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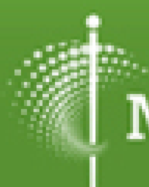

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Advanced article

Mitochondrial DNA Copy Number Alterations in Human Cancers

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Mitochondria are cytoplasmic organelles that participate in adenosine triphosphate (ATP) production through respiration and oxidative phosphorylation (OXPHOS), free radical formation and execution of apoptosis. Perturbation of mitochondrial function has been proposed as one vital hallmark of cancer cells. Besides plenty of germline and polymorphic sequence variations accumulated in both coding and noncoding regions of the mitochondrial genome (mitochondrial deoxyribonucleic acid (DNA); mtDNA), increased or reduced mtDNA copy number has also been increasingly described in a plethora of primary human malignancies. Altered mtDNA quantity may act as an important player in the multistep carcinogenesis of at least some types of human cancer by fuelling tumour initiation and/or advancement. In addition, mtDNA content

turnover in bodily liquids from cancer patients could be exploited as a novel molecular tool for early cancer screening and diagnosis.

Key Concepts:

- Disruption of mitochondrial function and the oxidative phosphorylation system may lead to cancer occurrence and development.
- MtDNA copy number changes have been frequently observed in a wide range of human malignancies.
- Aberrant mtDNA quantity is correlated with many key clinicopathological features of cancer patients.
- Alterations in mtDNA copy number have potential to affect cancer cell behaviours in vitro and in vivo.
- mtDNA content alterations could be considered as a novel biomarker for early cancer detection and diagnosis.

Introduction

Mitochondria are semi-autonomous multifunctional organelles in eukaryotic cells that play indispensable roles in energy metabolism, free radical formation, calcium buffering and apoptosis. Mitochondria carry their own unique genome, namely mitochondrial deoxyribonucleic acid (DNA; mtDNA), which exists in hundreds to thousands of copies in each individual cell and replicates independently of the cell cycle (Clay Montier *et al.*, 2009). Human mtDNA is a ~16.6kb, maternally inherited, closed-circular, double-helical molecule encoding 13 crucial polypeptide subunits of respiratory enzyme complexes, two ribosomal ribonucleic acids (rRNAs) and a set of 22 transfer RNAs required for intramitochondrial protein synthesis (Li *et al.*, 2012). The actual number of mtDNA is dependant on cellular energy demand and may vary substantially between distinct cell types (Clay Montier *et al.*, 2009). mtDNA contains a 1124bp noncoding fragment named displacement (D)-loop with key regulatory elements

for controlling mtDNA transcription and duplication (Li *et al.*, 2012). Because of limited DNA repair mechanism, the lack of protective histones and its proximity to high concentration of endogenous reactive oxygen species (ROS) generated in the mitochondrial inner membrane, mtDNA is extremely susceptible to oxidative damage and thereby have a much greater mutation rate (10–200 times) than nuclear DNA (nDNA; Cline, 2012).

Mitochondrial dysfunction has long been suspected to contribute to the occurrence and/or progression of human cancers. Given the utter importance of mtDNA in sustaining proper mitochondrial activity, intense research efforts have been made to uncover the possible link of mtDNA abnormality with cancer development. In addition to numerous sequence variations including point mutations, large-scale deletions and insertions in both protein-coding and control regions of mtDNA (Yu, 2012), mtDNA copy number alterations have been increasingly detected in a board spectrum of primary solid tumours and haematologic malignancies. This article summarises mtDNA content changes in common human neoplasms and briefly discusses the potential reasons behind mtDNA amount turnover as well as its causative involvement in driving malignant transformation and tumour advancement. The current state of our knowledge with regard to the putative utility of altered mtDNA quantity in tumour tissues and peripheral blood of cancer patients as a novel molecular biomarker for early cancer detection and diagnosis is also provided.

