are base substitutions; (2) mutations occur in all protein-coding mitochondrial genes; (3) the D-loop region is the most frequent site of somatic mutations across most tumor types; and (4) the presence of homoplasic mutant mtDNA in tumors suggests that they may play an important role in the development of tumors. It is noteworthy that depletion of mtDNA is also reported in many kinds of cancer[67]. As discussed above, in addition to several case-control studies, there are various in vitro studies concerning the molecular mechanism(s) of these molecular alterations.

In the future, a combination of these studies will be useful in the deciphering of the cancer puzzle.

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