Cell-free DNA in the Circulation as a Non-Invasive Biomarker for Breast Cancer

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Abstract

Breast cancer remains the most common cancer among women worldwide. Although standard screening methods such as clinical breast examination (CBE) and Full-Field Digital Mammography (FFDM) are non-invasive itself, their sensitivity and specificity is limited and they still require biopsy proof after suspicion. Quantitative as well as qualitative changes of circulating cell-free nuclear and mitochondrial DNA (ccf nDNA / ccf mtDNA) have been shown to have potential as biomarker for breast cancer. In the first part of this study, to evaluate the applicability of plasma ccf nDNA and mtDNA quantitative alterations as a biomarker for distinguishing between three study-groups (benign, malignant, healthy), multiplex real-time PCR and ROC (Receiver Operating Characteristic) curve analysis were performed. While the levels of ccf nDNA in the cancer group were significantly higher in comparison with the benign tumor group ($P < 0.001$) and the healthy control group ($P < 0.001$), the level of ccf mtDNA was found to be significantly lower in the two tumor-groups (benign: $P < 0.001$; malignant: $P = 0.022$). Using ROC curve analysis, we were able to distinguish between the breast cancer cases and the healthy controls using ccf nDNA as marker (sensitivity: 81%; specificity: 69%; $P < 0.001$) and between the tumor group and the healthy controls using ccf mtDNA as marker (sensitivity: 53%; specificity: 87%; $P < 0.001$). Our data suggest that both species might have a potential as biomarkers in breast tumour management. However, ccf nDNA seems to be the stronger biomarker regarding sensitivity and specificity. The second part of this work focused on finding breast cancer specific qualitative alterations in ccf nDNA and ccf mtDNA. Therefore MALDI-TOF MS based 40-plex assay was applied to investigate the mutational status of candidate cancer genes (CAN-genes) to evaluate their value as biomarker for diagnostic/therapeutic purposes. No mutations were found in the analyzed cell lines; only one breast cancer patient was found to be heterozygous at one locus within the ZFYVE26 gene which was also confirmed by single-plex assay. Sjöblom et al. / Wood et al. already showed that the vast majority of CAN-genes are mutated at very low frequency. Due to the fact that we only found one mutation in our cohort, we therefore assume that at the selected loci, mutations might be low-frequency events and therefore, more rarely detectable. However, further evaluation of the CAN-gene mutations in larger cohorts should be the aim of further studies. Compared to nDNA, the analysis of mtDNA mutations has some advantages such as higher mtDNA copy numbers and higher mutation rate. Using Sanger sequencing we identified 43 informative mutations within the HVR1 and HRV2 regions in breast cancer tissues; however we did not detect these

mutations in mtDNA of the corresponding plasma samples. This might be due to the fact that the amount of circulating mutant mtDNA is tiny in comparison to background wild-type mtDNA suggesting that more sensitive methods will be needed for this approach to be of clinical utility. Since breast cancer is frequently associated with a decrease in mtDNA content which we also demonstrated in the first part of this work, we finally focused on the molecular mechanisms that are hypothesized to underlie this decrease in mtDNA content. Therefore we investigated the the PGC-1 regulatory network. Reduced mRNA expression of PGC-1α and PGC-1β was not associated with down-regulation of target genes, such as NRF1, TFB1M and TFB2M. POLRMT was downregulated in breast cancer tissues what might be implicated in decrease in mtDNA content. PGC-1β down-regulation was neither correlated with oxidative DNA damage status nor with the expression of genes of the VHL/HIF-1/C-MYC-pathway. We suspect that PGC-1β promoter methylation might be causal for its down-regulation. Summing up, limited sensitivity and specificity can be considered as the bottleneck of using quantitative as well as qualitative alterations as marker. Overall, our data give new insight to the applicability of ccf nDNA and mtDNA as a biomarker for breast cancer and contribute to a better understanding of molecular mechanisms that are suspected to underlie quantitative alterations in breast cancer.

1. GENERAL INTRODUCTION

1.1 Cancer

In western societies cancer is the main cause of death directly following cardiovascular diseases. In 2008 the International Agency for Research on Cancer (IARC) reported 7.6 mi1lion death and 12.4 million incident cases. Estimations indicate that the number of individuals dying from cancer worldwide will exceed 11 million in the year 2030 [1]. Understanding the molecular mechanisms underlying the processes of cancer initiation, progression and metastization could allow for finding new prevention and treatment strategies, thereby reducing incidence and mortality.

In 1902 Theodore Boveri laid the foundation for modern cancer genetics, being the first to propose that cancer is a genetic disease that originates from a single cell which due to abnormal processes acquires chromosomal abnormalities that are passed on to all descendents of the primordial cell [2-3]. The occurrence of abnormalities in the genetic material in normal cells has been suspected to provide a growth advantage leading to their proliferation. As a consequence of genetic instability some proliferating cells might then acquire traits that confer a selective advantage over other cells and thereby permit clonal expansion of a predominant subpopulation [4]. As a consequence unrestrained growth of these cells may then lead to tumor formation.

Although Boveri already postulated the existence and the importance of cell cycle checkpoints, oncogenes and tumor suppressor genes at the beginning of the 20th century, it took many years of research to get closer insight into the cellular mechanisms that drive cells towards malignancy and to finally be aware that carcinogenesis is not the result of a single event but a multistep process, an idea primarily proposed by Nordling in 1953 and revisited by Knudson in 1971 [5-6]. Hanahan and Weinberg recently suggested that all cancers obtain the same functional capabilities during the carcinogenic process. They primarily proposed six hallmarks cells need to acquire in order to be capable of malignant transformation: self-sufficiency in growth signals; insensitivity to growth-inhibitory (antigrowth) signals; evasion of programmed cell death (apoptosis); limitless replicative potential; sustained angiogenesis and tissue invasion and metastasis [7]. Two novel hallmarks, namely reprogramming of energy metabolism and evasion of immune destruction, have evolved during the last decade [8] (Figure 1).

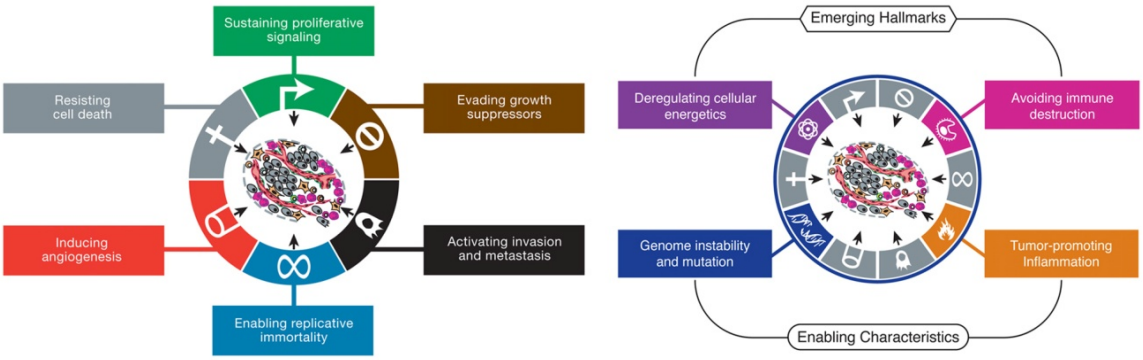

Figure 1: Established and emerging hallmarks of cancer. The original six hallmarks of cancer (left) have been complemented by two novel hallmarks (right) that have emerged during the last years as a consequence of remarkable progress in understanding the carcinogenic process. Figure 1 from references [7-8].

Yet, malignant transformation could not occur without impairment of DNA damage signaling and deficiencies in cellular DNA repair capacity. During the evolutionary process, organisms developed DNA repair systems as protective mechanisms against endogenous (oxidative stress) and exogenous (carcinogens, viruses) threats in order to guarantee the maintenance of genome stability and integrity. Precise DNA repair is favourable for an organism in terms of propagating genetic information precisely from one to the next generation. On the other hand, to certain extend error-prone DNA damage repair allows an organism to easier adapt to physical and biological changes thereby conferring a selective advantage. The latter provides the basis for the expression of a mutator phenotype which in turn may promote the acquisition of the above mentioned hallmarks ultimately inducing malignant transformation [9].

The concept of cancer arising as a consequence of accumulation of mutations, that confer growth advantage to certain cells thereby leading to their clonal expansion and finally to malignant transformation has been questioned within the past years. Instead a theory, the so called cancer stem cell (CSC) hypothesis, which was already proposed 150 years ago, recently regained popularity [10]. It postulates the idea that cancer could arise from a distinct population of cells with tumor-initiating capacity. John Dick revived the hypothesis in 1994 by proving that human acute myeloid leukemia (AML)-initiating cells possess stem cell characteristics, such as the capacity to self-renew and the ability of differentiation. Using a murine model system Dick et al. performed transplantation experiment which revealed that only a subset of AML cells had the potential to initiate leukemia [11]. Since then the CSC

hypothesis has attracted much attention and putative cancer stem cells (CSCs) have been identified in a variety of cancer types.

1.2 Breast cancer

1.2.1 Epidemiology

According to the World Health Organization (WHO) breast cancer is the most common cancer among women worldwide, accounting for nearly half a million deaths in 2008 [12]. Although in the majority of high-income countries breast cancer incidence and mortality rates have been stable or even declined during the last decade due to improved prevention strategies [13], in low- and middle-income countries both, incidence and mortality have risen. A major reason for this development can be attributed to the so called "Westernization" of these countries, which encompasses on the one hand socioeconomic changes that go along with increased life expectancy and access to reproductive control and on the other hand adaption to western habits including dietary changes and decreased exercise [14]. Furthermore access to screening programs in such countries is limited, often leading to breast cancer diagnosis in very late stages [15].

Risk factors of breast cancer are diverse. It has been shown that the probability of getting breast cancer is dependent on sex and increases with age [16], is higher in families with previous history of cancer or families known to be genetically susceptible to breast cancer (e.g. genetic alterations of BRCA1/BRCA2; TP53; ATM)[17-19] , is associated with an increase in certain hormone levels (e.g. estrogen, progesterone)[20] and is linked to dietary habits (e.g. alcohol consumption) [21] and environmental factors (e.g. carcinogens)[22].

1.2.2 Definition

The female breast is located on the anterior thoracic wall with the base extending from the second to the sixth rib; overlying the pectoralis major muscle of the chest, the serratus anterior muscle and the upper portion of the abdominal oblique muscle [23]. The breast is composed of glandular, adipose and connective tissue. The glandular part consists of 15 to 20 lobes each subdivided in several lobules which are again split in clusters of milk-secreting sacs, the so called alveoli. A lactiferous duct drains each lobe and dilates near the nipple forming the lactiferous sinus [24]. Breast cancer arises from the abnormal growth of cells in the breast tissue. It may develop in various parts of the breast, but most frequently breast

cancer originates in the glandular tissue, within the lobes/lobules (milk-producing glands) and the milk ducts.

1.2.3 Classification

Classification of breast cancer is performed according to various criteria such as histopathological characteristics, stage, grade and receptor status.

a. Histopathological classification

According to histopathological appearance two major types can be discriminated, ductal and lobular carcinomas. Ductal carcinomas which originate from epithelial cells in the lumen of the mammary duct are the most common type of breast cancer. According to the grade of invasiveness two major pathological subtypes can be distinguished: ductal carcinoma in situ (DCIS) and invasive ductal carcinoma (IDC). DCIS is the most common type of non-invasive breast cancer, accounting for approximately 20 % of screening-detected cases [25]. It is characterized by abnormal proliferation of epithelial cells which accumulate within the lumen of the mammary ducts without invading tissue beyond the epithelial basement membrane [26]. For this reason DCIS lacks the ability to metastasize to distant organs. Patients suffering from this type of cancer have been shown to have a 10-year survival rate close to 100%, even in the absence of treatment [27]. Nevertheless DCIS if left untreated is considered to be a precursor lesion to IDC; the most common type of invasive breast cancer. In contrast to DCIS, IDC is not confined to the mammary ducts. It invades the basement membrane, infiltrates into adjacent breast stroma and can metastasize to distant organs via the lymphatic system. Lobular carcinomas arise in the mammary lobules and terminal lobular ducts. Like ductal carcinomas lobular carcinomas can be divided into two major pathological subtypes: lobular carcinoma in situ (LCIS) and invasive lobular carcinoma (ILC). While LCIS is confined to the mammary lobules or lobes, ILC is invasive. ILC is the second most common type of invasive breast cancer and accounts for 8-14% of cases [28]. The aforementioned breast cancer types and other less common subtypes have been characterized in detail by the WHO in 2003 [29-30].

b. Classification according to the TNM-system

The TNM-System is the most widely used system for cancer staging world-wide. It is based on the evaluation of the size of the primary tumor (T); the infiltration of lymph nodes (N) and the metastization to distant organs (M). TNM classifications are cancer type specific

and correspond to a certain stage (I-IV). For breast cancer, stage 0 is considered benign and strictly confined. Stages I-IV are defined as malignant: Stage I is characterized by a primary tumor size <2cm without invasion of nodes or distant organs; Stages II/III may possess a tumor size from <2cm to any given size with or without lymph node involvement; Stage IV may have a tumor of any given size, any node status and always is of invasive nature [31].

c. Histological grading

The histological grade of the breast cancer provides information about the differentiation status of tumor cells under the microscope. The prognostic relevance of histological grading in breast cancer has been proven by various studies [32-33]. Several grading systems are used in practice, but most common is the Scarff-Bloom-Richardson grading system in which three different morphological aspects including nuclear grade, tubule formation and mitotic rate are evaluated. Each of the aforementioned features is assigned a score, ranging from 1 to 3 for each feature and ranging from 3 to 9 for all features together. The scores correspond to different grades. While the lowest grade corresponds to well-differentiated tumors, the highest grade stands for a poorly differentiated tumor [34].

d. Classification based on receptor status

Classification according to receptor status including Estrogen receptor (ER), Progesterone receptor (PR) and Her2/neu is important in terms of prognosis and treatment of patients [35]. Based on receptor status, breast cancer patients can be categorized into 3 distinct groups: *i.*) Hormone receptor positive: Approximately 75% of breast cancers are ER and around 65% are also PR positive and therefore responsive to selective modulators. *ii.*) Her2/neu positive: Merely about 20-25% of breast cancers overexpress the Her2/neu protein product. Her2/neu positive patients can undergo herceptin therapy, in which a recombinant monoclonal antibody (Trastuzumab) is directed against the receptor leading to G1 phase arrest and thereby to reduced cell proliferation. *iii.*) Hormone receptor and Her2/neu negative: Triple negative breast cancer occurs in around 10-17% of cases and is usually more aggressive and associated with poorer prognosis due to lack of targeted therapies [36-37].