Quantitative Changes of mtDNA Levels in Cancer

Accurate measurement of mtDNA quantity by taking the advantage of real-time polymerase chain reaction (PCR) techniques has hitherto revealed aberrant mtDNA copy number in diverse types of primary human malignancies,

and the change of mtDNA copies seemingly relies on the original tumour site (Yu, 2012), as either an increase in the vast majority of acute lymphoblastic leukaemia; colorectal carcinoma (CRC); endometrial cancer; oesophageal squamous cell carcinoma (ESCC); head and neck cancers; ovarian cancer; papillary thyroid carcinoma and prostate cancer or a reduction in advanced gastric cancer, breast cancer, Ewing's sarcoma (EWS), fibrolamellar carcinoma, hepatocellular carcinoma (HCC), nonsmall cell lung cancer (NSCLC), osteosarcoma and renal cell carcinoma (RCC; **Table 1**). It is gradually recognised that mtDNA quantitative level in tumour cells could be precisely modulated during the carcinogenic process by a complex mechanism(s), instead of easily modulated as a consequence of abnormal cell proliferation, because the change of mtDNA content in most tumour tissues are generally maintained within a certain stable range (≤ 2-folds) as compared to that in adjacent noncancerous counterparts (Lee *et al.*, 2005).

Table 1 Selected studies showing mtDNA copy number alterations in solid tumours

Cancer types	Cohort size	Patient characteristics		mtDNA copy number	References
		Age (average)	Ethnic background		
Breast	60	N/A	Asian	Decrease	Tseng *et al.* (2006)
	59	54	Asian	Decrease	Yu *et al.* (2007a)
	51	N/A	Caucasian	Decrease	Fan *et al.* (2009)
CRC	44	51	Asian	Increase	Feng *et al.* (2011)
Endometrial	65	59	Asian	Increase	Wang *et al.* (2005)
ESCC	72	60	Asian	Increase	Lin *et al.* (2010)
EWS	17	14	Asian	Decrease	Yu *et al.* (2010)
Gastric	31	N/A	Asian	Decrease	Wu *et al.* (2005)
HCC	61	N/A	Asian	Decrease	Lee *et al.* (2004)
	18	N/A	Asian	Decrease	Yin *et al.* (2004)
Head and neck	14	9	N/A	Increase	Kim *et al.* (2004)
NSCLC	29	64	Asian	Decrease	Lin *et al.* (2008)
Ovarian	42	52	Asian	Increase	Wang *et al.* (2006)
RCC	37	64	Caucasian	Decrease	Meierhofer *et al.* (2004)
Prostate	10	62	N/A	Increase	Mizumachi *et al.* (2008b)

Abbreviations: CRC, colorectal carcinoma; ESCC, oesophageal squamous cell carcinoma; EWS, Ewing's sarcoma; HCC, hepatocellular carcinoma; NSCLC, nonsmall cell lung cancer; RCC, renal cell carcinoma.

Mounting evidence has suggested that somatic mutations in the D-loop segment of mtDNA is a critical determinant leading to reduced mtDNA copies in some types of cancer

(Lee *et al.*, 2004; Yu *et al.*, 2007a, 2010). Although D-loop mutations do not directly trigger changes in the coding sequences and impair mitochondrial respiratory function, accumulated mutations in this transcriptionally active area have potentials to disturb the binding affinity of some pivotal inducers or mediators responsible for mtDNA transcription through the modification of the promoter sequences, eventually exerting a negative effect on the mtDNA transcriptional rate and resulting in decreased mtDNA amount in tumour cells (Clayton, 2000). Lowered mtDNA copies in cancer cells also plausibly stems from defective p53-mediated signalling. The p53 tumour suppressor not only sustains mitochondrial genetic stability via its interplay with mtDNA polymerase γ (POLG) but also serves as a mitochondria damage checkpoint (mito-checkpoint) to physically coordinate mitochondrial biogenesis (Kulawiec *et al.*, 2009). Hence, loss of *p53* expression enhances vulnerability of mtDNA to oxidative stress and disrupts redox and ROS homoeostasis in mitochondria, which may potentially elicit mtDNA reduction or depletion (Lebedeva *et al.*, 2009). Furthermore, the number of mtDNA copies was recently found to be considerably declined in breast cancer cell lines and clinical specimens harbouring the POLG mutations (Singh *et al.*, 2009), supporting a notion that reduction in mtDNA quantity in some cancer entities may be attributed to inefficient enzyme activity associated with mutant POLG. However, increment of mtDNA content in some forms of cancer is most likely derived from elevated oxidative stress, albeit that the underlying mechanisms still remain largely obscure. Previous research has illustrated that the accretion in mitochondrial mass and mtDNA level is correlated with enhanced oxidative stress in senescent human tissues (Lee *et al.*, 2002). The similar observation was made in cultured human lung fibroblast cells exposed to different oxidative stress-inducing agents (Lee *et al.*, 2000). Given that there are many common features between aging

and neoplastic cells, the above-noted scenario could also occur during the onset and progression of certain cancer types and increased mtDNA quantity can be explained as one of the feedback responses that compensate, at least partially, for metabolic malfunction in mitochondria carrying mutated mtDNA or deficient respiratory system.

