Expert Opinion

The mitochondrial genome: a biosensor for early cancer detection?

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Mutations in the mitochondrial genome have been reported as biomarkers for the detection of cancer. Hallmarks of cancer development include the accumulation of genetic alterations in the mitochondrial and nuclear genomes. Damage to mitochondria affects energy metabolism, generation of reactive oxygen species, apoptosis, cell growth and other processes that contribute to the neoplastic process. Furthermore, mitochondrial DNA mutations occur frequently in cancer. Little work has been done to link a pathway between mitochondrial mutations and cancer etiology. Volumes of work have been reported on the association of mitochondrial mutations and almost all types of cancer including the use of body fluids for early detection. This review examines the measurement of mitochondrial mutations for the application of detecting human tumor tissue.

Keywords: biomarkers, cancer, mitochondrial DNA

Expert Opin. Med. Diagn. (2007) 1(2):169-182

1. Introduction

The mitochondria and, in particular, the mitochondrial genome, has been intensely investigated from an oncology perspective [1,2]. There are many resonating reasons for careful consideration of mitochondrial metabolism in the oncogenic pathway. The mitochondria generate as much as 80% of the cellular fuel that fires metabolism. The numerous alternative oxidative pathways, available through this organelle, generate reactive oxygen species (ROS), which play a signaling role in other biologic cascades such as apoptosis; however, an excess of ROS within the mitochondria damages vital biomolecules such as DNA, lipids and amino acids. Removing excessive ROS is the responsibility of nuclear encoded mitochondrial enzymes. When ROS passes an intra-mitochondrial threshold without a reductive response, then either nuclear-to-mitochondria control of these detoxifying mechanisms is lost or overproduction of mitochondrial-based oxidation is occurring. Either scenario results in excess ROS species and subsequent molecular damage. The mitochondrial genome is not protected by histones and yet has DNA polymerase with low proofreading fidelity. Hence the mitochondrial genome is particularly vulnerable to the deleterious effects of ROS. This circumstance is probably the mechanism for the accelerated mutation rate of the mitochondrial genome in comparison with the nuclear genome in an evolutionary context. Moreover, point mutations and deletions occur at a phenomenal rate in a subset of tissue samples from a malignant milieu, where oxidative stress is attested [3,4]. Importantly, these lesions appear not only in obvious tumor, but also in adjacent histopathologically normal tissues. There are two explanations for this observation: i) tumor tissue influences and recruits surrounding cells; ii) tumors arise from extensive 'preconditioned' tissue termed a 'cancerization field'. An extensive review

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of literature suggests that this field effect is a common background mechanism that allows, rather than precludes, tumorigenesis [5].

Other biologic aspects that enhance the biosensor qualities of mitochondria in addition to its fast mutation rate exist. The mitochondrial genome has a cytoplasmic or maternal inheritance pattern. This precludes recombination such that somatic mutation events are the sole means of variation. In general, there are thousands of copies of the mitochondrial genome per cell, in contrast to the diploid state of the nuclear archive of information. Both low and high frequencies of alterations associated with pending disease are easily amplified and identified due to the mitochondrial mass advantage [6]. The authenticity of these lesions can be directly compared with the germplasm or blood of individual patients. To avoid misinterpretation of nuclear mitochondrial pseudogenes (numt), guidelines for testing for negative primer amplification against Rho^0 cells and libraries of pseudogenes have been reported [7,8]. The modest mitochondrial genome (16,568 bp) can be sequenced rapidly, reliably and inexpensively at a population level [9]. The human mitochondrial genome sequence has been available for some time [10]. Within the mitochondrial genome are 13 loci encoding proteins and enzymes that participate in the electron transport chain, and 2 rRNAs and 22 tRNAs involved in the translation of mitochondrial proteins. These coding gene sequences can be analyzed for alterations in their functional products [4]. Mitochondria are mediators of cell death or apoptosis. This process must be disabled for malignant growth.

As a result of these biological features, the mitochondrial genome has been characterized for its mutation behavior in a number of cancers. The following brief study synopses are meant to cover most of the studies presented in the recent literature; however, it is not exhaustive. Included works are oriented towards using the somatic mutation patterns of the mitochondrial genome as a biomarker for cancer detection or diagnosis. Although some of these studies are critiqued by Salas *et al.* [11] the authors have still reviewed them because the basis of the criticisms are not necessarily valid [12].

2. Mitochondrial studies and specific cancers

2.1 Bone

Guo and Guo [13] reported mitochondrial D-loop mutations in 14 out of 20 (70%) osteosarcomas in comparison with samples of the patient's blood. Importantly, polymerase chain reaction (PCR) primers were controlled for pseudogene amplification against Rho^0. Most of the mutations were found in hypervariable regions 1 and 2 (HV1, HV2).

2.2 Brain

Analyses of the mitochondrial genome in gliomas have been addressed in a small number of studies. In one study, Liang identified an increased copy number of mitochondrial genomes in gliomas, when compared with histologically normal brain tissue [14]. Large mitochondrial genome deletions were observed in the malignant tissues as well. In another study, Kirches *et al.* used laser-capture microdissection (LCM) to recover glioblastoma cells from 55 patients in parallel with adjacent normal tissues [15]. The homopolymer region (HPR; 303 – 315 bp) in the mitochondrial D-loop was analyzed for length variations, which were found in 5 out of 55 samples (9.1%). The entirety of the D-loop was sequenced in 17 glioblastomata and compared with the patient's blood. The frequency of patients with mutations increased to 35% (6 out of 17). In a similar study, Montanini *et al.* found somatic mutations in the D-loop of 15 out of 42 (36%) gliomas [16]. Analyses of the entire mitochondrial genome in 15 medulloblastomata found at least 1 mutation in 6 out of 15 (40%) tumors [17]. Seven matched cerebrospinal fluid samples harbored mutations, some of which were shared with the tumors. However, it is unclear whether the mutations in the cerebrospinal fluid samples are from tumor cells.