1.2.4 Pathophysiology

Like other cancers, breast tumorigenesis is driven by multiple factors including molecular changes on the genetic, epigenetic and protein level which may affect key

signalling-pathways involved in cell proliferation and growth, cell-cycle control and repair of DNA damage.

a. Genetic susceptibility

Approximately 5-10% of breast cancers are hereditary in nature. Genetic susceptibility to breast cancer is due to the inheritance of genetic variants of susceptibility genes. *BRCA1* and *BRCA2* are high-penetrance cancer genes and mutations in these genes account for the majority of hereditary breast cancers. *BRCA1/2* mutation carriers are estimated to have an average cumulative risk for breast cancer by the age of 70, of 65% and 45%, respectively [38]. Additionally, alterations in a number of genes within the *p53* regulatory network have been linked to increased breast cancer risk. Germline mutations of *p53* itself are associated with the Li-Fraumeni syndrome, an autosomal dominant cancer predisposition syndrome, characterized by the development of a variety of tumors at early onset age [39]. Association studies revealed that women suffering Li-Fraumeni syndrome primarily have an increased risk for breast cancer [40]. Other susceptibility genes include *ATM* [41], *CHEK2* [42] and *PTEN* [43].

b. Alterations of the p53-signalling pathway

A major signalling pathway that frequently exhibits alterations in cancer is the *p53* regulatory pathway which is induced in response to cellular stress events (DNA damage, hypoxia), securing the maintenance of genome stability and preventing malignant transformation by promoting functions in DNA repair, cell cycle arrest and apoptosis. Although the overall frequency of *p53* mutations in breast cancer is with approximately 20% less than in other cancers, mutations and loss of heterozygosity (LOH) in the *p53* gene are among the most common events in breast cancer [44-46]. In breast cancers lacking *p53* mutations, *p53* is affected indirectly via diverse other mechanisms. Two important upstream factors of the p53 protein, the tumor suppressors phosphatase and tensin homolog (PTEN) and $p14^{ARF}$, which protect p53 from human double minute (HDM2) ubiquitin ligase mediated degradation and thereby allow cells to respond to damage or mutation with an apoptotic response, have been found to be either mutated at a high frequency or completely lost in breast cancer patients [47-48]. In response to DNA damage ATM, another upstream regulator of p53, phosphorylates and thereby stabilizes the p53 protein directly or via a variety of mediators such as BRCA1 and Chk2 [49]. *ATM* heterozygous are known to have an increased risk for developing breast cancer [50-51]. Nevertheless, increasing evidence exists that there

is a role for ATM in sporadic breast cancer as well. Abnormal expression of *ATM* and *p53* and LOH in the region of the *ATM* gene located on chromosome 11q23.1 could be observed in sporadic breast cancer [52-53].

c. Alterations of the Ras/Raf/MEK/ERK and the PI3K/AKT- signalling pathways

Other pathways shown to be commonly altered in breast cancer include the Ras/Raf/MEK/ERK and the PI3K/AKT signalling pathways which regulate a variety of cellular processes, such as cellular growth and proliferation. The Ras/Raf/MEK/ERK cascade is induced by binding of growth factors to cognate receptors, which can lead to activation of Ras. After stimulation by activated Ras, Raf phosphorylates mitogen-activated protein kinase-1 (MEK1), which in turn phosphorylates and thereby activates extracellular-signal-regulated kinases 1 and 2 (ERK1/2) whose targets include genes involved in growth and cell cycle regulation such as p90 ribosomal S6 kinase ($p90^{Rsk-1}$) [54] and p21 cyclin-dependent kinase inhibitory protein-1 ($p21^{Cip1}$). On the other hand activated ERK functions through a feedback loop mechanism phosphorylating and regulating B-Raf, Raf-1 and MEK1. Aberrant activation of the Ras/Raf/MEK/ERK pathway in breast cancer has been linked to overexpression of growth factor receptors such as Her2/-neu and epidermal growth factor receptor (EGFR) [55-56] and to amplification and activating mutations of Ras [57] and Raf [58]. PI3K/AKT pathway is initiated through stimulation of receptor tyrosine kinases (RTKs) by growth factors such as EGFR, Her2/-neu and IGF-1 which activate phosphatidylinositol 3-kinases (PI3Ks). PI3K then triggers the generation of Phosphatidylinositol (3,4,5)-triphosphate (PIP3) in the membrane, which functions as a second messenger and activates downstream pathways that involve Akt and its downstream targets [59]. Deregulation of receptor tyrosine kinases and of PI3K/AKT pathway genes such as activating mutations in Ras [60], in the PI3K gene [61-62], Akt overexpression and inactivation or loss of the PTEN tumor suppressor gene [63] have been reported and implicated in the breast carcinogenic process.

d. Alterations in mitochondrial regulatory pathways

Alterations in mitochondrial regulatory pathways and impairment of mitochondrial function play a central role in cellular pathophysiology of cancer. In 1930 Warburg was the first realizing that cancer cells adapt to the tumor microenvironment by shifting their energy metabolism from oxidative phosphorylation (OXPHOS) to anaerobic glycolysis, known as the Warburg effect [64-65]. He hypothesized that this switch might occur as a result of impaired respiratory capacity. Mitochondria are the place of a variety of processes including

carbohydrate metabolism (TCA cycle), fatty acid oxidation, the urea cycle, gluconeogenesis, regulation of cytosolic NAD+, intracellular homeostasis of inorganic ions, and apoptosis [66]. Nowadays it is known that in cancer cells many of these processes exhibit alterations which are associated with cancer pathogenesis.

Like in other cancers, in breast cancer mtDNA point mutations, deletions and insertions have been observed throughout the mitochondrial genome e.g. in genes coding for OXPHOS complexes, NADH dehydrogenase subunits and 16sRNA [67]. A mutational hot-spot is the D-loop region which contains regulatory elements for mtDNA replication and transcription [68-69]. Mutations, deletions and insertions in the D-loop have thus been linked to decreased expression of mtDNA encoded genes and reduced mtDNA copy number in breast cancer [70]. In this context, especially decrease in abundance and activities of OXPHOS complexes I-V is a frequent event. Owens et al. reported decreased gene expression of OXPHOS complexes in human breast cancer cell lines and primary tumors. They also showed correlation between OXPHOS defects with the severity of breast cancer [71]. Analyzes of protein expression levels β-subunit of F1-ATPase revealed significant down-regulation and expression status of β-F1-ATPase was found to be a significant marker independent from clinical variables to assess the prognosis of breast cancer patients [72]. Alterations of OXPHOS components are often associated with increased generation of ROS. Inhibition of electron flux within the electron transport chain is known to lead to leakage of electrons mainly at complexes I and III; followed by a transfer of these electrons to O_2 and the generation of superoxide. On the one hand, there is evidence that chronically elevated oxidative stress together with other factors such as reduced mitochondrial membrane potential, increased matrix Ca^{2+} levels and mitochondrial permeability transition pore (mtPTP) induction leads to activation of apoptotic pathways; on the other hand ROS promotes carcinogenesis by causing mtDNA mutations, nDNA mutations in proto-oncogenes and tumor-suppressor genes and through driving cellular proliferation [73-74].

A frequent alteration that has been observed in many cancer types including colorectal cancer [75], gastric cancer [76], renal cell carcinoma [77] and breast cancer [70] is the reduction of mtDNA copy number. Reduced mitochondrial DNA copy number has been shown to be correlated with tumor progression and prognosis in breast cancer patients and might therefore serve as biomarker [78]. MtDNA mutations and altered mRNA expression of the genes involved in mitochondrial biogenesis have been suspected to be involved in the reduction of mtDNA content. The network of PGC co-activators are key players in a variety of mitochondria-related processes including mitochondrial biogenesis [79] (Figure 2).

Peroxisome proliferator-activated receptor - γ coactivators PGC-1α and PGC-1β interact with with a wide range of transcription factors, such as ERR α, β, γ and nuclear respiratory factors 1 and 2 (NRF-1/-2) which in turn regulate mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription factor A (TFAM) and the dimethyladenosine transferases 1 and 2 (TFB1M, TFB2M) . Under normal physiological conditions PGC-1 co-activators have an activating function in mitochondrial biogenesis. However, in cancer down-regulation of PGC-1α and PGC-1β has been observed frequently and has therefore been suspected to play a role in reduction of mtDNA content [80-82].

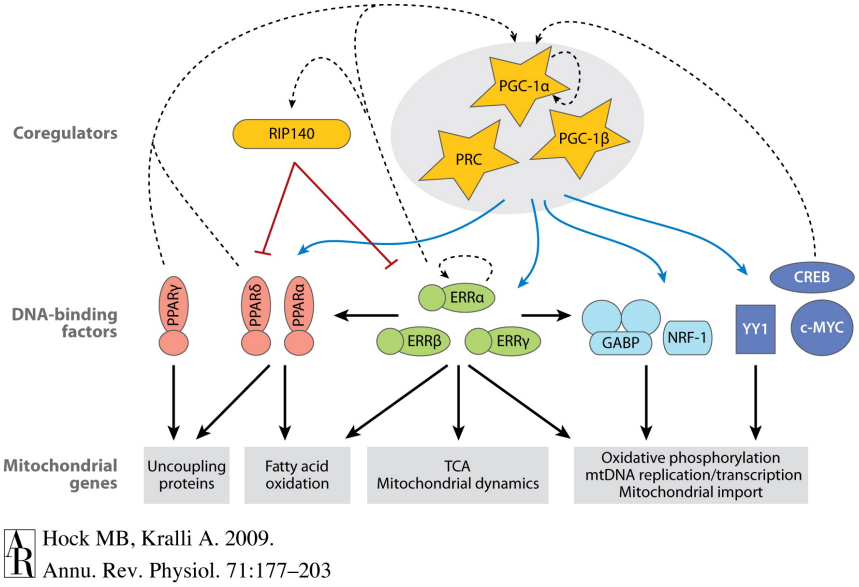

Figure 2: The PGC-coactivator network and its function in control of mitochondrial gene expression. Coactivators PGC-1α, PGC-1β, PRC and RIP140 interact with DNA-binding factors ERRα PPARα, NRF-1 and GABPα to regulate mitochondria-related processes. DNA-binding factors regulate expression of themselves as well as of coregulators and target genes. Abbreviations used: BAT, brown adipose tissue; CREB, cAMP response element–binding protein; ERR, estrogen-related receptor; GABPα, GA-binding protein α; NRF-1, nuclear respiratory factor; PPAR, peroxisome proliferator–activated receptor; PGC-1, PPARγ coactivator-1; PRC, PGC-1-related coactivator; RIP140, receptor-interacting protein 140;WAT, white adipose tissue. Figure 2 from reference [83].

1.3 Cancer Biomarkers

1.3.1 Definition

The Biomarkers Definitions Working Group of the US National Institute of Health's (NIH) defined a biological marker (biomarker) as: "A characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes,

or pharmacologic responses to a therapeutic intervention"[84]. Yet, until now there is no consensus on how the term "cancer biomarker" should be defined. If one considers the functions a biomarker should perform, a cancer biomarker could refer to: A biological entity that: *i.*) indicates the presence of malignancy, *ii.*) reflects the pathophysiological changes during the carcinogenic process or *iii.*) gives information about the response to anticancer therapy.

1.3.2 Classification

Regarding cancer biomarker classification, strategies are diverse. One commonly used classification is performed according to a biomarkers characteristics: Thereby one can distinguish imaging markers such as Positron emission tomography - computed tomography (PET-CT), Full-Field Digital Mammography (FFDM) and Magnetic Resonance Imaging (MRI) and molecular markers which are based on molecules such as DNA, RNA or Protein. Another popular classification scheme is based on the function a biomarker performs; one can for example discriminate between: prognostic (provides information regarding course of the disease/outcome irrespective of therapy), diagnostic (enables classification of individuals as diseased or non-diseased), and predictive biomarkers (assesses response to treatment)[85].

1.3.3 Current biomarkers for breast cancer

At present less than two dozen molecular cancer biomarkers are approved by the US Food and Drug Administration (FDA), among which only five are breast cancer biomarkers such as Cancer-Antigen 15/3 (CA15-3), Cancer-Antigen 27-29 (CA27-29), Cytokeratins, Oestrogen and Progesterone Receptor and HER2/NEU [86]. However, these biomarkers are so far mostly applicable for prognosis, therapy selection and monitoring. For screening and early diagnosis of breast cancer, clinical breast examination (CBE) and imaging markers such as Full-Field Digital Mammography (FFDM) are the standard clinical procedures [87]. Even though all of the above mentioned breast cancer biomarkers are routinely applied in the clinic, they have numerous disadvantages. Firstly, many of them are invasive, as they directly require biopsy or at least biopsy proof after suspicion, which goes along with physical and psychological stress of the patients. Furthermore, especially for screening methods, sensitivity and specificity are quite limited. For women under the age of forty CBE is the screening method of choice; women over forty years of age are usually screened with a combination of CBE and mammography. A meta-analysis study by Barton et. al estimated overall CBE sensitivity at about 54% [88]. For mammography sensitivity has been reported to range from

85% to 90% in women over fifty years of age and has been shown to decrease to about 75% in women between the age of forty and fifty [89]. Regarding the false-positive detection rate, a ten-year retrospective cohort study estimated a cumulative risk of false positive detection up to 49.1 % for mammography and up to 22.3 % for CBE, considering annually screening [90]. This indicates the need for more sensitive and specific non-invasive biomarkers for breast cancer in particular for screening and early detection.

1.4 Circulating cell-free nucleic acids as a potential cancer biomarker

In the past years circulating cell-free (ccf) nucleic acids such as nuclear and mitochondrial DNA (nDNA /mtDNA), RNA and micro RNAs (miRs) have attracted much attention within the scientific community and their potential value as cancer biomarker has been researched widely [91-92].

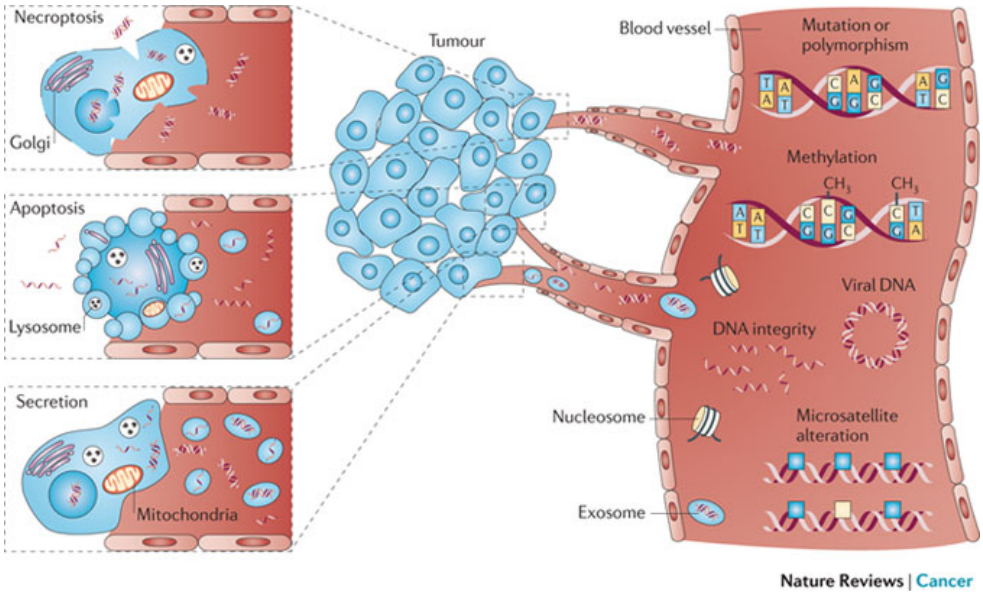

Figure 3. Cell-free nucleic acids in the circulation. Proposed release mechanisms of nucleic acids into the circulation by apoptosis, necrosis or active secretion (left)**.** Quantitative (nucleic acid content) and qualitative (mutations, methylation, DNA integrity, microsatellite instability) alterations detectable in the circulation (right). Figure 3 from reference [91].