Clinicopathological Correlation between mtDNA Copy Number Alterations and Cancer

The potential involvement of altered mtDNA quantity in the aetiology of malignant diseases has gained supports from a growing body of clinicopathological association studies. In an investigation of 18 HCC patients, the mean value of mtDNA copies was revealed to be remarkably reduced in females than males (Yin *et al.*, 2004). This interesting finding favours an idea that mtDNA quantity may be a key factor among others contributing to the difference in mortality rate and clinical manifestation between female and male HCC patients. For breast cancer, the authors' group and others have shown that decreased mtDNA amount in tumour tissues was intimately related with an older age of onset (≥50 years), a higher histological grade and negative status of progesterone receptor (Tseng *et al.*, 2006; Yu *et al.*, 2007a; Fan *et al.*, 2009). Patients bearing lower mtDNA levels exhibited noticeably poorer disease-free and overall survival rates in comparison with those with normal mtDNA quantity (Yu *et al.*, 20007a). Likewise, diminished mtDNA content was frequently identified in the ulcerated, infiltrating (Borrmann's type III) and diffusely thick (Borrmann's type IV) types of gastric carcinoma (Wu *et al.*, 2005). A high proportion of individuals afflicted with types III and IV gastric cancers tended to have poor prognosis and short 5 year survival after tumour resection. Moreover, declined mtDNA copy number in NSCLC was in a tight connection with the advancement of

tumour progression (Lin *et al.*, 2008). Reduced mtDNA content was also reported to be relevant to tumour metastasis in EWS (Yu *et al.*, 2010).

As aforementioned, mtDNA copy number is aberrantly upregulated in many human cancers. In a study involving a series of 44 CRC cases, elevated number of mtDNA copies displayed a significant association with tumour grade, indicating that mtDNA content alteration could be engaged in CRC progression (Feng *et al.*, 2011). It has been shown that the average value of mtDNA quantity in head and neck cancers was increased in a progressive fashion with histopathological grade from normal mucosal cells, mild, moderate and severe premalignant lesions, to invasive tumours (Kim *et al.*, 2004). Jiang *et al.* (2005) also evaluated mtDNA copy number change in salivary rinses collected from patients with primary head and neck squamous cell carcinoma. They found that tumours at advanced stages (III and IV) contained a much higher level of mtDNA content than low-grade ones (I and II). In agreement with these data, mtDNA content in ESCC was significantly linked with tumour aggressiveness and augmented stepwise from benign dysplasia to cancerous ESCC nests and then to metastatic lymph nodes (Lin *et al.*, 2010). Overall, the above data indicate that altered mtDNA level in tumour tissues has potential to be applied as a predictive or prognostic indicator for tracking tumour progression as well as monitoring patient's posttreatment outcome.

Implications of Altered mtDNA Amount in Prompting Malignant Phenotype

Although mtDNA content change appears to be a general hallmark of malignant cells and is significantly associated with many clinicopathological properties of patients, the question of whether quantitative mtDNA variation has a

primary and causative relationship with the pathogenesis of human cancers or just emerges as a secondary bystander effect reflecting nuclear genomic instability during tumourigenesis is poorly understood.

In recent years, an increasing number of functional analyses using various mtDNA-depleted (ρ^0) cell models have substantiated that mtDNA content change is sufficient enough to influence cancer cell behaviour, such as cell proliferation, apoptosis, hormone dependence, invasive and metastatic capabilities. For instance, elimination of mtDNA molecules drastically suppressed proliferation and tumourigenesis of T47D breast cancer cells both *in vitro* and *in vivo* (Yu *et al.*, 2007b). Growth retardness and decreased *in vivo* tumour-forming capacity were similarly observed in several other cancer cell lines devoid of mtDNA, such as mtDNA-depleted MOLT-4 leukaemia and HeLa cervical cancer cells (Armand *et al.*, 2004; Schauen *et al.*, 2006). More importantly, partial loss of mtDNA or administration with mitochondrial-specific inhibitors was proven to lead to invasive phenotypes and significantly upregulated markers of tumour invasion (e.g. *cathepsin L* and *transforming growth factor* (*TGF*)-β) in both noninvasive C2C12 myoblasts and low invasive A549 lung cancer cells (Amuthan *et al.*, 2002). Overexpression of several genes related to angiogenesis and vasculogenesis during tumour invasion, such as vascular endothelial growth factor and matrix metalloproteases, was detected in SK-Hep1 hepatoma ρ^0 cells (Cheon *et al.*, 2010). In line with these results, LNCaP prostate cancer and MCF-7 breast cancer cells could undergo an epithelial–mesenchymal transition to gain progressive tumour properties, such as higher aggressiveness and loss of hormone-dependent growth, during the process of mtDNA depletion by means of activating the Raf/MAPK and TGF-β signalling pathways (Naito *et al.*, 2008a). It was also reported that reduced number of mtDNA copies in PC3 prostate