2.3 Breast

A study investigating the entire mitochondrial genome for somatic mutations reported a relatively high frequency of 74% (14 out of 19) of breast tumors with alterations. Mutations were scored against matched histopathologic normal tissue [18]. Most of these mutations were located in the D-loop (22 out of 27 or 82%). However, a large deletion event, such as the 4977 common deletion (CD), was absent in these tumors. Likewise, Parrella *et al.* sequenced a subset of the mitochondrial genome (39%) of 18 primary tumors and detected somatic mutations in 61% (11 out of 18), in comparison with the germplasm [19]. Of these mutations, 58% occurred in the D-loop, mainly in the HPR. This prompted the investigators to interrogate only the HPR in an additional 46 breast tumors. Mutations were found in this limited region in 15% of the tumors. This indicates that methods that interrogate the mitochondrial genome, as opposed to the HPR, identify more somatic mutations. Rosson and Keshgegian investigated the differences between tumor and normal tissues in 15 patients using laser-capture microdissection and reported that all samples had mutations [20]. Unfortunately the revised Cambridge reference sequence (rCRS; [21]) was used as the control sequence, as opposed to blood.

The role of mitochondrial genome copy number and mutations was assessed in a group of 60 Taiwanese women by Tseng *et al.* [22]. Somatic mutations were determined by comparison of the sequences of benign versus malignant tissues. Potentially, this method may overestimate the number of overall mutations as sequence alterations can occur in normal-appearing tissues proximal to the tumor [23]. Using this reference method, mutations were found in 18 out of 60 (30%) tumors. A substantial number of these

(13 out of 18) had attested alterations in the HPR. The CD was 9.4-fold higher in 'normal' breast tissue when compared with tumor. Interestingly, mutations were associated with women ≥ 50 years of age ($p = 0.042$) and with tumors lacking expression of both estrogen and progesterone receptors. Women with mutations in the D-loop experienced poorer outcomes than those free of mutations. Finally, mitochondrial DNA (mtDNA) content was reduced in breast cancer tumors when compared with their corresponding normal controls. This same depletion is also attested by other studies [24,25].

Wang *et al.* evaluated mitochondrial microsatellite instability (mtMSI) in breast tumors [26]. MtMSI are mitochondrial genome loci with homopolymer or repetitive bases, such as the HPR. Out of 12 mtMSI loci, 4 indicated instability by length alterations in tumors. In total, 3 of these 4 markers are located in the D-loop, and the other is found in 12S rRNA. Out of 51 tumors, 15 (29%) demonstrated alterations. In another study, mtMSI loci within the D-loop were investigated [27]. Sequence size variants between bp 16108 and 16420 were characterized by 4 *Mnl* I sites. The other location was a $(CA)_n$ beginning at bp 514. Paired breast tumors and normal controls ($n = 40$) were queried for these markers. Alterations were observed in 17 out of 40 (42.5%) of mtMSI beginning at bp 514, and 19 out of 40 (47.5%) cases had length variants identified by *Mnl* I.

A study by Zhu *et al.* compared the mitochondrial genome mutation rates in normal and malignant breast tissues [28]. Nipple aspirate fluid recovered from the malignant breast was sequenced for mitochondrial genome positions 15331 to 2463. Of 15 cancer samples, 14 (93%) had sequence alterations. Most of these mutations were in coding regions and 11 were non-synonymous changes. Four nipple aspirate fluid samples recorded a single mutation each that was also found in the matched tumor.

2.4 Cervix

The incidence of mutations in the mitochondrial D-loop of 19 patients with cervical cancer was surveyed by Sharma *et al.* [29]. All tumors, with the exception of one, had mutations in the D-loop. This excessive frequency of mutations may be due to the direct comparison of the results to the reference sequence as opposed to blood. Such a comparison should consider only the polymorphic differences between individuals and does not represent somatic changes that have diverged from the inherited maternal DNA sequence. This suggests that the association of these mutations with the high incidence of human papillomavirus in these women may be unwarranted. In addition to their work on breast cancer, Wang *et al.* evaluated mtMSI in 71 cervical cancers [26]. Of the 71, 18 (25.4%) of these tumors demonstrated instability at 2 mtMSI loci. A 10-year retrospective study of invasive cervical cancer demonstrated that patients with eight or more mutations within the complex I subunits of the mitochondrial genome experienced a significantly worse outcome than those with fewer than eight mutations [30]. Overall, a third of the tumors had mutations.

2.5 Colorectal

Alonso *et al.* observed a mutation rate of 23% (3 out of 13) in HV1 and HV2, of the mitochondrial D-loop [31]. The HPR was the site of 60% of these mutations. A group of 45 colorectal carcinomas were analyzed for mitochondrial genome sequence alterations in *ND1, ND4, ND5, COII and CYTB* [32]. Of these tumors, 7 (16%) demonstrated mutations in complex 1 genes. This is less than half the frequency of mutations seen in an earlier study (20 out of 45 or 44%), in which mtMSI sites were indentified [33]. In contrast to other cancers, this group did not observe deletions in full-length mitochondrial genome amplifications, followed by restriction digest. Mitochondrial genome mutations were demonstrated in 7 out of 10 colorectal cancer cell lines, and identical homoplasmic mutations were present in the primary tumors from which the cell lines were derived [1]. Aikhionbare *et al.* demonstrated an increase in mitochondrial genome mutations between adenomas and colorectal cancers in comparison with matched surrounding normal tissues [34]. Once again, these data are problematic as the matched normal is used as 'normal control tissue'. Several studies indicate that mutations occur in normal-appearing tissue in proximity to a tumor (reviewed in [5]).