1.4.1 A brief history of ccf-nucleic acids

In the first half of the 20th century, Griffith and Avery proved that DNA contains the hereditary information. Although at that time the underlying mechanisms of the transforming principle were not clear, nowadays we know that it is based on the fact that nucleic acids can exist in a free form in circulation and bacteria can take up foreign DNA from the environment

[93-94]. In 1948 Mandel and Metais for the first time detected ccf- nucleic acids in plasma of human beings [95]. A link between pathophysiological conditions including systemic lupus erythematosus, rheumatoid arthritis, leukemia, and other diseases and the amount of ccf-nucleic acids could be made some years later [96-97]. The fact that there is an elevated ccf-DNA concentration in the serum of cancer patients compared to normal controls was first observed in the late 70´s by Leon et al. [98]. Since then the biological characteristics, physiological/pathophysiological behaviour and the potential of quantitative and qualitative alterations of ccf-DNA as biomarker for cancer have been investigated widely.

1.4.2 Biological characteristics and origin of ccf-DNA

Ccf-nucleic acids refer to extracellular DNA (nuclear and mitochondrial), RNA and miRNA fragments floating in fluids of the human body. While in healthy individuals concentrations range between 0 to around 100 ng/mL, in cancer patients levels between 0 to > 1000 ng/mL have been reported [99-100]. It has been estimated that in patients with a tumor of 100 g in size ($\approx 3 \times 10^{10}$ neoplastic cells), 3.3% of the tumor DNA is shed into the circulation daily [101]. However, this amount is usually not detectable since ccf-nucleic acid concentration is influenced by a variety of physiological mechanisms. Ccf-nucleic acids are usually cleared quite rapidly from the circulation with indicated half-lifes ranging from few minutes to several hours [102-103]. It is supposed that clearance of ccf-nucleic acids mainly takes place within liver, spleen, and kidney [104-105].

Since the discovery of ccf-DNA the question regarding its origin remained more or less unanswered. There have been diverse studies pursuing this issue, mainly focusing on active secretion, apoptosis and necrosis. Earlier research proved that cells are able to actively release nucleic acids into the circulation [106-108] (Figure 3). However, growing evidence indicates that the major source of ccf-DNA is apoptosis and necrosis. It is supposed that as a consequence of high cell turnover at tumor site which is accompanied by apoptosis and/or necrosis, intracellular material is released from the tumor into the circulation and can thus be detected in blood of cancer patients [109]. Apoptosis and necrosis are morphologically and biochemically distinct. Apoptosis is characterised by cell shrinkage, chromatin condensation and DNA fragmentation which is caused by specific endonucleases, cleaving the DNA into nucleosomal units with about 180-200 bp. In contrast to apoptosis DNA fragments which result as a consequence of necrosis are much larger [107]. Earlier analyzes of ccf-DNA using electron microscopy and sucrose-gradient sedimentation revealed fragment sizes between 500bp and >30 kb indicating necrotic as well as apoptotic fragments [110-111]. Using other

methods, some authors mainly detected small ccf-DNA fragments indicating an apoptotic origin [112]; others mostly reported large ccf-DNA fragments of primarily necrotic origin [101]. Jahr et al. found some evidence that both, apoptotic and necrotic tumor cells could be the source of ccf-DNA [113].

Irrespective of its release mechanisms, the fact that elevated levels of ccf-DNA have been found in patients with different cancer types and the fact that this ccf-DNA features the same alterations than the corresponding tumor DNA demonstrates that at least a not inconsiderable amount of ccf-DNA is of tumoral origin. For this reason both, changes in the level of ccf-DNA and tumor-associated alterations might have a potential as biomarker for the disease.

1.4.3 Quantitative and qualitative alterations in breast cancer

Quantitative as well as qualitative changes of ccf-DNA (ccf-nDNA and ccf-mtDNA) have been reported in patients with malignant and benign lesions in comparison with healthy individuals in various cancer types [114-119].

Breast cancer studies on quantitative alterations were mostly performed using plasma and serum. In breast cancer patients plasma mean ccf-DNA levels ranged between 115 ng/mL to 462 ng/mL; in serum mean ccf-DNA levels between 153ng/mL to 549 ng/mL could be observed [120]. Various studies evaluated the potential of ccf-DNA levels as diagnostic or prognostic marker for breast cancer. In our study we could distinguish between malignant, benign and healthy individuals using levels of plasma ccf-nuclear and mitochondrial DNA; although sensitivity and specificity was limited [115] . A potential as diagnostic marker was also confirmed by other groups [114, 121]. Prospective analyzes of plasma tumor DNA at diagnosis in breast cancer patients showed that ccf-DNA is also a valuable predictor of disease-free survival and may be used as a prognostic factor in these patients [122-123].

Studies in breast cancer patients determining the concordance of tumor-specific DNA alterations between the primary tumor and blood mainly focused on mutations in oncogenes and tumor-suppressor genes, on methylation status and microsatellite alterations. Mutations in the p53 gene within exons 5 to 8 have been abundantly detected in tissue of primary tumor and in several cases could be also identified in the corresponding plasma [123-125]. Shao et al. showed that primary tumor and plasma DNA p53 mutations could be used as significant prognostic factors for both relapse-free and overall survival [126]. Methylation status of a variety of genes in breast tumor tissue and paired plasma or serum was also assessed in a subset of studies. Major analyzes were done for *P16INK4A, RASSF1A, APC* and *BRCA1* [127-129]. Fiegl et al. were able to use *RASSF1A* methylation patterns to monitor efficacy of

adjuvant systemic breast cancer treatment. Disappearance of *RASSF1A* DNA methylation in serum throughout treatment with tamoxifen indicated a response, whereas persistence or new appearance showed resistance to adjuvant tamoxifen treatment [130. Müller et al. used methylation status to assess the value of a set of genes (ESR1, APC, HSD17B4, HIC1, and RASSF1A) as prognostic markers. Patients with methylated serum DNA for RASSF1A and/or APC were found to have the worst prognosis [131]. Microsatellite alterations including allelic imbalance and LOH were analyzed as well. In many cases these markers were detectable in tumor tissue and circulation [132-133]. Silva et al. prospectively examined tumor and plasma DNA of a total of 147 breast cancer patients using six polymorphic markers (D17S855, D17S654, D16S421, TH(2), D10S197, and D9S161) and mutations in the TP53 gene and demonstrated that their detection in tumor plasma DNA was a predictor of disease-free survival [122]. The potential of both, quantitative and qualitative changes, as a biomarker for cancer management in general is discussed in our review [134].

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2. AIM OF THE WORK

Quantitative alterations of ccf-nDNA and mtDNA as a breast cancer biomarker

The discovery that tumors are capable of shedding cancer-specific nucleic acids (DNA, RNA, miRs) into the circulation has opened up new avenues for using these specimens as non-invasive biomarker for cancer management. Alterations in the level of cell-free nucleic acids in the circulation have been observed in malignant and benign in comparison to healthy individuals in a variety of cancers and have therefore been suggested as biomarker for the disease. In the present study, to evaluate the applicability of such quantitative changes as a biomarker for breast cancer we raised the following questions: (1) Is there a difference in the amount of plasma ccf- nuclear and mitochondrial DNA between cancerous, malignant and healthy subjects? (2) If yes, could such quantitative alterations have a value as a non-invasive biomarker to distinguish between these conditions?

Qualitative alterations as breast cancer biomarkers

Various types of qualitative alterations have been observed in circulating nucleic acids and in many cases these alterations were also present in the primary tumor tissue of the patient. Such cancer-specific qualitative alterations have been proposed as a potential diagnostic tool. Unfortunately at present, no universal cancer-specific qualitative alterations neither for the disease cancer in general nor for special cancer types are available. The identification of qualitative alterations specific for breast cancer could permit the development of a highly sensitive and specific clinical approach for the management of the disease. As in our and in various other studies quantitative alterations of plasma ccf-nDNA and mtDNA showed a limited value as a biomarker due to too low sensitivity and specificity, we therefore focused on finding breast cancer specific qualitative alterations. In this context we developed a MALDI-TOF MS 40-plex assay for the detection of nuclear DNA mutations within breast cancer candidate genes (CAN-genes) with the aim to evaluate the value of these CAN-genes as a biomarker for breast cancer. Additionally, we sequenced the hypervariable regions within the mtDNA D-loop in 10 breast cancer patients and healthy controls with the aim to determine somatic mtDNA mutations within the D-loop region in breast cancer tissues and to evaluate whether it is possible to detect these mutations in the matched plasma samples.

Molecular mechanisms underlying decreased mtDNA content in breast cancer patients

In 1926 Otto Warburg set up the theory that cancer is a disease resulting from impaired respiration caused by mitochondrial dysfunction. Since then various factors have been suspected to contribute to mitochondrial dysfunction and reduced mtDNA content in cancer including mtDNA mutations, deregulation of pathways involved in mitochondrial bioenergetics and biogenesis and increased generation of ROS. Since in our first study we found decreased mtDNA content in the plasma of patients with breast cancer in comparison with healthy controls, in the last part of this work we focused on investigating the molecular mechanisms that are hypothesized to underlie a decrease in mtDNA content in breast cancer patients. The first part aimed to investigate the intergenomic cross-talk between reduced mtDNA content, mtDNA mutations and methylation status of breast cancer related genes. In a second part we analyzed the PGC-1 regulatory network to evaluate its implication in the regulation of mtDNA content in cancer patients and healthy individuals.

3. PUBLICATIONS, MANUSCRIPTS AND PRELIMINARY DATA

3.1. Published review article:

Cell-free DNA in the Circulation as a Potential Cancer Biomarker

Authors: Corina Kohler*, Zeinab Barekati, Ramin Radpour and Xiao Yan Zhong

Journal: Anticancer Res. 2011 Aug; 31(8):2623-8.

Summary: Quantitative and qualitative changes of circulating cell-free nucleic acids (DNA and RNA) in bodily fluids have been suggested as a potential biomarker for a variety of cancers. However, to be applied in a clinical setting several obstacles, such as limited sensitivity and specificity have to be overcome. This review focuses on evaluating the potential of both, quantitative and qualitative changes, as a biomarker for cancer management.

***Author contributions:** *Corina Kohler* wrote the manuscript.

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Review

Cell-free DNA in the Circulation as a Potential Cancer Biomarker

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Abstract. *In the course of the search for new biomarkers, circulating cell-free DNA (ccf-DNA) has become a popular target of interest. An elevated level of ccf-DNA has been detected in the circulation of cancer patients in comparison with healthy controls. Since ccf-DNA in cancer patients often bears similar genetic and epigenetic features to the related tumor DNA, there is evidence that some of the ccf-DNA originates from tumoral tissue. This, and the fact that ccf-DNA can easily be isolated from the circulation and other body fluids of patients, makes it a promising candidate as a non-invasive biomarker of cancer. Yet ccf-DNA-based cancer tests have not come to fruitful clinical applications. This review evaluates the potential of ccf-DNA alterations as a biomarker for cancer management by addressing the question of how large the gap between ccf-DNA and the ideal cancer biomarker is.*

Worldwide, cancer is the third most common cause of death, directly following cardiovascular diseases and infectious parasitic diseases. According to data published by the World Health Organisation (WHO) in the world cancer report, cancer deaths will increase dramatically in the forthcoming years. Whereas there were 7.4 million cancer deaths in 2004, it has been estimated that in 2030, there will be 11.8 million dying as a consequence of cancer (1).

During the last few years, much research has been carried out to find new cancer biomarkers with the aim of reducing cancer mortality. However, most of the cancer biomarkers currently available are not sensitive or specific enough to be applied in routine clinical approaches. Additionally, for many cancer types, such as lung or breast cancer, invasive procedures are still necessary to obtain material for pathological analyses. To simplify cancer management, much effort has been made in the search for biomarkers which allow non-invasive assessment, screening, disease classification and monitoring.

Circulating cell-free DNA (ccf-DNA) represents such a non-invasive biomarker, as it can easily be isolated from human plasma, serum and other body fluids (2). Mandel and Métais demonstrated the existence of ccf-DNA in human plasma as early as 1947 (3). The fact that there is an elevated level of ccf-DNA in the circulation of cancer patients in comparison with healthy controls was primarily discovered 30 years later by Leon *et al.* (4) and was confirmed in numerous studies (5-7). After the findings by Leon *et al.*, it took more than a decade until it was shown that ccf-DNA often exhibits the same alterations as DNA derived from related tumoral tissue (8). A huge variety of alterations, such as mutations in oncogenes and tumor suppressor genes (9), microsatellite variances (10), and epigenetic alterations, such as promoter hypermethylation (11), have since been reported and plenty of studies have been conducted investigating the potential of ccf-DNA as a non-invasive diagnostic tool for cancer management (12, 13).

Although ccf-DNA was discovered more than half a century ago, ccf-DNA-based cancer tests have not yet been developed for clinical application. The main progress has been observed in the field of prenatal medicine, where ccf-DNA has been successfully used for fetal Rhesus D genotyping (14) and for the detection of paternally inherited genetic disorders (15) from maternal plasma and serum. However, the applicability of ccf-DNA as a biomarker for cancer management still needs extensive evaluation. This review discusses the potential for commonly analyzed ccf-DNA alterations to be used as biomarkers and compares their characteristics with that of the ideal cancer biomarker.

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Alterations of ccf-DNA and their Potential for Use as Cancer Biomarkers

Quantitative alterations in ccf-DNA. The fact that there is an elevated ccf-DNA concentration in the serum of cancer patients when compared with healthy controls was first observed in the late 1970s using radioimmunoassay (4). Although elevated levels of ccf-DNA in serum and plasma were detected in various studies and in many cancer types, interpretation of such results requires special consideration. Firstly, a certain level of ccf-DNA can also be observed in healthy individuals. Therefore, it is crucial to gain closer insight into the mechanisms that lead to ccf-DNA release and to establish a baseline that allows a reproducible discrimination between healthy and diseased individuals. In addition, it has been shown that there are various parameters influencing ccf-DNA levels, such as sample preparation (16) and speed and effectiveness of clearance of ccf-DNA from the circulation (17). Finally, an elevated level of ccf-DNA was found not only in the circulation of cancer patients, but also in patients with other physiological conditions, such as myocardial infarction (18), physical trauma (19) and inflammatory disorders (20), which makes it difficult to evaluate the extent to which ccf-DNA in the circulation of a patient is cancer specific. Nevertheless, research on how qualitative ccf-DNA changes could be used in a clinical approach is ongoing, as there are promising results for the use of ccf-DNA content in combination with other well-known tumor markers (21).

Qualitative alterations in ccf-DNA. Cancer is a disease mainly caused by the accumulation of genetic and epigenetic alterations and both have been observed in DNA isolated from tumoral tissue and in the corresponding ccf-DNA (22). In recent years, the research in this field has mainly been driven by these findings, since they promise to provide a basis for the development of new approaches in which cancer-specific ccf-DNA alterations may be used as a non-invasive biomarker.

Mutations in oncogenes and tumor suppressor genes. The regulation of cell proliferation, cell differentiation and apoptosis is subjected to special control mechanisms that keep these processes in balance and thereby allow a regular course of the cell cycle. Such mechanisms are controlled by those genes that are involved in the regulation of the cell cycle, specifically DNA repair genes, tumor suppressor genes and diverse growth-regulating genes. Genetic alterations in these genes can cause disturbance of the cell cycle, which often results in cell transformation and thereby promotes carcinogenesis (23).