cancer cells was coupled with an increased migration onto the basement membrane protein laminin-1 (Moro *et al.*, 2008), suggestive of a possible association of mtDNA content with metastatic competence of PC3 cells.

mtDNA copy number alterations have been shown to facilitate tumour cells in gaining increased resistance against many common anticancer drugs. SK-Hep1 ρ^0 cells displayed a reduced sensitivity to doxorubicin and two other oxidative stressors, menadione and paraquat (Park *et al.*, 2004). The investigators proposed that the adaptive overexpression of major antioxidant enzymes following chemotherapeutic treatment may help cancer cells arm themselves in order to counteract oxidative stress and host anticancer surveillance. Similarly, mtDNA depletion in CRC cells was documented to significantly potentiate tolerance against cisplatin, doxorubicin and 5-fluorouracil (5-FU) and somatic mutations in the D-loop may be accountable for resistance to 5-FU-based adjuvant chemotherapy (Lievre *et al.*, 2005; Qian *et al.*, 2005). In addition, mtDNA-deficient T47D breast cancer cells and HCT-8 colon cancer cells had a pronouncedly decreased drug intake after exposure to doxorubicin, vincristine and paclitaxel as compared to their respective parental cells, probably owing to upregulated expression of ABC transporter P-glycoprotein (Lee *et al.*, 2008; Yu *et al.*, 2009). Acquisition of a multidrug resistant phenotype, together with overproduction of reduced form of glutathione, was observed in osteosarcoma 143B cells lacking mtDNA after induction of several cytotoxic agents, including doxorubicin and daunomycin (Ferraresi *et al.*, 2008). It is worthy emphasising that the impact(s) of mtDNA content change on chemotherapy sensitivity alters with the type of cancer, and the discrepancy might be ascribed to the difference of basal mtDNA copy number in distinct tumour types. As an example, the increase in mtDNA quantity was able to evoke acquired docetaxel resistance in laryngeal

cancer HEp2 cells by both enhancing F_0-adenosine triphosphatase (ATPase) activity and downregulating ROS generation (Mizumachi *et al.*, 2008a).

In breast and prostate cancer cells, change in mtDNA amount was reported to determine hormone dependence. Androgen-independent C4-2 prostate cancer cell line, which was derived by inoculation of androgen-dependent LNCaP cells in castrated mice, had a significant reduction in number of mtDNA copies and an accumulation of large-scale mtDNA deletions (Higuchi *et al.*, 2006). Reconstitution of normal mtDNA molecules into the mtDNA-depleted androgen-independent mutant clones reestablished their dependence on androgen. mtDNA content was substantially declined in 4-hydroxytamoxifen-resistant MCF-7 breast cancer cells as compared to their wild-type counterparts (Naito *et al.*, 2008b). mtDNA depletion in MCF-7 cells induced their resistance to both 4-hydroxytamoxifen and the oestrogen receptor antagonist ICI182780, whereas transfer of normal mtDNA into normal mtDNA into MCF-7 ρ^0 cells was capable to restore their susceptibility to antiestrogen treatment (Naito *et al.*, 2008b).