The D-loop of 365 colorectal cancer samples was sequenced and compared with matched 'healthy colon tissues' [35]. Sequence results were compared with the rCRS to determine polymorphisms and differences between tumor and normal sequences were scored as somatic mutations. D-loop alterations were observed in 142 out of 365 (39%) of these samples. Moreover, 132 out of 142 mutations occurred in the HPR. Interestingly, the 3-year survival rate was significantly lower ($p = 0.05$) for those patients with D-loop mutations. These mutations may play a role in stage III colon cancer resistance to fluorouracil-based adjuvant chemotherapy. Guleng *et al.* analyzed the HPR in a cohort of 95 colorectal cancer patients and 95 control patients without gastrointestinal cancer and made appropriate comparisons with the patients corresponding blood [36]. Homopolymer regions in *ND1* and *ND5* genes were also analyzed. Out of the 95 cancer patients, 32 out of 95 (34%) had alterations in the HPR compared with 2 out of 95 (2%) for the non-cancerous controls.

MtMSI at the HPR was assessed in 133 colorectal cancers in comparison with matched non-cancerous tissues [37]. Alterations were found at this locus in 8% (11 out of 133) of the cases. In contrast to the results of Lièvre *et al.* [35], no correlation was found between clinicopathologic characteristics and mutation frequencies. Another study assessing mtMSI was performed by Kim *et al.* [38]. This study analyzed 9 mtMSI sites in the D-loop and an additional 5 in coding regions in 48 colorectal cancers. In addition, adjacent stromal tissues were recovered for a comparative

determination of mutation rates to that of epithelial-derived tumors. Normal colonic mucosa from each participating patient was utilized as control material. Including both stroma and tumor, 16 (33%) of the patients incurred mutations; however, there was no statistical association between stroma and tumor, suggesting separate mutation processes for these tissues.

2.6 Endometrial

The mutation characteristics of a portion of the mitochondrial genome in endometrial carcinomas, were investigated by Liu *et al.* [39]. A total of 50 primary endometrial carcinomas and paired histopathologic normal control tissues (cervical or lymphocytes) were amplified and sequenced through the D-loop, as well as *12S* and *16S rRNA* genes. Over half of the carcinomas had at least 1 alteration (28 out of 50 or 56%), when compared with controls. Most of the changes (78%) were located in the D-loop, with 44% of those mutations occurring in the HPR.

In addition to their work on breast and cervical cancers, Wang *et al.* evaluated mtMSI in 62 endometrial tumors [26]. Of the four gynecologic cancers investigated by this group, endometrial cancer had the highest frequency of instability. Over all, 30 out of 62 (48.4%) of the samples had changes. In a follow-up study, using LCM to recover cells from different areas of an endometrial tumor, Wang *et al.* demonstrated a heterogenous mutation pattern among the samples [40]. Moreover, most of the mutations were heteroplasmic. As detection methods advance, most mtDNA mutations associated with cancer may prove to be low level heteroplasmies.

A total of 40 endometrial tumors were collected in parallel with normal tissue (cervix, omentum or blood) for mtDNA mutation analysis by Semczuk *et al.* [41]. A total of 1623 bases of the mitochondrial genome were sequenced including all or parts of HV1, *16SrRNA, tRNA^{leu1}, ND2, ND3, ND4, ND4L* and *ND5*. Mutations were found at low frequencies in early (3 out of 32; 9%) and advanced endometrial lesions, but did not correlate with other clinicopathologic parameters. In contrast to Semczuk *et al.* [41], Pejovic *et al.* [42] discovered mutations in 5 out of 8 (63%) uterine serous carcinomas, using single-strand conformation polymorphism (SSCP) analysis. Malignant tissues were compared with adjacent, matched, histopathologically normal endometrium.

2.7 Esophageal

Hibi *et al.* were the first to investigate mitochondrial D-loop alterations in association with esophageal cancers (EC) [43]. Of 37 tumors, only 2 (5%) had mutations compared with adjacent normal tissue. In a follow-up study to Hibi *et al.* [43], Kumimoto *et al.* also examined the somatic mutation rate of EC in a group of 38 tumors [44]. This group found changes in 13 out of 38 (34.2%) tumors, which is almost 7-fold greater than that seen by Hibi *et al.* [43]. Most of the alterations were in the HPR. Abnet *et al.* analyzed 21 EC for the presence of mutations in the D-loop and the frequency of the CD [45]. EC were compared with matched, adjacent normal tissue, as well as blood. All samples were genotyped using the ABIAmpFISTR Profiler Plus PCR multiplex assay as an added quality control measure. Mutations were found in 7 out of 21 (33%) of EC. In comparison, the CD was observed in 17 out of 20 (85%) tumors as well as in 19 out of 20 (95%) of the adjacent normal mucosa, indicating a 'field effect', which has the potential for early diagnosis. The presence of mitochondrial genome D-loop mutations in Barrett's epithelium and Barrett's carcinoma was studied by Miyazono *et al.* [46]. Changes were found in 8 out of 20 (40%) patients with these histologies. Interestingly, mutations were observed in both tumor and epithelium, suggesting a 'field effect'.