Many groups have tried to discover mutations in such genes in cancerous tissues and confirm them in corresponding plasma or serum ccf-DNA. Analyses were mainly carried out for well-known proto-oncogenes such as *KRAS*, and tumor suppressor genes, such as *TP53* and *APC* (9). Garcia *et al.* detected mutations in exons 5-8 of the *TP53* gene in 73.2% of patients suffering from primary breast carcinoma. Among these, 42.9% exhibited molecular changes in plasma DNA (24). Another study which evaluated the same *TP53* exons detected *TP53* mutations in 36% of breast cancer patients and 65.1% of these patients also had mutations in their plasma DNA (25). *TP53* mutations in cancerous tissue and corresponding plasma were also found in other types of cancer including colorectal and ovarian cancer (26, 27).

Several studies also confirmed the presence of *KRAS* mutations in tissue and in ccf-DNA of patients. In pancreatic carcinoma patients, the *KRAS* gene was verifiably found to be mutated in 80% to 90% of the cases. Castells *et al.* showed that these mutations were also present in 27% of plasma DNA samples of patients with pancreatic ductal adenocarcinoma (28). Kopreski *et al.* determined *KRAS* mutations in colorectal cancer patients: 83% of the patients in whom *KRAS* mutations were found in the cancerous tissue also had mutations in their plasma ccf-DNA. However, in this study, *KRAS* mutations were also found in the plasma of some patients that had high risk factors but did not test positively for colorectal cancer (29). To increase the sensitivity, some groups therefore studied *KRAS* mutations and tumor marker levels simultaneously. As the detection of mutations in ccf-DNA is completely independent of the results obtained by using a common tumor marker, such a combined detection has the advantage of having a much higher sensitivity. Combining the detection of *KRAS* mutations in ccf-DNA and the analyses of the tumor marker CA19-9, *KRAS* mutations were found in 70.7% of patients with pancreatic carcinoma, while no *KRAS* mutation was detected in healthy controls. Additionally, the level of CA19-9 was elevated in 73.2% of pancreatic carcinoma patients. In total, 90.2% of the patients tested positively (30).

Microsatellite alterations. Beside mutations in oncogenes and tumor suppressor genes, microsatellite alterations, such as microsatellite instability (MSI), and loss of heterozygosity (LOH) have been observed for ccf-DNA as well (10, 31). Microsatellites are highly polymorphic repetitive sequences, consisting of multi-nucleotide repeats. As a consequence of mutations in DNA repair genes, some microsatellites show abnormal length referred to as MSI. LOH occurs when, due to mutations, the normal function of one allele is lost. In cancer, such LOH is often found in tumor suppressor genes, which most likely contributes to neoplastic transformation. As MSI and LOH are both suggested to play a fundamental role in carcinogenesis, numerous studies have tried to find such alterations in ccf-DNA. Testing a panel of 12 microsatellite markers, Beau-Faller and colleagues found

alterations in 88% of the plasma samples of lung cancer patients, whereas all control samples were negative for such changes (32). Using two markers, one to detect MSI (D21S1245) and another one to find LOH (*FHIT* locus) microsatellite alterations were observed in 56% of non-small cell lung cancer tumors and in 40% of the related plasma samples (33). Goessl *et al*. conducted a study to identify microsatellite alterations in renal malignancies. In 80% of all renal malignancies, a deletion of DNA sequences on chromosome 3p which led to LOH occurred. By the use of several highly polymorphic microsatellite markers spanning the chromosomal region between 3p26 and 3pl4, they demonstrated that there is LOH in one locus in 63% and in more than one locus in 35% of plasma samples of cancer patients (34).

Epigenetic alterations. The epigenetic code bears information additional to that of the genetic code. Epigenetic modifications are known to play a role in many cellular processes, including chromatin remodeling, imprinting, gene silencing, X chromosome inactivation and carcinogenesis (35). The best examined epigenetic modification doubtless is that of DNA methylation. In cancer, aberrant DNA methylation is often found in the promoter region or at regulatory sites of genes which are involved in cell cycle regulation, growth or apoptosis (36). While promoter *hyper*methylation of tumor suppressor genes results in gene silencing, promoter *hypo*methylation of proto-oncogenes can lead to gene activation (37). Concordant methylation patterns in tissue of primary tumors and corresponding plasma or serum have been found for a huge number of genes and various types of cancer including breast (38), ovarian (39), cervical (40), and lung cancer (41). However, the methylation pattern seems to be subject to factors such as age and gender. Previous studies on monozygotic and dizygotic twins revealed divergent methylation patterns with increasing age (42) and between genders (43). Additionally, there is no defined methylation signature for healthy individuals, which makes it difficult to really determine cancer-specific methylation patterns.

Ccf-DNA – The Ideal Cancer Biomarker?

In the course of technological and medical progress, the demand for biomarkers has increased enormously during the last decade. According to the FDA, the ideal cancer biomarker should meet multiple requirements in order to make it attractive for routine clinical use (44):

(a) The first premise is the direct association of the biomarker with the disease in general or at least with a specific disease state. Regarding the direct association of ccf-DNA with cancer, it should be mentioned that although significantly elevated levels of ccf-DNA have been found in many studies and in patients with several cancer types, one has to consider that an undefined part of DNA present in the circulation is of non-tumoral origin and is thus not directly associated with the disease.

Considering qualitative alterations in ccf-DNA, it is obvious that at present, there are no cancer-specific alterations that show high enough sensitivity or specificity to be used as a marker in clinical applications. Therefore, the first hurdle that has to be overcome is to find cancer-specific genetic and epigenetic alterations which are not present in non-cancer-derived ccf-DNA. The question as to which alterations should be targeted for an approach is difficult to answer. Epigenetic alterations are highly frequent in cancer but their disadvantage is that, for example, methylation patterns are quite heterogenous between different cancer and tissue types and individuals (45). Genomic mutations seem to be excellent candidates, as they can be easily analyzed by high-throughput multiplex approaches. However, finding such cancer-specific mutations seems to be the critical point. Firstly, the mutational background of cancer is enormously complex, which makes it quite difficult to find cancer-specific mutations. Secondly, each cancer type probably possesses cancer-type specific mutations (46) which would limit the marker to a certain cancer type.

(b) The ideal cancer biomarker preferably should cover the whole continuum or at least a part of the cancer management process ranging from the assessment of predisposition to monitoring of disease recurrence (47). Although there are no clinical applications to date, it is undeniable that ccf-DNA has a huge potential as a cancer biomarker. Cancer specific ccf-DNA alterations theoretically could be implemented for the whole continuum of cancer management. As a risk assessment marker, a level could be set for the assessment of a probable cancer risk, enabling clinicians to take appropriate provisions before the onset of the disease. For cancer screening in asymptomatic patients, ccf-DNA alterations could be used to detect cancer at the earliest stage possible, thereby improving outcome. Determining the frequency of aberrant methylation of four candidate genes, adenomatous polyposis coli (*APC*), glutathione *S*-transferase P (*GSTP1*), ras association domain family 1 isoform A (*RASSF1A*), and retinoic acid receptor, beta 2 (*RARB2*) in the plasma of women with breast cancer, Hoque *et al*. was able to successfully detect 33% of early-stage tumors (48). Ccf-DNA may also be usable for categorization of disease stages, as was shown by Fujiwara *et al*., who demonstrated a significant correlation of a combination of plasma LOH microsatellite markers with progression of different clinical stages of disease in melanoma patients (49). As a prognostic marker, ccf-DNA alterations could be helpful in stratifying patients for treatment. Müller *et al*. evaluated several prognostic DNA methylation markers

in the serum of cervical and breast cancer patients of which two, *APC* and *RASSF1A*, proved to be independent prognostic parameters in breast cancer patients (40). Finally, as a marker of recurrence, ccf-DNA alterations could facilitate assessment for disease recurrence in individuals who previously suffered from cancer. It was shown that plasma tumor DNA levels are significantly higher in patients with colorectal cancer, and that there is a progressive decrease in the follow-up period in tumor-free patients, and increase in patients with recurrence or metastasis (50).

(c) In all stages the ideal biomarker should provide 100% sensitivity and 100% specificity. For several reasons, quantitative and qualitative ccf-DNA alterations seem to provide too low a sensitivity and specificity to reliably discriminate between cancerous and healthy individuals, as has been shown by several studies (28, 51, 52). Firstly, ccf-DNA is also present in the circulation of healthy individuals and the physiological factors which influence the levels of ccf-DNA are relatively unknown, which makes it difficult to establish a clear baseline and to interpret the results in a correct way. Secondly, even though mutations can be detected in cancerous tissue and the corresponding serum or plasma, some studies found mutations in healthy individuals as well, increasing the probability of false-positive detection (29). Nevertheless, a possibility to overcome low sensitivity and specificity may be the use of combined measurement of ccf-DNA alterations with common tumor markers. Various reports reported an improved sensitivity and specificity for a combined use of quantitative, as well as qualitative ccf-DNA alterations with well-known markers such as prostate-specific antigen (53), carcino embryonic antigen (21) and CA19-9 (30).

(d) On behalf of the user, as well as of the patient, the biomarker should be as non-invasive as possible. Unfortunately at present, many diagnostic tests for cancer require biopsy-proof for confirmation. Even though there is no routine ccf-DNA-based cancer test, the fact that ccf-DNA can easily be obtained from the patient by extraction from a simple blood (serum/plasma) or urine sample would make it an ideal non-invasive biomarker. Its non-invasive nature not only brings the advantage of easy access to the specimen for the clinician, but at the same time also a reduction of the physical and psychological stress to the patient.

(e) Since economical aspects gain importance when selecting clinical tools, the cost benefit of the biomarker should be reasonable. The major advantage of the use of ccf-DNA is its ease of access, which in comparison to biopsies, is concomitant with an enormous reduction of cost. However, for quantitative analysis, costs would be dependent on the method of choice and a reasonable cost benefit should be considered.

Conclusion

Considering all aspects, ccf-DNA seems only partially to meet the attributes that characterize the ideal cancer biomarker. Even though quantitative as well as qualitative ccf-DNA alterations are to a certain extent associated with cancer, one has to realize that at present none of these alterations can be considered absolutely cancer-specific and that the low sensitivity and specificity of known alterations do not allow use in a clinical setting. However, the attractiveness of using ccf-DNA as a biomarker lies in its non-invasive nature and a combined use with common already established tumor markers could be the first step to a clinical approach to its use.

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Levels of Plasma Circulating Cell Free Nuclear and Mitochondrial DNA as Potential Biomarkers for Breast Tumors

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Summary: Quantitative changes of circulating cell-free (ccf) nuclear and mitochondrial DNA (nDNA / mtDNA) have been shown to have potential as biomarker for breast cancer. Multiplex real-time PCR was used to investigate the levels of ccf nDNA and mtDNA in plasma samples from patients with malignant and benign breast tumors, and from healthy controls. To evaluate the applicability of plasma ccf nDNA and mtDNA as a biomarker for distinguishing between the three study-groups, ROC (Receiver Operating Characteristic) curve analysis were performed. Our data suggest that both species might have a potential as biomarkers in breast tumour management. However, ccf nDNA seems to be the stronger biomarker regarding sensitivity and specificity.

***Author contributions:** *Corina Kohler* was involved in experimental design, performing the experiment, data analysis and writing of the manuscript.

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Levels of plasma circulating cell free nuclear and mitochondrial DNA as potential biomarkers for breast tumors

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Abstract

Background: With the aim to simplify cancer management, cancer research lately dedicated itself more and more to discover and develop non-invasive biomarkers. In this connection, circulating cell-free DNA (ccf DNA) seems to be a promising candidate. Altered levels of ccf nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have been found in several cancer types and might have a diagnostic value.

Methods: Using multiplex real-time PCR we investigated the levels of ccf nDNA and mtDNA in plasma samples from patients with malignant and benign breast tumors, and from healthy controls. To evaluate the applicability of plasma ccf nDNA and mtDNA as a biomarker for distinguishing between the three study-groups we performed ROC (Receiver Operating Characteristic) curve analysis. We also compared the levels of both species in the cancer group with clinicopathological parameters.

Results: While the levels of ccf nDNA in the cancer group were significantly higher in comparison with the benign tumor group ($P < 0.001$) and the healthy control group ($P < 0.001$), the level of ccf mtDNA was found to be significantly lower in the two tumor-groups (benign: $P < 0.001$; malignant: $P = 0.022$). The level of ccf nDNA was also associated with tumor-size (<2 cm vs. >2 cm<5 cm; 2250 vs. 6658; Mann-Whitney-U-Test: $P = 0.034$). Using ROC curve analysis, we were able to distinguish between the breast cancer cases and the healthy controls using ccf nDNA as marker (cut-off: 1866 GE/ml; sensitivity: 81%; specificity: 69%; $P < 0.001$) and between the tumor group and the healthy controls using ccf mtDNA as marker (cut-off: 463282 GE/ml; sensitivity: 53%; specificity: 87%; $P < 0.001$).

Conclusion: Our data suggests that nuclear and mitochondrial ccf DNA have potential as biomarkers in breast tumor management. However, ccf nDNA shows greater promise regarding sensitivity and specificity.

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Introduction

In several branches of biomedical research the quest for new disease-related biomarkers has become one of the main objectives [1-3]. When it comes to discover and develop new biomarkers, oncology seems to be the most ambitious field. During the last few years a lot of research has been done identifying new cancer biomarkers with the aim to identify high risk individuals, detect cancer at an early stage, predict outcome, monitor treatment and screen for disease recurrence [4]. In this respect the focus is now mainly directed towards the identification of non-invasive cancer biomarkers [5,6].

In the case of breast cancer, there are only a few non-invasive biomarkers for screening, predicting prognosis and monitoring that have come to routine clinical application [7]. Current established methods for routine breast cancer screening firstly encompass non-invasive methods including clinical breast examination and imaging techniques like mammography and ultrasonography [8]. However, when pathological changes are suspected these techniques generally have to be followed by histopathological analysis for which invasive procedures, such as biopsies, are needed.

Lately, the discovery of circulating cell-free DNA (ccf DNA) has sparked the interest of scientists as it opens up a new possibility for non-invasive analysis of tumor derived genetic material. Both ccf nuclear DNA (nDNA) and mitochondrial DNA (mtDNA) have become a matter of investigation and qualitative as well as quantitative alterations in these two determinants have been implicated in cancer [9]. Changes in the level of ccf nDNA and mtDNA have been found in plasma and serum of patients with various cancer types [10,11]. In breast cancer patients it has been shown that ccf nDNA levels are elevated in plasma as well as in serum when compared to healthy controls [12,13]. On the other hand, mtDNA levels were mostly found to be decreased in breast cancer patients in comparison to healthy controls [14,15].

To investigate the potential of ccf nuclear and mitochondrial DNA as a marker for clinical application we examined the level of both species in malignant and benign tumor groups and healthy controls.

Materials and methods

The study was performed at the Laboratory for Prenatal Medicine and Gynecological Oncology/Department of Biomedicine, Women's Hospital Basel and approved by the local institutional review board (Ethic commission beider Basel). Written consent forms were collected from all patients who were involved in this study.

Study cohort and sampling procedure

The blood samples used in this study were collected in a time period from 2005 to 2007 in either the Women's Hospital of the University of Basel or the Women's Hospital of Liestal. In total 148 women were included in the study. Most of the women were European Caucasians. All blood samples were taken before any surgical interventions or therapeutic treatments. Patients' data (age, tumor size, lymph node involvement, extent of metastasis, estrogen receptor, progesterone receptor and Her2neu - status) were obtained from the pathological reports. The blood samples were processed and the DNA was extracted according to a standardized protocol as previously described elsewhere [16]. DNA was quantified using a Nanodrop spectrophotometer (Thermo scientific).