Potential Diagnostic Value of mtDNA Copy Number Alterations in Cancer

Examination of mtDNA quantitative levels in peripheral blood extracted from patients has recently emerged as a very promising candidate molecular strategy for predicting cancer propensity of high-risk populations as well as auditing malignant progression with many apparent strengths (e.g. shorter length, simpler organisation and higher abundance of mtDNA molecules relative to nDNA) over the methods based on epigenetic and genetic changes in the nuclear genome (**Table 2**). As exemplified in RCC, low mtDNA quantity in isolated lymphocytes was shown to be relevant with a markedly elevated risk of developing cancer as compared to

those with high mtDNA level (Xing *et al.*, 2008). In addition, a statistically significant dose–response relationship was identified between lower mtDNA content and higher RCC risk in a trend analysis. In a prospective cohort study, Lan *et al.* (2008) assessed mtDNA copy number in whole blood cells of 104 patients with non-Hodgkin lymphoma (NHL) and paired healthy controls. They found that individuals carrying higher mtDNA content tended to have a greater propensity to develop NHL, and the association of mtDNA quantity and NHL risk was particularly obvious for the cases diagnosed with the chronic/small lymphocytic lymphoma subtype. A population-based study of 227 prospectively collected lung cancer cases and matched healthy subjects revealed a significant dose–response relationship between mtDNA copy number and subsequent cancer risk, with a pronounced effect in the highest mtDNA copy number quartile (Hosgood *et al.*, 2010). In consistence with these studies, augmented mtDNA amount in peripheral blood samples was also found to be positively correlated with increased susceptibility to breast cancer, CRC and pancreatic cancer (Shen *et al.*, 2010; Qu *et al.*, 2011; Lynch *et al.*, 2011). Using multiplex real-time PCR assays, Xia *et al.* (2009) demonstrated that mtDNA content in whole blood from stage I breast cancer patients was considerably decreased in comparison with individuals affected by advanced diseases (stage II–IV). The author put forward a notion that low mtDNA quantity at the early stage of breast cancer development could accrue at later phases probably for counteracting impaired respiratory function and thereby tracing peripheral blood mtDNA content may act as a novel molecular marker for tracing tumour progression.

Table 2 Selected case-control studies showing the association between mtDNA quantitative level in peripheral blood and cancer risks

Cancer types	Participants	Ethnic background	mtDNA quantity in peripheral blood	Multivariable OR (95% CI)	References
Breast cancer	103 cases 103 controls	Caucasian + Black	Increase	4.7 (2.5–8.9)	Shen *et al.* (2010)
CRC	320 cases 320 controls	Asian	Increase	2.0 (1.4–2.8)	Qu *et al.* (2011)
Lung cancer	227 cases 227 controls	Caucasian	Increase	2.4 (1.1–5.1)	Hosgood *et al.* (2010)
NHL	104 cases 104 controls	Caucasian	Increase	1.3 (1.1–1.6)	Lan *et al.* (2008)
Pancreatic cancer	203 cases 656 controls	Caucasian	Increase	1.6 (1.0–2.7)	Lynch *et al.* (2011)
RCC	260 cases 281 controls	Caucasian	Decrease	1.5 (1.1–2.2)	Xing *et al.* (2008)

Abbreviations: CI, confidence interval; CRC, colorectal carcinoma; OR, odd ratio; NHL, non-Hodgkin lymphoma; RCC, renal cell carcinoma.

In the past several years, the discovery of circulating cell-free (ccf) mtDNA in the plasma or serum samples of cancer patients has attracted tremendous attentions and ignited great interests in delving its potential applicability for early tumour detection. The level of ccf mtDNA in the plasma of patients with benign and malignant breast cancer was markedly reduced compared to that of the age-match healthy group (Kohler *et al.*, 2009). The receiver operating characteristic curve analysis showed that using plasma ccf mtDNA quantity as a fingerprint can distinguish cancer cases from the cancer-free healthy population. Ellinger *et al.* (2009, 2012) showed that ccf mtDNA copy number was much higher in the serum of patients suffering from prostate and urologic cancers (bladder, testicular and renal cell cancers) than that in healthy volunteers and serum ccf mtDNA content allowed a sensitive and specific discrimination between the two groups. The authors and other groups also noticed similar upregulation of ccf mtDNA content in the serum specimens from patients with epithelial ovarian cancer and EWS relative to the healthy group (Zachariah *et al.*, 2008; Yu *et al.*, 2012). Quantitative analysis of ccf mtDNA content could further be utilised by clinicians to follow patient outcome after treatment. Prostate cancer patients (~70% of the cohort were metastatic diseases) carrying high plasma ccf mtDNA copy number had a significantly shorter disease-specific survival than people with low ccf mtDNA

level (~35% vs. ~73% cumulative survival) (Mehra *et al.*, 2007). Moreover, elevated ccf mtDNA content in the serum of patients with localised prostate cancer has been reported as an independent prognostic factor for predicting biochemical recurrence after prostatectomy (Ellinger *et al.*, 2008). Collectively, notwithstanding that the fundamental mechanism(s) underlying altered ccf mtDNA quantity in peripheral blood of cancer patients has yet to be uncovered at this moment, its potential as a novel noninvasive predictive and prognostic marker for at least some types of solid tumours seems obvious, in particular for patients who present with normal levels of established nuclear markers.