2.8 Gastric

Habano *et al.* determined that '*mitochondrial gene mutations, which are associated with the mtMSI phenotype, may play a specific role in the tumorigenesis of intestinal-type gastric carcinomas*' [47]. Primary gastric carcinomas (n = 62) were surgically recovered from 60 patients, in parallel with matched normal tissue. A subset of protein coding regions *(ND1, tRNA^{leu}, ND4, ND5, COXII, CYTB, ATPase 8* and *6)* and the HPR were sequenced. SSCP was used to identify sites of mismatch, which were then sequenced directly. Of these samples, 11 out of 62 (18%) had mutations in the HPR and/or coding regions. One of the coding region changes caused truncation of *ATPase 6*, whereas another had a similar effect on *ND5*. Nuclear microsatellite instability (nMSI) was observed in 7% of these samples. In a similar study, Maximo *et al.* evaluated mitochondrial genome mutations in 32 gastric carcinomas and matched normal tissue [48]. Using SSCP, the authors characterized the mutation patterns in five mitochondrial genes (*ND1, ND5, COI, tRNA^{Leu}* and *tRNA^{Ser}*). In addition, part of the D-loop was sequenced as well. In a previous study, these samples were interrogated for the CD, which was found in 17 out of 32 (52%) [49]. Using the associated normal tissue as control, mitochondrial genome changes were seen in 26 out of 32 (81%) samples. Most changes occurred in the D-loop and *ND1* and *ND5* genes. Both *tRNA* genes were free of mutations. Interestingly, there was an inverse relationship between the CD and occurrence of other mitochondrial genome mutations ($p = 0.080$).

Zhao *et al.* investigated D-loop alterations in 20 gastric cancers and matched normal mucosa with regard to ROS, cell cycle and apoptosis [50]. Mutations were present in 7 out of 20 (35%) of the samples. Of 18 total changes, only 4 were found in mtMSI or the HPR. Proliferation, apoptosis and ROS production were increased in mutation-positive samples when compared with controls ($p < 0.05$). Depletion of mtDNA, changes in the D-loop and the frequency of the CD were analyzed in 31 gastric cancers and the corresponding benign mucosa [51]. Of these samples, 48% (15 out of 31) presented alterations in the D-loop, 10 of which were in

the HPR. The CD was present in 17 out of 31 (55%) of the normal mucosa and in only 3 (9%) tumors. Although D-loop alterations were not associated with any clinical aspects, mtDNA depletion was significant for both Borrmann's type III and IV gastric carcinomas. Kose *et al.* surveyed the HPR in 96 gastric cancers in comparison to normal paired tissues and found 14 of these tumors (15%) to have changes in this region [37].

Dani *et al.* observed a higher incidence of the CD in normal (100%) compared with malignant foci (79%) [24]. Another comparison of tumor and corresponding normal tissues (n = 107) identified a higher frequency of the CD in non-tumoral tissues (16.82%) than in gastric carcinomas (5.6%) [52]. In a follow-up study of 71 gastric carcinoma patients, Kamalidehghan *et al.* demonstrated the presence of multiple large-scale mitochondrial genome deletions at varying frequencies in both normal and tumor tissues [53].

2.9 Head and neck

Ha *et al.* investigated mutations in the HPR of 137 premalignant lesions from 93 patients [54]. A moderate number of patients (34 out of 93; 37%) had alterations in the target sequence. The authors concluded that these mutations have utility in the early identification of malignancy. A series of 109 head and neck squamous cell carcinomas (HNSCC) were investigated for alterations in the D-loop [55]. Mutations were found in 21% of these tumors. The most frequent alteration was in the HPR. There was no association with prognosis or post therapy outcome, suggesting that D-loop changes in these tumors may only be an indicator of malignancy. Prior *et al.* characterized somatic mitochondrial genome changes in *ND2* and the D-loop in 30 oral, smoking-related tumors (squamous cell carcinoma [SCC]) and paired normal tissues [56]. Out of a total of 30, 20 (67%) lesions had mutations, mostly in the D-loop as opposed to *ND2*. Only 6 point mutations were noted in *ND2*. Most of the changes were at nucleotide pairs 146 (8 patients), 152 (9 patients), 186 (12 patients), HPR (11 patients) and in *ND2* at 4917 (4 patients).

Poetsch *et al.* investigated the frequency of mutations in *ND1, ND5* and a portion of the D-loop as well as the CD [57]. Sixty seven primary HNSCC and matched normal tissues from 56 patients, as well as 40 metastatic deposits from 23 of the 56 patients were investigated. Blood was used as a control and no differences were found between blood and normal tissues. Of the primary tumors, 24 (42%) presented mtMSI in the HPR. Moreover, in 10 metastatic tumors, the sequence was identical for both the primary tumor and the metastatic site, yet different from paired normal samples. MtMSI was absent in 6 primary tumors. A total of 12 primary tumors had somatic mutations that were absent in paired normal. The CD was found in 17 (31%) of the primary tumors and 4 metastatic deposits. The CD was also found in 28% of both malignant and paired normals. Interestingly, in 15 metastatic sites, the deletion was absent. Deletion frequency was not correlated with age. The frequency of the CD was also studied in 58 patients with nasopharynx carcinoma (NPC) in parallel with 31 paired blood samples [58]. The CD was found in 49 out of 58 (85%) tumors, in contrast to 26% of the blood samples. Moreover, the ratio of deleted to full-length mitochondrial genomes was threefold higher in advanced, compared with early-stage NPC. The authors concluded that increasing levels of the CD parallels tumor progression. In another study, 35 tumor samples from patients with laryngeal squamous cell cancer were characterized for the CD [59]. Overall, 20 out of 35 (57%) harbored the deletion, which seemed to correlate with tumor differentiation status. Pure populations of oral cancer, precancerous cells, precancerous stroma, cancer stroma and lymph nodes were laser-capture microdissected for analysis of the CD [60]. The levels of the deletion were much higher in lesions than in lymphocytes, and were even much higher in precancerous lesions than in cancer. Similarly, the stroma of precancerous lesions harbored more deletions than cancer stroma.