The study cohort (n = 148) was divided into 3 groups: 1) malignant disease group (n = 52); 2) benign disease group (n = 26) and 3) healthy control group (n = 70). For groups 1 and 2 the diagnoses were all biopsy-confirmed. The healthy control group used in this study neither had a history of cancer nor suffered from any other severe diseases.

qPCR

For the simultaneous quantification of ccf nDNA and mtDNA from plasma a multiplex qPCR was performed using the Glyceraldehyd-3-phosphat-dehydrogenase (*GAPDH*) and the mtDNA encoded ATPase 8 (*MTATP 8*) reference genes.

The gene IDs, the amplicon length, the annealing temperature and the sequence information of primers and probes for the *GAPDH* and the *MTATP 8* reference genes are shown in table 1.

qPCR was carried out in 25 μl of total reaction volume containing 7 μl H_2O, 12.5 μl TaqMan® Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA), 0.75 μl of each of the above mentioned 10 μM primers (Microsynth, Balgach, Switzerland), 1 μl of a 5 μM FAM-labeled *MTATP 8*-probe and 0.5 μl of a 5 μM VIC-labeled *GAPDH*-probe (both probes from Applied Biosystems, Rotkreuz, Switzerland). For each reaction 1 μl of DNA was added. qPCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Branchburg, New Jersey, USA) under the following conditions: an initiation step for 2 minutes at 50°C is followed by a first denaturation for 10 minutes at 95°C and a further step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Data collection and processing

The threshold cycle (Ct) values were obtained by the ABI Prism 7000 software. Each sample was analyzed in dupli-

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Table 1: Quantitative PCR (qPCR) for *GAPDH* and *MTATP8*

Gene	Gene ID	Sequences of primers and probes (5' → 3')		Length of primer/probe	Amplicon lengths (bp)
GAPDH	2597	Forward	CCC CAC ACA CAT GCA CTT ACC	21	97
		Reverse	CCT AGT CCC AGG GCT TTG ATT	21	
		Probe	(MGB) TAG GAA GGA CAG GCA AC (VIC)	17	
MTATP8	4509	Forward	AAT ATT AAA CAC AAA CTA CCA CCT ACC	27	78
		Reverse	TGG TTC TCA GGG TTT GTT ATA	21	
		Probe	(MGB) CCT CAC CAA AGC CCA TA (FAM)	17	

Quantitative PCR (qPCR) for *GAPDH* and *MTATP8* was carried out in a total reaction volume of 25 μl containing 7 μl H_2O, 12.5 μl TaqMan® Universal PCR Master Mix, 0.75 μl of each of the shown 10 μM primers, 1 μl of a 5 μM FAM-labeled *MTATP 8*-probe and 0.5 μl of a 5 μM VIC-labeled *GAPDH*-probe and 1 μL of template. The reaction was performed at the following conditions: initiation for 2 minutes at 50°C, followed by a first denaturation for 10 minutes at 95°C and a further step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

cate and one negative control was included in every run. For calibration a standard calibrator curve with known genomic DNA concentrations ranging from 3.125 × 104 to 10 pg/μL with a dilution factor of 5 (including 31250, 6250, 1250, 250, 50 and 10 pg/μL) was used. The efficiency of the multiplex assay for amplifying both nDNA and mtDNA simultaneously was measured in our previous study using standard curves generated by dilution series [17]. The standard curves had average slopes at approximately -3.3 (~100% efficiency). *GAPDH* and *MTATP8* levels were normalized by data obtained from the amplification of HPLC-purified single-stranded synthetic DNA oligonucleotides (Microsynth) specifying a 97-bp *GAPDH* amplicon and a 79-bp *MTATP8* gene amplicon with concentrations ranging from 5×10^7 copies to 5×10^2 copies. The concentrations of ccf nDNA were calculated according to the standard curves, using known concentration of human genomic DNA. The results were expressed as genome-equivalent (GE) per mL of plasma by using the conversion factor of 6.6 pg of DNA per cell. Genome equivalents were calculated as follows:

$$c = Q \times V_{DNA} / V_{PCR} \times 1 / V_{EX}$$

For the calculation of the concentration (c) in genome equivalents (GE/mL) the DNA quantity (Q) obtained by qPCR was multiplied with one fraction consisting of the volume of eluted DNA ($V_{DNA;}$ 80 μl/sample) divided by the sample volume used for PCR (V_{PCR}; 2.5 μl/reaction) resulting in a factor of 32 and with another fraction consisting of the unit (1 ml) divided by the volume of extracted plasma (V_{EX} = 400 μl) resulting in a factor of 2.5.

The content of mtDNA was calculated using the delta Ct (ΔCt) of an average Ct of mtDNA and nDNA (ΔCt = CtnDNA - CtmtDNA) in the same well as an exponent of 2 ($2\Delta^{Ct}$). Relative quantities of ccf mtDNA could be estimated using an equation of GE (nDNA) × fold-change mtDNA and expressed also as GE per mL of plasma.

Statistical Analysis

All statistical analyses were performed using SPSS 17.0 (SPSS Inc., Chicago, USA). The normality distribution of the data was determined using the Shapiro-Wilk-Test. The data were not normally distributed. For comparison of ccf nDNA and mtDNA levels between the three groups (malignant disease group, benign disease group and healthy control group) the Mann-Whitney-U-Test was performed. For the comparison of the ccf nDNA and mtDNA levels with other established prognostic factors the Mann-Whitney-U-Test and the Kruskal-Wallis-Test were used. *P*-values ≤ 0, 05 were considered statistically significant.

Results

Comparison of plasma ccf nDNA and mtDNA levels between the three study-groups

We compared the levels of plasma ccf nDNA and mtDNA, analyzed by multiplex real-time PCR, between the malignant disease group, the benign disease group and the healthy control group. The level of ccf nDNA in the malignant disease group was significantly higher in comparison with the benign disease group (4678 vs. 1359, Mann-Whitney: $P < 0.001$) and the healthy control group (4678 vs.1298, Mann-Whitney: $P < 0.001$). No significant difference could be found in the level of nDNA between the benign disease group and the healthy controls (1359 vs. 1298 Mann-Whitney: $P = 0.830$).

In contrast to the ccf nDNA determination, a decreased level of ccf mtDNA was found in the malignant disease group when compared with the healthy control group

(205013 vs. 522115, Mann-Whitney; $P = 0.022$) and the benign disease group (205013 vs. 73977; Mann-Whitney: $P < 0.001$). However, in the benign disease group the level of ccf mtDNA is even significantly lower than in the malignant disease group (73977 vs. 205013; Mann-Whitney: $P < 0.001$). The median of plasma ccf nDNA and mtDNA in the three study groups is shown in Table 2. The comparison of the ccf nDNA and mtDNA levels between the study groups is depicted in Fig. 1.

Correlation between the level of plasma ccf nDNA and mtDNA with clinicopathological parameters

For the malignant disease group, the association between the level of ccf DNA and other established clinical parameters, including tumor size, lymph node involvement, extent of metastasis and the status of estrogen receptor (ER), progesterone receptor (PR) and Her2/neu were analyzed.

Association between plasma ccf DNA level and tumor size in the malignant disease group

The level of ccf nDNA was significantly lower in patients with breast cancer with a tumor size of less than two centimeters (<2 cm; n = 21) than in those with a tumor size between 2 and 5 centimeters (>2 cm<5 cm; n = 25) (2250 vs. 6658; Mann-Whitney: $P = 0.034$). Only four patients with a tumor size of more than five centimeters (>5 cm) were recruited. There was no significant difference in the level of ccf nDNA between patients with a tumor size of more than five centimeters (>5 cm) and a tumor size from 2 to 5 centimeters (2 cm-5 cm). No correlation between the levels of ccf mtDNA and tumor size could be found. The correlation between ccf nDNA level and the tumor size is depicted in Fig. 2.

Association between plasma ccf DNA level and lymph node involvement, extent of metastasis, receptor status of ER, PR and Her2/neu amplification in the malignant disease group

In the malignant disease group no statistical significance in the level of ccf nDNA nor mtDNA between node negative and node positive patients, extent of metastasis and receptor status of ER, PR and Her2/neu amplification could be found.

The applicability of plasma ccf nDNA and mtDNA as marker for the discrimination between the three study groups

To evaluate the applicability of ccf plasma nDNA and mtDNA as a marker for distinguishing between malignant disease group, benign disease group and healthy control group, we performed ROC (Receiver Operating Characteristic) curve analysis. For the identification of the optimal cut-off point we used the Youden index (J). J is the maxi-

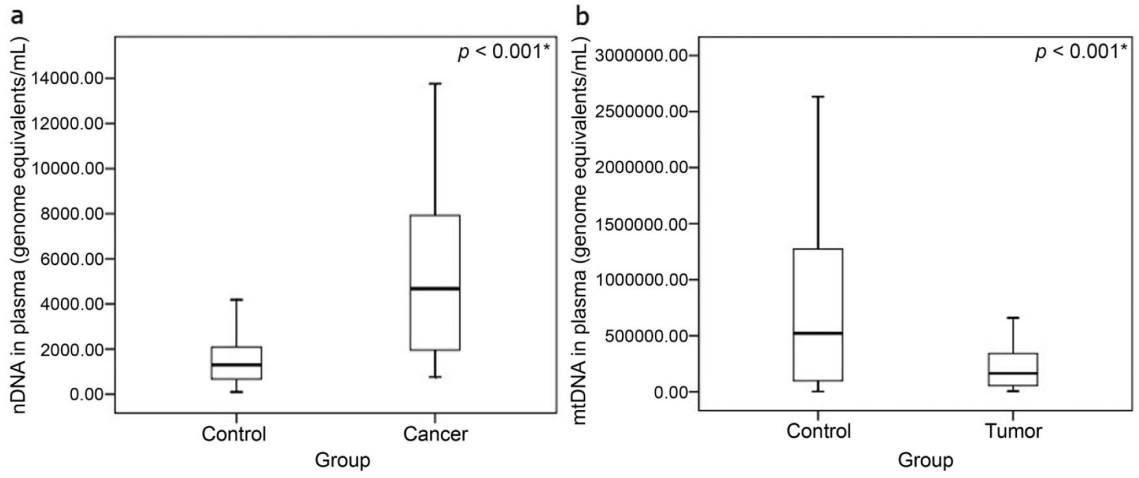

Figure 1

Boxplot for the comparison of the ccf nDNA and mtDNA levels between the study- groups. A) Boxplot for comparison of ccf nDNA levels between the malignant disease group and the healthy control group. Level of ccf nDNA in the cancer group was significantly higher in comparison with the healthy control group ($P < 0.001$). As no significant difference was found in the level of ccf nDNA between the benign disease group and the healthy controls the comparison is not shown in the figure. B) Boxplot for comparison of ccf mtDNA levels between the tumor group (including the malignant and benign cases) and the healthy control group ($P < 0.001$). Decreased levels of ccf mtDNA was found in both, the benign disease group and the malignant disease group, when compared to the healthy control group. (* significant correlation; Mann-Whitney-U-Test).

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Table 2: Concentrations (GE/mL) of plasma ccf nDNA and ccf mtDNA in the 3 study-groups; expressed as median.

Group	Total no. of patients	Age (mean ± S.D.)	Median Ccf nDNA(GE/mL)	Median Ccf mtDNA (GE/mL)
Malignant disease group	52	64 ± 15	4678	205013
Benign disease group	26	41 ± 16	1359	73977
Control group	70	53 ± 14.6	1298	522115

mum vertical distance between the ROC-curve and the diagonal reference line and is defined as J = maximum (sensitivity) + (specificity) - 1. The Youden index allows the selection of an optimal cut-off point under the assumption that sensitivity and specificity are equally weighted [18].

ROC curve analysis using ccf nDNA for the discrimination between the malignant disease group and the healthy control group

Level of ccf nDNA in the malignant disease group was significantly higher in comparison with the healthy control group, but no significant difference was found in the level of ccf nDNA between the benign disease group and the healthy controls. For discriminating between the malignant disease group and the healthy control group, an optimal cut-off point was indicated at 1866 GE/ml for plasma ccf nDNA with a sensitivity of 81% and a specificity of 69% (AUC = 0.80, $P < 0.001$, 95% confidence interval = 0.732-0.885). The ROC-curve for discrimination between the malignant disease group and the healthy control group using ccf nDNA is shown in Fig. 3.

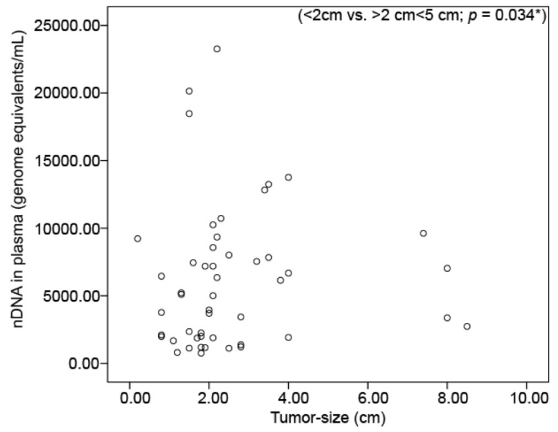

Figure 2
Scatterplot for correlating levels of ccf nDNA between breast cancer patients with a tumor size > 5 cm; (n = 4), >2 cm<5 cm; (n = 25) and < 2 cm; (n = 21). Significant difference in the levels of ccf nDNA could be found between tumors with a tumor size of >2 cm<5 cm and tumors with a tumor size of < 2 cm ($P = 0.034$). For the group of the tumor size > 5 cm, only 4 cases were recruited. (* significant correlation; Mann-Whitney-U-Test).

ROC curve analysis using ccf mtDNA for the discrimination between the breast tumor group and the healthy control group

Decreased levels of ccf mtDNA was found in both the benign disease group and the malignant disease group when compared to the healthy control group. For discriminating between the breast tumor group (malignant and benign) and the healthy control group an optimal cut-off point was indicated at 463282 GE/ml for ccf nDNA with a sensitivity 53% and a specificity of 87% (AUC = 0.68, P < 0.001, 95% confidence interval = 0.589-0.768). The ROC-curve for discrimination between the breast tumor group and the healthy control group using ccf mtDNA is shown in Fig. 3.

Discussion

According to our knowledge, our study is the first to find increased levels of ccf nDNA and simultaneously decreased levels of ccf mtDNA in plasma samples from patients with breast tumor compared to healthy controls. The former shows a probable diagnostic value in discriminating between breast cancer and healthy controls with a sensitivity of 81% and specificity of 69%, the latter reveals possible relevance in distinguishing between breast tumors (malignant and benign) and normal controls with a sensitivity of 53% and specificity of 87%.