Considering the fact that there is presently no solid approach for setting early cancer screening, monitoring aberrant mtDNA copy number in peripheral blood indeed opens a new window of opportunity for noninvasive evaluation of tumour-derived genetic materials in cancer patients. Once rigorously validated in future studies, tracking mtDNA quantitative changes may effectively supplement existing detection criteria and ultimately enhance the sensitivity and specificity of cancer diagnostics.

Concluding Remarks

At the beginning of the twentieth century, Otto Warburg pointed out that neoplastic cells adapt a unique metabolic machinery by preferentially utilising aerobic glycolysis over oxidative phosphorylation as a major source for energetic and anabolic purposes, even in the presence of sufficient oxygen. He also hypothesised that compromised mitochondrial respiratory function could be the origin of cancer. In light of the irreplaceable role played by mtDNA integrity in governing proper mitochondrial activities, unravelling the detailed implications of mtDNA abnormalities in many aspects of carcinogenesis has already turned into an important task for cancer researchers. The accumulating

evidences outlined in this article illustrate a tight association of mtDNA content change with various clinicopathological factors of cancer patients and strongly suggest an active role of abnormal mtDNA copy number, in addition to frequent mtDNA sequence alterations, in conferring malignant phenotypes at various phases of tumourigenesis through multiple potential mechanisms (**Figure 1**). Continued insights into the functional significance of altered mtDNA quantity in the aetiology of human cancers is hoped to cultivate significant translational merits by aiding in establishing new methodologies for cancer prevention, detection and treatment.

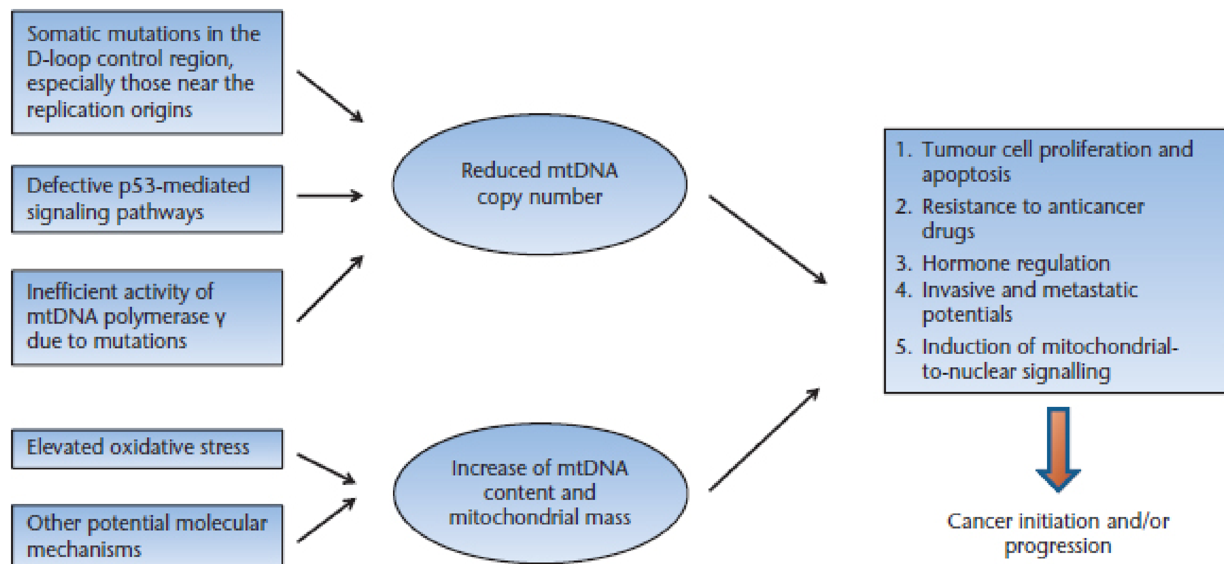

Figure 1 Schematic diagram depicting the molecular mechanisms underlying mtDNA copy number alterations in tumour cells and how they contribute to cancer development.

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