With recent technological advancements, it is now easy to sequence the entire mitochondrial genome. Indeed, recent microarray-based resequencing of the entire mitochondrial genome was conducted on a panel of 83 HNSCC and mutations were present in 41 (49%) of the tumors [61]. Sequencing of dysplastic adjacent tissues of one tumor identified an identical mutation in both tumor and margin samples, suggesting that mtDNA mutations occurred early in this tumor, and could be involved in disease progression.

mtDNA copy number or content alterations have also been studied in head and neck cancers. Oral rinse samples were collected from 94 patients with HNSCC as well as 656 individuals without HNSCC for copy number analysis using *COI* and *COII,* and normalized to nuclear β-actin levels. The authors found that the copy number of these genes was elevated in the saliva of HNSCC patients and was also associated with advanced-stage disease [62]. An earlier study utilizing tumor tissues with the same methodology concluded that mtDNA content increases in parallel with histopathologic grade [63].

2.10 Liver

Shao *et al.* examined the frequency of a 4981-bp deletion of the mitochondrial genome in 27 surgically resected hepatocellular carcinomas (HCC), corresponding non-cancerous control tissue and 27 hepatocellular nodular hyperplasias (HNH) [64]. Using real time PCR, this deletion was found in 23 out of 27 HCCs (85%), 22 out of 27 HNHs (83%) and 15 out of 27 adjacent normal samples (57%). These results suggest that mitochondrial deletions play a role in the development and progression of HCC. Normalized levels of the CD were significantly higher in HCC than in non-cancerous control and HNH tissues.

Nishikawa *et al.* characterized the mitochondrial D-loop mutation profile in HCC samples, in comparison with matched normal samples from 71 individuals [65]. A total of

59 individuals had chronic hepatitis C, whereas the remaining 12 had chronic hepatitis B. In addition, control liver tissue was recovered from 7 individuals without hepatitis B and HCC, yet had other liver conditions. Moreover, these researchers precluded all potential pseudogene contribution by testing their PCR primers against DNA from cells absent of mtDNA. In an initial study phase, the entire mitochondrial genome was sequenced from five samples (two poorly differentiated HCCs and two matched normal tissues, as well as one noncancerous control liver tissue). The region of sequence between bp 100 – 600 harbored the greatest number of mutations and was sequenced in the remaining samples. A total of 28 (39%) of the 71 cancer patients had a greater number of mtDNA mutations in cancerous than in non-cancerous tissues. Interestingly, a 'field effect' is suggested by the higher incidence of mutations in normal liver tissue from liver with malignancy, when compared with liver samples free of HCC.

An important clinical aspect of HCC is the ability to differentiate between relapse and a second primary tumor. In an attempt to resolve this dilemma, 19 patients with multiple HCCs were collected, including adjacent histologically normal controls [66]. These samples were characterized by mitochondrial D-loop sequencing. Matched plasma from each patient was also investigated for corresponding D-loop alterations. Overall, 13 of the 19 patients (68%) presented with a D-loop mutation. Three patients demonstrated identical mutations in multiple tumors, suggesting tumors were monoclonal in origin. Plasma was available from 10 out of 13 mutation positive individuals. Interestingly, the corresponding HCC mutations were found in mtDNA from the matched plasma in 8 out of 10 (80%) of the cases. Lee *et al.* also analyzed the mutation rate in the D-loop of 61 HCCs and the adjacent normal tissue [67]. A total of 24 of the 61 demonstrated alterations (39.3%) in the D-loop and a significant decrease in mtDNA copy number was observed in 60.5% of cancer patients. This reduction was consistent with those HCCs which demonstrated mutations in the D-loop and was associated with mutations near the heavy strand origin of replication. The authors concluded that mtDNA depletion and D-loop mutations are early events in HCC. A study involving 54 HCCs with 47 surrounding normal tissue samples and 5 samples with metastatic foci, but lacking inflammation, were sequenced for D-loop aberrations. Interestingly, the number of alterations increased as differentiation decreased, demonstrating a progressive pattern [68].

Another study found the levels of mtDNA to be lower in HCCs than in normal matched tissue [69]. In addition, 4 out of 18 HCCs (22%) had somatic mutations in the D-loop. The trend of reduced mtDNA content was only found in females. MtDNA content in 31 HCCs and corresponding non-malignant samples were analyzed for correlations with pathologic findings and prognosis [70]. MtDNA copy number was significantly reduced in HCCs compared to normal corresponding samples ($p = 0.002$), and also correlated significantly with tumor size ($p = 0.014$) and cirrhosis ($p = 0.048$). There was no significant association of copy number or D-loop mutations with clinicopathologic parameters.

2.11 Lung

Suzuki *et al.* interrogated a 336-bp fragment of HV2 that contained the HPR in 55 non-small cell lung cancers with their matched, histologically normal tissue [71]. Changes were found in 11 out of 55 (20%) of these samples. Most of these alterations were homoplasmic. D-loop and rRNA mutations were characterized in 202 non-small cell lung cancers and associated normal lung tissues [72]. Overall, 70 of these samples (35%) presented with mutations. Moreover, the levels of D-loop mutations were associated with high-grade tumors. Finally, this increased mutation load is associated with lymph node metastasis. Given these results, the authors suggest that preoperative analysis of the D-loop region, using biopsy material, has clinical utility. Atypical adenomatous hyperplasia (AAH) is often found in close proximity to lung adenocarcinoma and is thought to be a precursor to malignant lesions. Morandi *et al.* investigated the differences and similarities, in part, by sequencing the D-loop region in associated tumor and AAH [73]. The mutation patterns were independent, suggesting that the tumors and corresponding AAH are genetically different in agreement with the concept of field cancerization. In another study, 37 matched lung cancer and adjacent tissue, as well as 20 normal samples from individuals without lung cancer were scored for the CD [74]. The CD was present in 20 out of 37 (54.1%) of lung cancers, 22 out of 37 (59.5%) of adjacent normal and 6 out of 20 (30%) of normal lung tissue free of malignancy. These authors determined there was no statistical difference between these tissues.