For ccf nDNA, our previous studies indicated that in comparison with other potential circulating biomarkers involved in malignancy, such as nucleosomes, vascular endothelial growth factor (VEGF) and its soluble receptor (sVEGFR1), the ccf DNA showed more sensitivity and specificity in discriminating between breast cancer and normal controls [19,20]. Recently, Diehl et al, explored the possibility of using ccf tumor derived DNA for the management of colorectal cancer [21]. Patients with detectable ccf tumor DNA suffered from relapse, whereas subjects without ccf tumor DNA did not experience tumor recurrence. The ccf tumor DNA detection seems to be more reliable for predicting relapse than the standard biomarker, carcinoembryonic antigen (CEA), used for the management of colorectal cancer [22]. It was also reported that

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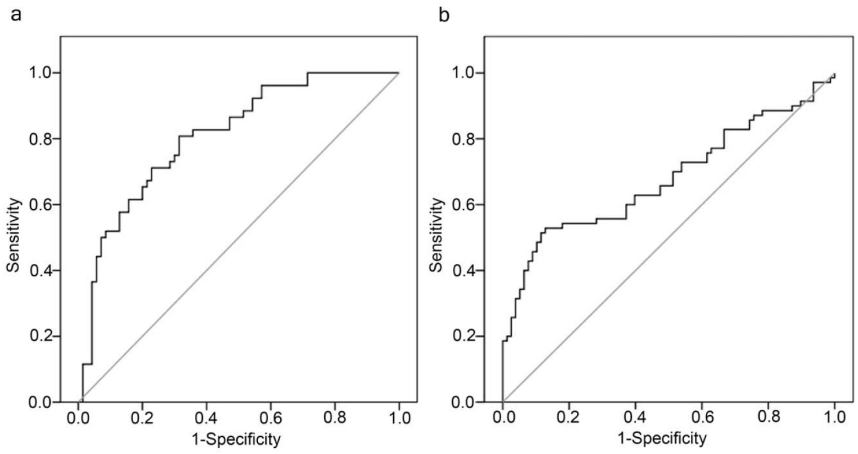

Figure 3
ROC curves using ccf nDNA and mtDNA for discriminating between the study-groups. A) ROC curve of ccf nDNA for discriminating between the cancer group and the healthy control group (sensitivity = 81%; specificity = 69%). B) ROC curve of ccf mtDNA for distinguishing between the tumor group and the healthy control group (sensitivity = 53%; specificity = 87%).

the levels of ccf DNA could be changed after therapy in breast cancer [23,24]. The observations suggest that determination of ccf DNA in cancer may prove a useful tool in the management of the condition.

In this study, we found high levels of ccf plasma DNA related to tumor size. This finding can be supported by investigations in the field of prenatal medicine. Placenta has been regarded as "pseudomalignant" and placental derived ccf fetal DNA in maternal circulation can be used for risk-free prenatal diagnosis [25-27]. The concentration of placental derived ccf fetal DNA in maternal blood increases with the progress in gestational weeks and with respect to placental size [28]. Using fetal specific DNA sequences, ccf fetal DNA could be detected from the 5th gestational week, and the results were reliable by the 8th gestational week with an accuracy of 100% in fetal DNA determination [29,30]. The results imply that by using tumor specific genetic alterations as marker, tumor derived ccf DNA may be detectable at an early stage with confined tumor growth and size.

For mtDNA, both down-or up-regulation in cancer patients has been shown in the past, and many attempts to explain both events have been made. While up-regulation of mtDNA in cancer patients was only demonstrated in a few cases [31], many studies including this one found decreased mtDNA levels in cancer patients [32,33]. One explanation for lower mtDNA copy numbers in cancer patients might be ascribed to mutations or deletions occurring as a consequence of exposure of mtDNA to reactive oxygen species (ROS) which are a by-product of respiration and oxidative phosphorylation. Especially in the D-Loop region which controls replication and transcription of mtDNA, such mutations and deletions may lead to changes in transcription and replication rate and finally result in a decrease of mtDNA levels in cancer patients [34]. In this study we found lower levels of mtDNA in the benign group when compared with the cancer group. In benign tumors depletion of mtDNA could be a mechanism of tumor cells to escape apoptosis and to finally promote cancer progression [35]. On the other hand, the relative increase of mtDNA levels in the cancer group compared to the benign disease group might be a compensatory mechanism of the cells to respond to the decline in respiratory function [36].

We showed that levels of ccf nDNA where significantly elevated in breast cancer patients in comparison with a benign disease group and a healthy control group, while levels of ccf mtDNA were significantly elevated in the breast tumor group (malignant and benign) when compared to the healthy control group. Regarding ccf nDNA levels, our results are confirmed by the findings of other studies which also found altered levels of ccf nDNA in cancer patients. For ccf mtDNA however, both down- as well as upregulation of ccf mtDNA levels in cancer patients have been reported and therefore grant further investigations of mtDNA content in different cancer and

tumor types, in order to clearly establish whether mtDNA levels are cancer type or tumor specific.

To conclude, both ccf nDNA and mtDNA levels allowed for discrimination between the different study groups. While ccf nDNA could be used for discriminating between patients with breast cancer and healthy controls, ccf mtDNA could be used for distinguishing between patients with breast tumors (malignant and benign) and healthy controls. Altogether this suggests that ccf nDNA has potential as a cancer specific biomarker, whereas ccf mtDNA may rather serve as a tumor biomarker.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

CK carried out the simultaneous quantification of ccf nDNA and mtDNA from plasma as well as the statistical data analysis and drafted the manuscript. RR, ZB and RA participated in data analysis and helped to draft the manuscript. NB and CD were responsible for the patient recruitment and the clinical study. JB, EW, WH and XYZ participated in the design and in the coordination of the study. All authors read and approved the final manuscript.

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3.3 Published research article 2:
Assessing the Value of CAN-Genes using MALDI-TOF MS

Authors: Corina Kohler*, Björn Tavelin, Alex Xiu-Cheng Fan, Ramin Radpour, Zeinab Barekati, Fabio Levi, Xiao Yan Zhong, Per Lenner and Paolo Toniolo

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Summary: As in our previous study quantitative alterations of plasma ccf-nDNA and mtDNA showed a limited value as a biomarker due to too low sensitivity and specificity, we additionally focused on finding breast cancer specific qualitative alterations. Therefore MALDI-TOF MS based 40-plex assay was applied to investigate the mutational status of candidate cancer genes (CAN-genes) to evaluate their value as biomarker for diagnostic/therapeutic purposes. No mutations were found in the analyzed cell lines; only one breast cancer patient was found to be heterozygous at one locus within the ZFYVE26 gene which was also confirmed by single-plex assay. Sjöblom et al. / Wood et al. already showed that the vast majority of CAN-genes are mutated at very low frequency. We therefore assume that at the selected loci, mutations might be low-frequency events and therefore rarely detectable. However, further evaluation of the CAN-gene mutations in larger cohorts should be the aim of further studies.

***Author contributions:** *Corina Kohler* was involved in experimental design, performing the experiments, data analysis and writing the manuscript.

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ORIGINAL PAPER

Assessing the value of CAN-gene mutations using MALDI-TOF MS

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Abstract
Purpose To identify cancer-linked genes, Sjöblom et al. and Wood et al. performed a genome-wide mutation screening in human breast and colorectal cancers. 140 CAN-genes were found in breast cancer, which in turn contained overall 334 mutations. These mutations could prove useful for diagnostic and therapeutic purposes.
Methods We used a MALDI-TOF MS 40-plex assay for testing 40 loci within 21 high-ranking breast cancer CAN-genes. To confirm mutations, we performed single-plex assays and sequencing.
Results In general, the mutation rate of the analyzed loci in our sample cohort was very low. No mutation from the 40 loci analyzed could be found in the 6 cell lines. In tissue samples, a single breast cancer tissue sample showed heterozygosity at locus c.5834G>A within the ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26).
Conclusions Sjöblom et al./Wood et al. already showed that the vast majority of CAN-genes are mutated at very low frequency. Due to the fact that we only found one mutation in our cohort, we therefore assume that at the selected loci, mutations might be low-frequency events and therefore, more rarely detectable. However, further evaluation of the CAN-gene mutations in larger cohorts should be the aim of further studies.

Keywords Breast cancer · CAN-genes · MALDI-TOF MS · Multiplex assay

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Background

The decoding of the human genetic code at the beginning of the twenty-first century officially heralded the age of genomics and laid a solid foundation for the future molecular research (Collins and McKusick 2001; Venter et al. 2001). Casting a retrospective glance at the last few

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Table 1 Clinical data of patients

Patient data divided into two subgroups according to pathological tumour type. *ER positive,* Estrogen receptor positive, *PR positive,* Progesterone receptor positive

Histological type	Total no. of patients	Age (years) mean ± S.D. (range)	Stage			ER positive	PR positive
			1	2A	2B		
Invasive ductal carcinoma	14	49 ± 10.8 (28–63)	11	2	1	5	4
Medullary carcinoma	5	50 ± 7.9 (36–56)	1	4	0	0	0

years, the achievements of the human genome project accounted strongly for the development in the research of genetically determined diseases. Identifying disease-related genes and understanding their involvement in pathogenesis is one of the fundamental goals currently pursued by the scientists from different fields (The Wellcome Trust Case Control Consortium 2007; Nessling et al. 2005).

Because cancer is one of the leading causes of death worldwide and has a primarily genetic determination, cancer research now dedicates itself to the search for genes that are involved in carcinogenesis. It is already known that mutations affecting some oncogenes and tumor suppressor genes, such as TP53 and BRCA1/2, are linked with an increased cancer risk (Soussi and Lozano 2005; Walsh et al. 2006). However, to date, there are only a few genes for which the existence of such a linkage is scientifically confirmed.

For this reason, high-throughput mutation profiling using large-scale sequencing approaches has been conducted and numerous candidate genes have been identified. Sjöblom et al. and Wood et al. conducted a sequencing-based genome-wide mutation screening in human breast and colorectal cancer with the aim of identifying genes linked with these cancers (Sjoblom et al. 2006; Wood et al. 2007). By testing nearly every well-annotated gene in both cancer types and by finally using stringent statistical criteria, they identified 280 genes overall that were mutated at a significant frequency, calling them candidate cancer genes (CAN-genes). The discovery of these CAN-genes in breast cancer could prove useful for diagnostic and therapeutic applications.

To evaluate the probable applicability of these CAN-genes for clinical purposes, we used MALDI-TOF MS 40-plex assay to test 40 single-nucleotide variants on 21 high-ranking CAN-genes in 6 breast cancer cell lines and in tissues of 19 breast cancer patients and 55 healthy controls.

Methods

Cell lines and culture conditions

MDA-MB-231, MCF-7, and HS578T were grown in DMEM (high glucose with L-glutamine). BT549 and T47D were cultured in RPMI 1640 medium, and SKBR3 was grown in McCoy's 5A. All media were supplemented with 10% FCS and 1% penicillin–streptomycin. The cells were maintained in a humid incubator at 37°C with 5% CO_2.

Study cohort

The study was performed at the Laboratory for Gynecological Oncology/Department of Biomedicine, Women's Hospital Basel and approved by the Ethical Committee of the University of Umeå. Patient information can be obtained from Table 1.

DNA extraction

For DNA extraction from cell lines and paraffin-embedded tissue samples, the High Pure PCR Template Preparation Kit (Roche Diagnostics, Germany) was used according to the manufacturer's protocol. Before extraction, cells were washed with 1× PBS. For patient samples, paraffin-embedded tissue sections were pretreated as proposed in the kit manual. The DNA was finally eluted in 100 µl elution buffer and stored at −20°C until further use. DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (Biolab, Mulgrave, VIC, Australia).

Assay design for detection of CAN-gene mutations

Wood et al. defined 140 genes containing more than 334 mutations as CAN-genes for breast cancer using cancer mutation prevalence (CaMP) score, which is calculated based on the likelihood that the amount of mutations in any gene is higher than those expected from a background mutation rate. Of the 140 CAN-genes, we selected 21 genes containing 40 loci (Table 2). For the design of the capture and the extension primers, the DNA sequences containing the CAN-gene mutations were entered in the software MassArray Assay Design version.3.1 (Sequenom, San Diego, CA, USA). Information about sequences and mass of capture and extension primers can be obtained from Supplementary 1 (Table 1a).

Genotyping using MALDI-TOF MS

For SNP genotyping, the iPLEX Gold assay (Sequenom, San Diego, CA, USA) was used. The assay consists of 3

Table 2 First CAN-gene assay

Gene	CCDS accession	CaMP score	Nucleotide (genomic)	Nucleotide (cDNA)	Amino acid (protein)
THBS3	NM_007112	3.5	g.chr1:151978713A>G	c.2863A>G	p.R955G
SP110	NM_004509	3.81	g.chr2:230907125T>C	c.23T>C	p.M8T
TLN1	NM_006289	2.98	g.chr9:35690282C>T (homozygous)	c.6566C>T	p.A2189V
ZNF646	NM_014699	3.06	g.chr16:30999156A>T	c.4010A>T	p.N1337I
TRIOBP	NM_001039141	3.54	g.chr22:36443733A>T	c.670A>T	p.R224W
ZNF569	NM_152484	3.28	g.chr19:42609051C>G	c.85C>G	p.Q29E
XDH	NM_000379	3.76	g.chr2:31500578C>G (homozygous)	c.2371C>G	p.R791G
VEPH1	NM_024621	5.34	g.chr3:158461684_158461683delAA	c.2443_2444 delAA	Fs
XDH	NM_000379	3.76	g.chr2:31501422C>T	c.2287C>T	p.L763F
ZFYVE26	NM_015346	3.06	g.chr14:67302874G>A	c.5834G>A	p.R1945Q
ZNF569	NM_152484	3.28	g.chr19:42597140A>G	c.260A>G	p.E87G
TP53	NM_000546	55.19	g.chr17:7519167A>G (homozygous)	c.488A>G	p.Y163C
ZFP64	NM_199427	3.39	g.chr20:50134614G>C	c.1827G>C	p.K609 N
TP53	NM_000546	55.19	g.chr17:7520091C>G	c.321C>G	p.Y107X
TP53	NM_000546	55.19	g.chr17:7518335A>T (homozygous)	IVS5-2A>T	Sp
ZFYVE26	NM_015346	3.06	g.chr14:67321561C>A	c.3491C>A	p.A1164E
TMEM123	NM_052932	4.7	g.chr11:101777989A>C	c.259A>C	p.N87H
TRIOBP	NM_001039141	3.54	g.chr22:36436328T>A	c.515T>A	p.V172E
VEPH1	NM_024621	5.34	g.chr3:158581776G>T	c.998G>T	p.S333I
TP53	NM_000546	55.19	g.chr17:7519095G>C	IVS4 + 1G>C	Sp
TIMELESS	NM_003920	2.96	g.chr12:55101032C>G	c.3022C>G	p.Q1008E
TP53	NM_000546	55.19	g.chr17:7517747C>T (homozygous)	c.916C>T	p.R306X
TP53	NM_000546	55.19	g.chr17:7517831C>T	c.832C>T	p.P278S
TLN1	NM_006289	2.98	g.chr9:35693800C>T	c.6329C>T	p.A2110 V
TG	NM_003235	5.84	g.chr8:134030233C>G (homozygous)	c.5264C>G	p.P1755R
TP53	NM_000546	55.19	g.chr17:7518937C>T	c.637C>T	p.R213X
TMPRSS6	NM_153609	3.28	g.chr22:35810313G>A	c.668G>A	p.R223H
TECTA	NM_005422	4.56	g.chr11:120504208T>A (homozygous)	c.2312T>A	p.I771N
TACC2	NM_206862	2.65	g.chr10:123834397C>G	c.2392C>G	p.L798V
TECTA	NM_005422	4.56	g.chr11:120494285G>A	c.851G>A	p.R284H
TG	NM_003235	5.84	g.chr8:133994534C>G	c.4220C>G	p.S1407X
TG	NM_003235	5.84	g.chr8:133968052A>G (homozygous)	c.1253A>G	p.D418G
TDRD6	NM_001010870	2.69	g.chr6:46764550C>G	c.726C>G	p.F242L
TMEM123	NM_052932	4.7	g.chr11:101777516T>C	c.509T>C	p.M170T
TG	NM_003235	5.84	g.chr8:134103511G>T	c.6970G>T	p.A2324S
TCF1	NM_000545	3.48	g.chr12:119900103G>A	c.1721G>A	p.S574N
SLC6A3	NM_001044	3.66	g.chr5:1456172A>C	c.1632A>C	p.R544S
TECTA	NM_005422	4.56	g.chr11:120505627delA	c.2438delA	Fs
TDRD6	NM_001010870	2.69	g.chr6:46769240G>C	c.5416G>C	p.E1806Q
SULF2	NM_018837	3.84	g.chr20:45728625T>C	c.1591T>C	p.Y531H

The table shows the genes analyzed in the MALDI-TOF MS 40-plex assay, the associated CCDS accession number, the cancer mutation prevalence score (CaMP score), the nucleotide and genomic position, and the affected amino acid. To optimize the signal-to-noise ratio, the 40 CAN-gene positions have been divided into four mass groups indicated by the parting lines

major steps: 1. Capture PCR (amplification of the amplicon containing the locus of interest); 2. Shrimp alkaline phosphatase (SAP) treatment (removal of unincorporated dNTPs); and 3. iPLEX reaction (Primer extension).

1. The Capture PCR was carried out in a 10 µl-PCR volume containing 1 µl DNA (10 ng/µl), 1.625 mM $MgCl_2$, 500 µM dNTP mix, 0.5U Hotstart Taq DNA polymerase (Quiagen), and primer mix (containing all

40 amplification primer pairs). PCR amplification was performed using a Mastercycler gradient (Eppendorf, Germany) under the following conditions: Preincubation at 94°C for 15 min, followed by 45 cycles of 94°C for 20 s, 56°C for 30 s, and 72°C for 1 min, and a final extension step at 72°C for 3 min.

2. For the removal of unincorporated dNTPs, SAP treatment using shrimp alkaline phosphatase (Sequenom) was performed at 37°C for 40 min and 85°C for 5 min, followed by a final cooling to 4°C.
3. For the iPLEX reaction, a PCR cocktail mix was made using iPLEX buffer (10×), 0.4 µl iPLEX termination mix, 0.08 µl iPLEX enzyme, and the primer mix (consisting of all 40 extension primers). As there is an inverse relationship between peak intensity and analyte mass that influences the signal-to-noise ratio, a classification of different mass groups is required when performing high-plex assays. To ensure an optimal signal-to-noise ratio, we divided our assay into 4 different mass groups (7, 9.66, 10.33, and 14 µM). The PCR was carried out in a Mastercycler gradient (Eppendorf, Germany) using a 200-short-cycle program consisting of two cycling loops. The first loop of five cycles is located within a second loop of 40 cycles. The PCR starts with a first denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 94°C for 5 s, primer annealing at 52°C for 5 s, and extension at 80°C for 5 s. Within these 40 cycles, the primer annealing and extension step is repeated 5 times resulting in a total of 200 cycles. After the 200 cycles, a final extension is done at 72°C for 3 min, and the product is cooled down to 4°C.

To optimize the mass spectrometric analysis, the iPLEX reaction products were desalted using clean resin and then dispensed on a 384-element SpectroCHIP bioarray by a Nanodispenser (Sequenom). For measuring the assay reproducibility, each sample was run in duplicate and found mutation(s) were reconfirmed with single-plex assay and sequencing. For the processing and analysis of the iPLEX SpectroCHIP, the MassARRAY Compact system and the MassARRAY Workstation software version 4.0 (Sequenom) were used.

Sequencing analysis

The PCR was carried out in 25 µl total volume containing 17.4 µl H_2O, 2.5 µl 10× PCR buffer (Quiagen), 0.5 µl dNTP mix (25 mM) (Quiagen), 1.25 µl of each primer, 0.1 µl Hotstart Taq (Quiagen), and 2 µl DNA. The PCR was performed under the following conditions: initial denaturation at 94°C for 15 min, followed by 45 cycles at 94°C for 20 s, 60°C for 30 s, and 72°C for 1 min, and a final extension at 72°C for 3 min. Removal of primers and dNTPs was done using Exo1/SAP treatment. To 25 µl of the PCR, 0.3 µl Exo1, 0.6 µl Exo1 buffer (10×) (both Fermentas), 3 µl SAP (Sequenom), and 2.1 µl H_2O were added and incubated under the following conditions: 37°C for 40 min and 85°C for 5 min, followed by a final cooling to 4°C. Sequencing was done by Microsynth (Balgach, Switzerland). Information on primers used for sequence analysis is listed in Supplementary 1 (Table 1b).

Results

Quality of the multiplex assay

In the previous studies, the sensitivity and specificity of the method itself has been proven by us as well as by other research groups (Garritsen et al. 2009; Thomas et al. 2007; Xiu-Cheng Fan et al. 2008). Therefore, we only analyzed several assay quality parameters including call rate and call probability. For every analyzed locus, these parameters are automatically calculated by the software MassArray Typer (Sequenom, Inc.). Assay quality was also assessed by visual analysis of MALDI-TOF spectrograms. The peak pattern was quite homogenous, with some slight variations in peak intensity (Fig. 1a).

Mutational analysis of breast cancer cell lines and patient samples

Using the MALDI-TOF MS-based 40-plex assay, we analyzed the mutational status of 40 variants on 21 CAN-genes in 6 human breast cancer cell lines and tissues of 19 breast cancer patients and 55 healthy controls; no CAN-gene mutations have been found at the analyzed loci in any of the cell lines. Regarding the tissue samples, only a single breast cancer tissue sample showed heterozygosity at locus c.5834G>A within the ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26). This heterozygosity could also be confirmed using a single-plex assay and sequencing (Fig. 1b, c).

Discussion

Using MALDI-TOF MS 40-plex assay, we evaluated 21 CAN-genes and 40 loci in 6 breast cancer cell lines and in tissue samples of 19 breast cancer patients and 55 healthy controls. The frequency of the mutations in our sample cohort was very low. No mutation was found at the analyzed loci within the 6 cell lines. Only one breast cancer patient was found to be heterozygous at one locus within

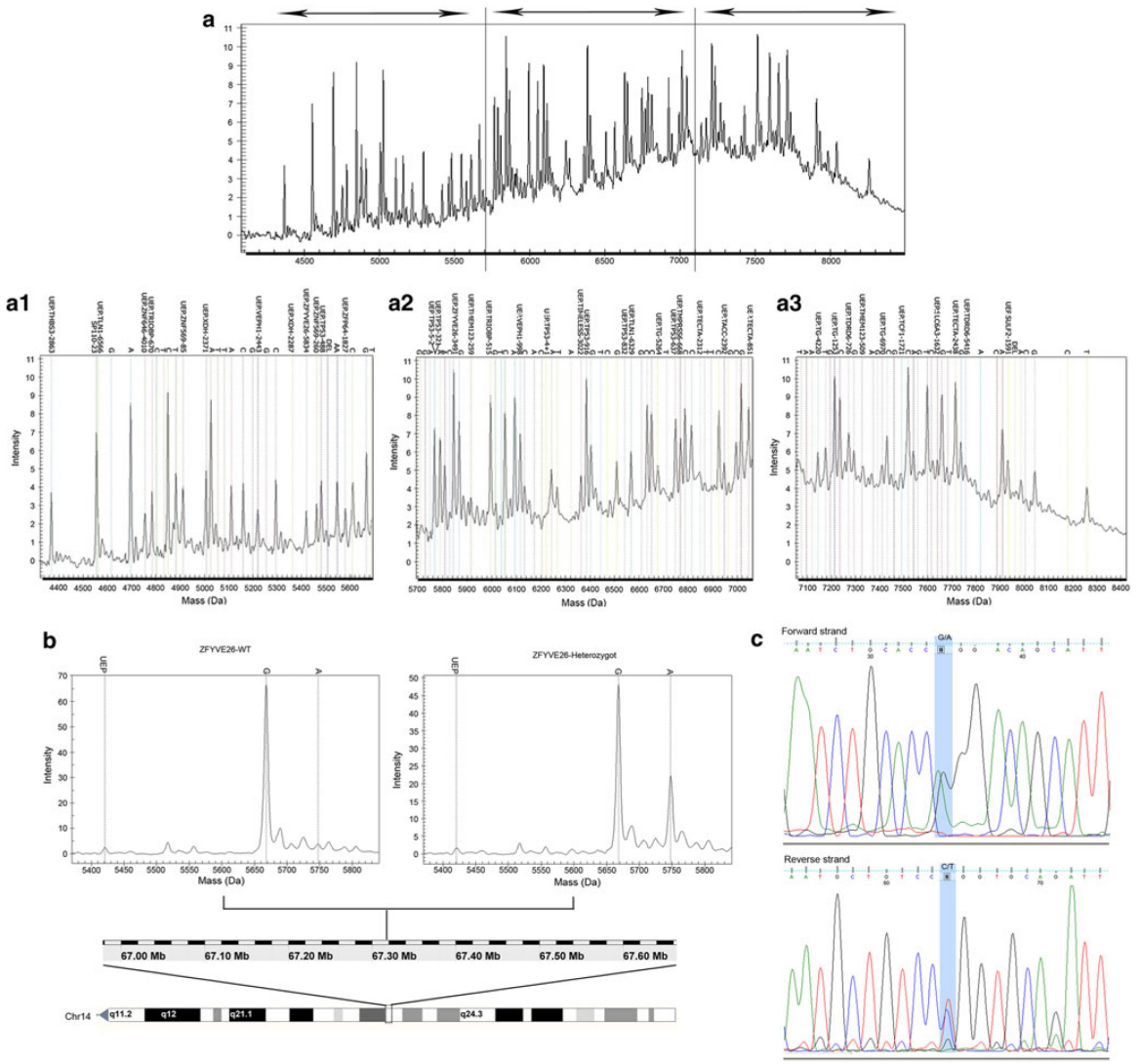

Fig. 1 Spectrograms of one 40-plex assay and a single-plex assay/sequencing confirming heterozygosity in ZFYVE26 gene (Zinc finger FYVE domain-containing gene 26). **a** Spectrogram of one 40-plex assay with detailed view in the augmentations a1, a2, and a3. The *dashed lines* represent the unextended primers (UEP, followed by gene name and nucleotide position of the allele) and the respective alleles. **b** Single-plex assays for two patients; one homozygote for G and the other heterozygote for G/A at locus g.chr14:67302874 within the ZFYVE gene. **c** Sequencing results for confirmation of heterozygosity at locus g.chr14:67302874 within the ZFYVE gene

the ZFYVE26 gene, which was also confirmed by single-plex assay and by sequencing. Defects in ZFYVE26 are known to be the cause of spastic paraplegia autosomal recessive type 15 (Goizet et al. 2009). However, ZFYVE seems to play a role in cytokinesis as well. Sagona et al. showed that depletion of the ZFYVE26 protein in HeLa cells led to the arrest of cells in cytokinesis (Sagona et al. 2010). A direct association of the gene with breast cancer has not been reported.

To get an idea whether others had already described cancer-related mutations at the same positions that we analyzed in this study, we investigated the COSMIC (Catalogue of Somatic Mutations in Cancer) database, Sanger (http://www.sanger.ac.uk/genetics/CGP/cosmic/), which contains information about previously reported somatic mutations in cancer. Interestingly, it seems that most of the CAN-gene loci analyzed in this study have only been analyzed by Sjöblom et al until now, and most of

them have not been directly implicated in breast cancer so far. Only few of the positions, which were included in our study, have previously been associated with breast cancer, and mutations at those loci have been found by other groups. Alsner et al. analyzed the heterogeneity in TP53 mutations in 315 breast cancer patients. Using tumour material from those patients, they identified 74 TP53 mutations. At position c.488, which is located in the exon 5 of the TP53 gene and was also analyzed in our study, they found a base exchange from A>G (Alsner et al. 2000).

To conclude, it is widely accepted that the landscapes of cancer are quite complex (Vogelstein and Kinzler 1993). Sjöblom et al. and Wood et al. showed that just a humble amount of genes are mutated at higher frequencies, while most of the genes are mutated at a relatively low frequency in cancer. Although our study included high-frequency as well as low-frequency genes/loci, one have to consider as well the mutational inter- and intra-variations within/between cancer types, which makes it more difficult to select the real driver mutations out of a background of passenger mutations. To really get a conclusion about the probable value of these CAN-genes for a diagnostic/therapeutic purpose, evaluation of a higher number of CAN-genes/CAN-gene loci in a larger cohort should be the aim of further studies.

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Conflict of interest The authors declare that they have no conflict of interests.

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3.4 Published research article 3:

Mutations of Mitochondrial DNA as Potential Biomarkers in Breast Cancer

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Summary: Compared to nDNA, the analysis of mtDNA mutations has some advantages such as higher mtDNA copy numbers and higher mutation rate. We used sanger sequencing with the aim to determine somatic mtDNA mutations within the D-loop region in breast cancer tissues and to evaluate whether it is possible to detect these mutations in the matched plasma samples. We therefore sequenced the two hypervariable regions HVR1 and HVR2, which are located in the D-Loop and are known as mutational hotspots in ten paired tissues and plasma samples from breast cancer patients.

***Author contributions:** *Corina Kohler* was involved in experimental design, performing the experiment, data analysis and writing of the manuscript.

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Mutations of Mitochondrial DNA as Potential Biomarkers in Breast Cancer

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Abstract. Background: Alterations of mitochondrial DNA (mtDNA) have been found in cancer patients, therefore informative mtDNA mutations could serve as biomarkers for the disease. Materials and Methods: The two hypervariable regions HVR1 and HVR2 in the D-Loop region were sequenced in ten paired tissue and plasma samples from breast cancer patients. Results: MtDNA mutations were found in all patients' samples, suggesting a 100% detection rate. Examining germline mtDNA mutations, a total of 85 mutations in the D-loop region were found; 31 of these mutations were detected in both tissues and matched plasma samples, the other 54 germline mtDNA mutations were found only in the plasma samples. Regarding somatic mtDNA mutations, a total of 42 mutations in the D-loop region were found in breast cancer tissues. Conclusion: Somatic mtDNA mutations in the D-loop region were detected in breast cancer tissues but not in the matched plasma samples, suggesting that more sensitive methods will be needed for such detection to be of clinical utility.

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Key Words: MtDNA mutation, D-loop region, breast cancer, plasma, mitochondrial DNA.

Human mitochondrial DNA (mtDNA) is a circular double-stranded molecule of 16.5 kb which contains 37 genes which code for 12S and 16S rRNAs, 22 tRNAs and 13 polypeptides (1). Approximately 10^3 to 10^4 copies of mtDNA are present per cell, out of which the majority is homoplasmic, showing genetic homogeneity within each individual. MtDNA is subject to a higher mutation rate than nuclear DNA because of the lack of histones and DNA repair mechanisms and due to its proximity to reactive oxygen species, which are generated during oxidative phosphorylation in the inner mitochondrial membrane (2). MtDNA mutations have been frequently observed in all types of human cancer and have thus been implicated in the carcinogenic process. Most mtDNA mutations have been found in the displacement loop (D-loop) region, where the origin of replication and the promoter is located. The D-loop region is a non-coding region which harbors two hypervariable regions (HVR1 and HVR2; positions 16024-16383 and 57-372) (3). Both germline and somatic mtDNA mutations preferentially take place at these hypervariable sites, which are considered as mutational hotspots (4). Alterations have been reported in cancer of the head and neck, lung, bladder, uterine cervix and breast in the form of point mutations, deletions, insertions, and mitochondrial microsatellite instabilities (5).