2.12 Ovarian

The presence of mtDNA mutations in cases of ovarian cancer was studied by Liu *et al.* [75]. In 15 primary tumors with matched normal tissues as controls, mutations were found in 3 (20%). A total mitochondrial genome analysis of 10 ovarian cancers, in parallel with matched normal, increased the mutation frequency to 60% (6 out of 10). These authors suggest that a 3-kb region (*16SrRNA, 12SrRNA, CTYB* genes and the D-loop) contains the appropriate sequencing targets for detecting mitochondrial genome mutations in ovarian cancer. A similar study demonstrates an accelerated mutation rate within a 3.3-kb region of the mitochondrial genome (D-loop, *12SrRNA, $tRNA^{phe}$, $tRNA^{val}$, $tRNA^{ser}$, $tRNA^{asp}$, $tRNA^{lys}$, COX I, COX II, ATPase 6* and *ATPase 8*) [76]. This particular study shows an unusually high number of variants. These numbers are misleading, however, as only 10 out of 102 tumors were compared with matched normal tissue. The remaining differences are attributed to comparisons with the published NCBI mitochondrial genome sequence and the mtDNA database, as opposed to blood. Van Trappen *et al.* looked at mutations in the D-loop in 52 samples from 35 ovarian cancer patients, 17 of which had

bilateral tumors [77]. Matched control tissue was used from each patient. Somatic mutations were found in 9 out of 35 tumors (26%). Of the 17 bilateral tumors, 4 harbored different mutations indicating different clonal origins of these tumors. Wang *et al.* investigated a suite of 12 mtMSI loci in 73 ovarian tumors and matched normal tissues [26]. Alterations were detected in 16 out of 73 (22%) of these tumors at 2 mtMSI sites (np 303 and np 514).

2.13 Pancreas

Navaglia *et al.* characterized the mitochondrial D-loop of 19 pancreatic adenocarcinomas [78]. mtDNA derived from the patient's blood was used as a germline control for determining somatic mutations. Only 3 out of 19 (16%) had mutations. The T, rather than C, allele at bp 16519 correlates with a shorter life expectancy for those with pancreatic cancer. Mu *et al.* [79] found a high incidence of mitochondrial D-loop alterations (11 out of 12 or 93%) in both premalignant and malignant lesions. Moreover, an increase in D-loop somatic changes was observed with disease progression.

2.14 Prostate

It is not surprising that the mitochondrial genome has a 'hyper-mutatgenesis' rate in prostate tumors [80]. The normal metabolic state of the prostate is geared for low energy production. Briefly, high concentrations of zinc in the mitochondria inhibit aconitase, which blocks the conversion of citrate to isocitrate, reducing the generation of NADH and $FADH_2$, compounds that are required to generate ATP through the electron transport chain. This reduction results in a low level of ROS production in normal prostatic tissue [4,81,82]. One of the indicators of a metabolic shift towards prostate cancer is a sharp decline in zinc levels resulting in 'normal' biologic execution of both the Krebs cycle and the electron transport chain. It is possible that this metabolic modulation causes a 'surge' of ROS, resulting in quantifiable damage to the mitochondrial genome. For example, several studies report relatively high mutation rates in the D-loop and other coding genes of the mitochondrial genomes from prostate tumors [4,83-85]. Moreover, a study attempting to sequence the mitochondrial genome in its entirety identified mutations in 91.7% (21 out of 24) of malignant prostate samples [23]. In particular, Petros *et al.* demonstrated the accelerated growth pattern of prostate tumors with a mtDNA *ATP6* mutation (T8993G) [4]. Not only were these tumors larger in size in murine models, but increased ROS production was also noted. Deletions of the mitochondrial genome are also observed to increase in frequency with age in the malignant prostate [86], and together with mtDNA depletion, might confer androgen independence [87]. Furthermore, a 3.4-kb deletion has been used to discriminate between malignant and benign prostate tissues with positive and negative predictive values of 75 and 80%, respectively [88].

2.15 Renal

Meierhofer *et al.* investigated the mitochondrial genome mutation rate in 15 renal cell carcinomas (RCCs) in comparison with normal cortex tissue obtained from nephrectomies [89]. Out of these paired samples, 7 tumors (46%) had at least 1 heteroplasmic change. Non-synonymous mutations were found in *ND2, COI, ND5* and *ND4L* regions. In particular, one carcinoma had an A3243G alteration, which is also a base alteration associated with mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes. Moreover, four mutations were present in ribosomal genes, with four more in the D-loop. Wada *et al.* determined the somatic mutation rate in 21 Japanese patients with RCC by sequencing the mitochondrial genome and assessing the levels of 8-OHdG, a marker of oxidative DNA damage [90]. RCC mitochondrial D-loop sequences were compared with similar sequences from matched 'normal' kidney tissues. Only 3 out of 21 (14%) RCC sequences had mutations. The HPR was a frequent site of mutation. Interestingly, 8-OHdG levels were elevated in malignant tissues, in comparison with matched normal; however, in many of these tumors, D-loop alterations were absent.

Chromophobe RCCs are defined by well known chromosomal losses [91]. Damage sustained by the mitochondrial genome in these malignancies were characterized by complete mitochondrial genome sequencing of 8 chromophobe RCCs. Sequence alterations were seen in 5 out of 8 (60%) tumors. All mutations were heteroplasmic with the exception of a homoplasmic 9-bp deletion. Normal kidney tissues from individual patients were used as comparative material. Tumor development often coincides with end-stage renal disease (ESRD). Nagy *et al.* studied 9 tumors from 6 ESRD kidneys and their associated normal tissues by sequencing the mitochondrial genome [92]. Somatic alterations were found in 7 out of 9 (79%) tumors. Interestingly, most transitions involved purines, in contrast to the more frequent pyrimidine exchanges observed in other studies.

2.16 Skin

Skin is a tissue that is often exposed to a potent mutagen in the form of ultraviolet radiation (UVR) in sunlight and mtDNA deletion mutations have previously been shown to accumulate in skin with cancer and photoageing (reviewed in [93]). Quite recently, Birch-Machin and others have pioneered the use of mtDNA damage as a highly sensitive biomarker of UV exposure in human skin (reviewed in [93]). UVR is known to induce ROS generation and in this regard, mitochondrial ROS generation causes various types of damage within the cell known to contribute to the cancer and ageing phenotype [93,94]. In addition, it has been recently shown that the accumulation of an ageing dependent mtDNA mutation is accelerated by exposure to sunlight in human skin [95].

Durham *et al.* were the first to investigate both mitochondrial genome deletions and mutations in non-melanoma skin cancers (NMSC) comprised of basal cell carcinomas (BCC)

and SCC [96]. Using long range PCR, both the major and minor deletion arcs were amplified from a suite of BCCs and SCCs along with corresponding normal sites. A plethora of deletions were observed in the major arc (20), whereas a smaller number occurred in the minor arc (4). A deletion ~ 4 kb in size was found in all samples that produced deletions. Interestingly, the CD was identified in the dermis of 4 out of 5 BCCs, but in only 2 out of 5 of corresponding tumors. Moreover, the CD was absent in the epidermis of all BCCs. The pattern for SCCs is less clear, yet the CD is found in 40% of tumor, dermal and epidermal samples respectively. There were no differences in the sequence profiles between tumor and perilesional skin in four areas of the mitochondrial genome (D-loop, *ND1, ND5* and *16SrRNA*), with the exception of a heteroplasmic site in one SCC. In a follow up to this work, Harbottle *et al.* identified the ~ 4-kb deletion as a 3895-bp deleted sequence in the minor arc of the mitochondrial genome [97,98]. They demonstrated the association of the 3895-bp deletion with NMSC; for example, the deletion was identified in 8 out of 10 NMSC samples used by Durham *et al.* [96] as opposed to 4 out of 10 when targeting the CD [98]. Not surprisingly, the 3895-bp deletion was found to increase at body sites with greater sun exposure [99].

In the literature, there are only three studies (two are predominantly cutaneous studies) that have begun to investigate mtDNA damage in melanoma [100-102]. A total of 69 melanoma resection specimens and corresponding normal tissue were analyzed for both mutations and sequence alterations in 2 HPRs located in a 157-bp segment of the D-loop (270 – 425). Mutations, or changes in the HPRs appeared in 11 out of 69 (16%) of these samples [100]. An intriguing study by Takeuchi *et al.* investigated the mutation rate of the D-loop in 12 melanoma samples in parallel with corresponding plasma [102]. DNA recovered from peripheral blood lymphocytes were used as controls. Overall, 5 out of 12 (42%) tumors had D-loop alterations. Corresponding mutations were found in 2 out of 5 matched plasma samples, suggesting that freely circulating mtDNA, as a detection tool, merits further investigation. Although interesting, these studies could be considered preliminary in the sense that they have used relatively insensitive mutation screening methods based on automated DNA sequencing to investigate mtDNA mutations in a minor proportion of the entire mitochondrial genome (see general comments in Section 3).

Eshaghian *et al.* looked at the deletion spectrum of 29 NMSCs and their tumor-free margins [103]. Using long extension PCR, 98% of the mitochondrial genome was amplified and resolved using field inversion gel electrophoresis. Older patients had a higher frequency of deletions, consistent with cumulative lifelong sun exposure. Moreover, tumor margins always had more deletions than the corresponding lesion, which contained little or no deletion(s). Another study, reported by Kamenisch *et al.* [104] confirmed the findings of Eshaghian *et al.* [103]. In an expanded set of BCCs and SCCs the CD presents a lower incidence in tumor lesions when compared to epidermal sites peripheral to the tumor ($p = 0.0005$ for both SCCs and BCCs). The collection of appropriate tumor and normal epidermis was closely controlled through LCM. Moreover, the mean age of the SCC group was 82, whereas the BCC cohort was 73 years, suggesting that long-term UV exposure is consistent with NMSC.

A recent study by Birket has discovered frequent genetic linkage between the 3895-bp mtDNA deletion and the T414G mutation [95]. This linkage indicates that mtDNA mutations such as these are unlikely to be distributed equally across the mtDNA population within the skin tissue, increasing their likelihood of exerting focal effects at the cellular level.

2.17 Thyroid

A study of mitochondrial mutations in 21 cases of thyroid tumors found alterations in 3 cases of papillary thyroid carcinomas (compared with matched normal) [105]. Other tumor types were free of mutations. A total of 72 thyroid cancers (papillary, medullary, anaplastic, follicular and insular carcinomas) were surveyed in the HPR. A low frequency of changes (6.9%) was found in these samples, when compared with adjacent normal controls [106]. An investigation analyzing both somatic mutations and the frequency of the CD was performed by Máximo *et al.* [107]. In this study, 32 out of 66 (48%) of tumors had at least one alteration in the portion of the mitochondrial genome sequenced. Interestingly, tumors with D-loop mutations had an increased frequency of the CD. In addition, when present, the CD was at a higher frequency in the lesion when compared with adjacent parenchyma. In a follow-up study, Máximo *et al.* characterized 3 mtMSI loci (HPR, D514, D568) in 66 thyroid lesions from 59 patients [108]. Overall, 30 of these samples were benign adenomas. A total of 32 (49%) of these tumors presented somatic lesions with the following loci distributions: HPR (20), D514 (15) and D568 (3). This study demonstrates the utility of targeting homopolymer and dinucleotide elements in the mitochondrial genome as indicators of somatic mutations. Papillary (PTC) and follicular (FTC) thyroid carcinomas were investigated for potential somatic mutations in their mitochondrial genomes using corresponding normal tissues as control [109]. The entire mitochondrial genomes of paired PTC tissues and three paired FTC tissues were analyzed using high-performance liquid chromatography and sequencing. Mutations were found in three out of seven PTCs (49%) and all three FTCs [109].

3. Expert opinion

The preceding outlined studies are replete with data suggesting biomarker potential for the mitochondrial genome. For example, in some studies, mutations in the mitochondrial genome, as opposed to their absence, indicate a worse clinical

outcome for cervical, breast and colorectal cancers. In contrast, the presence and frequency of the CD in 'normal' tissues, adjacent to malignant foci, suggests a possible 'cancerization field effect' as observed in many other cancers [5]. This suggests an important early detection opportunity, which is a vital component for successful intervention [110]. There are indications that mitochondrial genome alterations modulate with tumor progression, another important clinical aspect not always obvious from histology. Moreover, if the early appearance of mitochondrial genome mutations in a cancerization field is linked to tumor progression, it will be necessary to concede that molecular pathways leading to neoplasia, malignancy and tumor progression may not always correspond with histopathologic classifications. There may be reluctance to move away from this current 'gold standard'; however, many studies suggest that malignant transformation begins within the molecular machinery, before there are noticeable histologic changes indicating that altered morphology may be late in the process.

There are some limitations with the current trends in mitochondrial biomarker studies. There is an unusual lack of concern for the well known problem of numt co-amplification. Without this precaution, any conclusions as to biomarker utility are unsure. This is especially problematic when mutations are scored when mitochondrial depletion is attested. A simple test of intended amplification primers against total DNA extracted from Rho^0 cells, as noted by Parfait *et al.* almost 10 years ago, precludes this concern [8]. The use of histologically normal-appearing tissue, in proximity to tumor as the only normal control sample, is inappropriate. The concept of a 'cancerization field effect' occurred in the literature in 1953 and was alluded to as early as World War II. Many of the studies reviewed here use this strategy of matched or paired normal malignant and 'normal' tissues. Using this approach, it cannot be known whether the mutation has occurred in the affected or unaffected material. The mutation, by definition is scored as tumor in origin, blinding observation of any field effect. Using mitochondrial sequence from peripheral white blood cells as the comparative genetic material avoids these problems. Furthermore, some studies use the rCRS as the comparative sequence. Although this strategy will standardize the results to the reference sequence, it is significant for polymorphic genetic information and provides no information on somatic mutation load. In some instances, the rCRS serves as a template for determining both synonymous and non-synonymous mutations. Again, sequences require comparison within their own genetic context, which is corresponding, matched white blood cells to avoid false interpretation. Much work has been done on the HPR, however, these regions are difficult for polymerases to accurately read; such work requires careful replication.

Sequencing the entire mitochondrial genome produces an overall accurate mutation rate; however, more attention to quality control of this process must be a priority. Phylogenetic analysis is one useful method for detecting sequencing errors [111]. Important criticism has been published on this topic [11]; however, specific points of this appraisal require comment. For example, Salas *et al.* indicate that mitochondrial genome point mutations found in tumors that are also population-specific mutations result from sample switching or contamination and are unrelated to tumorogenesis [11]. Many of the studies here suggest otherwise, particularly those that assess the frequency of large-scale deletions. These mutations attest to an evolutionary active molecule in a real-time sense, particularly when supported by point mutation data originating from the same samples [48,49]. Rapid modulation of point mutations in malignant tissue is clearly attested [12]. Moreover, many of these 'shared' mutation sites may have a common evolutionary function [112]. It has long been established that mutational 'hot spots' are a feature of genomes [113]. The mitochondrial genome, in particular, has an accelerated mutation rate and this overlap should not be surprising; however, phylogenetic analysis is an appropriate and important method for elevating quality control in all studies using mitochondrial genome sequencing data.

Conventional sequencing methods are insensitive for the detection of low level heteroplasmic mutations that offer potential for early detection. However, novel sequencing technologies including MitoChip resequencing, denaturing high-performance liquid chromatography and pyrosequencing allow sensitive detection of heteroplasmy below 2% [114,115].

Much of the analyses of the CD cited here produced a presence or absence result. The application of real time, quantitative PCR assay has been used to discriminate between benign and malignant tissues in prostate cancer indicating that the frequency of mitochondrial mutations has important clinical utility when analyzed in detail [88].

Declaration of interest

Certain commercial equipment, instruments, materials or companies are identified in this paper to specify adequately the experimental procedure. Such identification does not imply recommendation nor endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are the best available for the purpose.

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