Cancer-derived mtDNA alterations have also been detected in plasma and serum of patients with diverse types of cancer (6-7). Somatic mtDNA mutations may therefore serve as biomarkers for the disease.

In the present study we used sanger sequencing with the aim of determining somatic mtDNA mutations within the D-loop region in breast cancer tissues and to evaluate whether it is possible to detect these mutations in matched plasma samples.

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Table I. Clinical data of breast cancer patients.

Variable	Cases
Age, years	
<50	4
≥50	6
Postsurgical stage	
I and II	6
III and IV	4
Histological grading	
G1	2
G2	4
G3	4
Lymph node involvement	
Positive	7
Negative	3
Distant metastasis	
M0	10
M1	0
ER	
Positive	8
Negative	2
PR	
Positive	9
Negative	1
Her2	
Positive	9
Negative	1

Materials and Methods

Sample collection. The study was approved by the local Institutional Review Board. A total of 10 different paraffin-embedded tissue samples were collected from 10 patients with breast cancer from China. Paraffin-embedded sections were examined by two experienced pathologists. Ten matched plasma samples were collected before any invasive procedures were performed or any therapy was administered. Breast cancer characteristics, postsurgical-stage, histological grading and hormone receptor status from the breast cancer patients are listed in Table I.

mtDNA extraction. DNA extraction of tissues was performed from 3-5 sections of each 10 μm thick paraffin-embedded sample (around 0.01-0.02 g of tissue) and matched plasma DNA was extracted from 400 μl plasma sample using High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to a standardized protocol as described elsewhere (8, 9). DNA was quantified using a Nanodrop spectrophotometer (Biolab, Mulgrave, VIC, Australia).

Amplification and sequencing of mtDNAThe primer sequences used for amplification and sequencing are presented in Table II. Primer pairs, mtHVR-1 mtHVR-2, were used for amplification of HVR1 and HVR2 respectively. These primers also served as sequencing primers.

Polymerase chain reaction (PCR) for HVR1 and HVR2 was performed under the following conditions: 10 min activation of the Amplitaq (Amplitaq Gold, Perkin Elmer, Weiterstadt, Germany) at 95˚C, followed by 30 cycles of denaturation at 94˚C for 30 s, annealing at 56˚C for 60 s, and extension at 72˚C for 60 s. The PCR was performed in a 50 μl total reaction volume containing 0.1 μM of each dNTP, 1.25 Units of Taq polymerase, 2 pM of each primer, 1.5 mM $MgCl_2$, 12.5 μl mtDNA (1:10 diluted in water) and 5 μl 10× PCR Buffer (Roche Molecular Systems, Branchburg, New Jersey, USA) and water. PCR was performed using a thermal cycler (Biometra Trio Thermoblock; Biometra, Göttingen, Germany). Ten microliters of amplified product was visualized and analyzed using a 2% agarose gel (Seakem ME; FCM Bioproducts, Biozym, Göttingen, Germany) with ultraviolet illumination after ethidium bromide staining. If the PCR was successful, the resulting products were further purified using spin columns from the High Pure PCR product purification kit (Boehringer Mannheim, Mannheim, Germany) to remove remaining amplification primers, dNTPs and PCR buffer, and then sequenced.

Purified PCR products were applied to the fluorescent dye terminator cycle sequencing reaction with a cycle sequencing kit (Big Dye Terminator; Applied Biosystems, Darmstadt, Germany). Thereafter, the sequencing products were purified with a spin kit (DyeEx; Qiagen, Hilden, Germany) and analyzed on a sequencer (ABI 3130; Applied Biosystems, Darmstadt, Germany). Nucleotide sequence alignment was performed with computer software (DNA Star; DNASTAR, Inc., Madison, WI, USA). All sequencing reactions were performed in both directions and tested for concordance. Sequence differences found in plasma from patients with breast cancer were recorded as germline mtDNA mutations. Sequence variations only found in breast cancer tissues were scored as somatic mtDNA mutations. Each polymorphism was checked against the Mitomap database (http://www.mitomap.org/).

Results

Germline mtDNA mutations in the D-loop regionIn total, 85 germline mtDNA mutations in the D-loop region were found: 31 of these mutations were detected in both tissues and matched samples of plasma, the other 54 germline D-loop region mtDNA mutations were only found in the plasma samples. Detailed information about the germline mutations which were found in HVR1 and HVR2 in this cohort is shown in Table II. A summary of total germline mutations and germline mutations per case can be obtained from Table III and IV, respectively. Example sequencing results are depicted in Figure 1.

Somatic mtDNA mutations in the D-loop regionIn total, 43 somatic mtDNA mutations in the D-loop region were found in breast cancer tissues. Each patient had two or more somatic mtDNA mutations in the D-loop region, with four patients having five or more. Detailed information about the somatic mutations which were found in HVR1 and HVR2 in this cohort is presented in Table II. A summary of total somatic mutations and somatic mutations per case can be obtained from Table III and IV, respectively. Example sequencing results are depicted in Figure 1.

Discussion

In cancer, mitochondria frequently exhibit genetic and functional alterations (10). Compared with nuclear DNA, the analysis of mtDNA mutations exhibits certain advantages,

Table II . Sequences of primers used for amplification and sequencing.

Locus	Sequence	Fragment size	Primer localization
mtHVR-1F	5′ TCCACCATTAGCACCCAAAGC 3′	522	L 15976
mtHVR-1R	5′ TCGGATACAGTTCACTTTAGC 3′		H 16497
mtHVR-2F	5′ GGTCTATCACCCTATTAACCAC 3′	422	L 8
mtHVR-2R	5′ CTGTTAAAAGTGCATACCGCCA 3′		H 429

Table III. Germline mtDNA mutations in the D-loop region in breast cancer.

Case no.	Locus	Variation (P+, T+)	Case no.	Locus	Variation (P+, T–)	Case no.	Locus	Variation (P+, T–)
1	16223	C>T	1	16290	C>T	7	16223	C>T
1	16362	T>C	1	16319	G>A	7	16266	C>T
1	73	A>G	1	152	T>C	7	16362	T>C
1	263	A>G	1	235	A>G	7	146	T>C
2	16223	C>T	2	16051	A>G	7	150	C>T
2	73	A>G	2	16189	T>C	7	489	T>C
2	150	C>T	2	16362	T>C	8	16093	T>C
2	263	A>G	2	456	C>T	8	16136	T>C
3	73	A>G	2	489	T>C	8	16217	T>C
3	263	A>G	3	16304	T>C	8	16218	C>T
4	73	A>G	3	198	C>T	8	58	T>C
4	263	A>G	4	16126	T>C	8	94	G>A
5	16172	T>C	4	16163	A>G	9	16189	T>C
5	73	A>G	4	16186	C>T	9	16217	T>C
5	150	C>T	4	16189	T>C	9	16261	C>T
5	263	A>G	4	16294	C>T	9	16299	A>G
6	16223	C>T	4	152	T>C	9	16311	T>C
6	16362	T>C	4	195	T>C	9	193	A>G
6	73	A>G	5	16129	G>A	10	16129	G>A
6	263	A>G	5	16140	T>C	10	16162	A>G
6	489	T>C	5	16166	A>G	10	16172	T>C
7	16172	T>C	5	16189	T>C	10	16304	T>C
7	73	A>G	5	16217	T>C	10	548	C>T
7	263	A>G	5	16274	G>A			
8	73	A>G	5	16293	A>G			
8	150	C>T	5	16335	A>G			
8	263	A>G	5	195	T>C			
9	73	A>G	6	16234	C>T			
9	263	A>G	6	146	T>C			
10	73	A>G	6	217	T>C			
10	263	A>G	7	16189	T>C			

P+, T+: Germline mtDNA mutations, found in breast cancer tissues and matched plasma samples; P+ , T–: germline mtDNA mutations, only found in breast cancer plasma samples

Table Ⅳ. Summary of germline and somatic mtDNA mutations.

P+	T+	31
P–	T+	43
P+	T–	54

P+, T+: Germline mtDNA mutations, found in both breast cancer tissues and matched plasma samples; P+, T–: Germline mtDNA mutations, only found in breast cancer plasma samples; P–, T+ : Somatic mtDNA mutations, only found in breast cancer tissues.

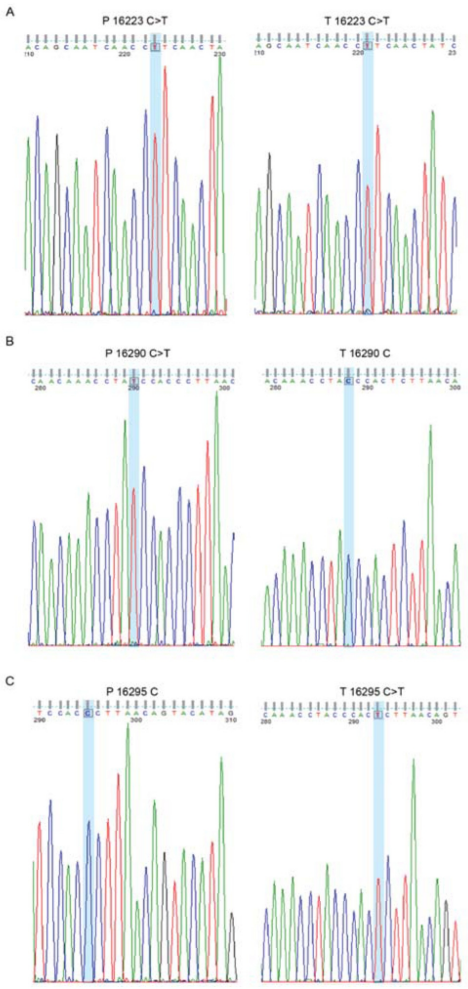

Figure 1. Representative sequencing results showing examples of germline and somatic mutations. Germline mtDNA mutations detectable in plasma (P) and tissue (T) (locus 16223) (A) and in plasma but absent from tissue (locus 16290) (B). Somatic mtDNA mutation detectable in tissue but absent from plasma (locus 16295) (C).

Table V. Frequency of germline and somatic mtDNA mutations per case.

Case	no.	1	2	3	4	5	6	7	8	9	10
P+	T+	4	4	2	2	4	5	3	3	2	2
P–	T+	5	3	8	2	4	2	5	3	5	6
P+	T–	4	5	2	7	9	3	7	6	6	5

P+, T+: Germline mtDNA mutations, found in both breast cancer tissues and matched plasma samples; P+, T–: Germline mtDNA mutations, only found in breast cancer plasma samples; P–, T+: Somatic mtDNA mutations, only found in breast cancer tissues.

such as higher mtDNA copy numbers and higher mutation rate (2). Tumor-derived mutant mtDNA can be detected in the cell-free fraction of the blood (plasma, serum) of individuals with cancer; somatic mtDNA mutations may therefore serve as potential markers.

Somatic mtDNA mutations in breast cancer were reported to be accumulated in the hypervariable regions HVR1 and HVR2 within the D-loop region (2, 11-12). In our study, germline and somatic mtDNA mutations within these two regions were found in all patients' samples. Kumimoto et al. reported 70.7% germline mutations in HVR1 in esophageal tumor cells (13). Zhu et al. identified 155 polymorphisms in 15 breast cancer tissues and matched nipple aspirate fluid. Fifty-five of the polymorphisms (35%) were located in the D-loop region (14). In the present study, we found all patients had germline mtDNA mutations in the D-loop region of breast cancer tissues, with 36.5% also being detected in the matched plasma samples; the rest were only present in the plasma samples. Examination of somatic mtDNA mutations in the D-loop region in human bladder, head and neck, and lung primary tumors revealed a high frequency of somatic mtDNA mutations, ranging between 46%-64% (2). We had an even higher mtDNA mutation frequency in our cohort. What is proposed as mitochondrial hypermutagenesis, possibly mediated by cellular oxidative stress, due to a burst of multiple mtDNA mutations (15). As also evidenced in our data, some individual sites act as mutational hot spots (e.g. positions 146, 150, 152, 195, 16189, 16311 and 16362) (16).

Using sanger sequencing we were unable to find mutant mtDNA in plasma samples. This might be due to the fact that the amount of circulating mutant mtDNA is tiny in comparison to that of the background wild-type mtDNA. Therefore research groups are currently trying to develop new approaches that have a higher sensitivity. Lo et al. developed the digital relative chromosome dosage (RCD) to assess fetal chromosomal aneuploidy (trisomy 21) in maternal plasma (17). To reliably monitor tumor dynamics in patients with colorectal cancer who were undergoing surgery or chemotherapy, Diehl et al. used BEAMing (beads, emulsion, amplification and magnetics), a method that uses magnetic bead-based PCR combined with a flow cytometric approach which enables the measurement of the ratio of mutant to wild-type fragments (18). Recently, several groups performed large-scale parallel sequencing approaches to analyze alterations of circulating cell-free DNA (ccf-DNA) in plasma/serum (19-20). Using the 454/Roche high-throughput

Table VI. Somatic mtDNA mutations in the D-loop region in breast cancer.

Case no.	Locus	Base change	Reference sequence	Hypervariable segment
1	16295	C>T	C	1
1	146	T>C	T	2
1	199	T>C	T	2
1	489	T>C	T	2
1	311-15	Insert C	C	2
2	16129	G>A	G	1
2	16257	C>T	C	1
2	16261	C>T	C	1
3	16126	T>C	T	1
3	16223	C>T	C	1
3	16234	C>T	C	1
3	16290	C>T	C	1
3	16319	G>A	G	1
3	189	A>G	A	2
3	235	A>G	A	2
3	303-09	Insert CC	C	2
4	16304	T>C	T	1
4	207	G>A	G	2
5	16145	G>A	G	1
5	16245	C>T	C	1
5	16257	C>T	C	1
5	16261	C>T	C	1
6	16189	T>C	T	1
6	16274	G>A	G	1
7	16145	G>A	G	1
7	16162	A>G	A	1
7	16304	T>C	T	1
7	548	C>T	C	2
8	16223	C>T	C	1
8	16325	T>C	T	1
8	16362	T>C	T	1
9	16140	T>C	T	1
9	16223	C>T	C	1
9	16362	T>C	T	1
9	298	C>T	C	2
9	489	T>C	T	2
10	16140	T>C	T	1
10	16189	T>C	T	1
10	16217	T>C	T	1
10	16274	G>A	G	1
10	146	T>C	T	2
10	150	C>T	C	2

GS-FLX platform, Beck et al. compared repetitive elements in serum circulating nucleic acids (CNAs) of 38 women with ductal carcinoma with 67 healthy controls. The serum CNA pool of patients with breast cancer contained significantly more repetitive elements than the serum CNA of the healthy controls and using receiver operating characteristic curve analysis they were able to distinguish between the two groups with a specificity of 95% and a sensitivity of 90% (21). Although the above mentioned technologies allow for a highly sensitive detection of ccf-DNA alterations, their applicability for routine diagnostics is still limited due to laborious procedures and high costs. Some time will pass before these technologies become technically mature and make it to clinical application.

In the present study, using sanger sequencing, we identified 43 informative mutations, in total, within the HVR1 and HVR2 regions in breast cancer tissues. However, we were not able to detect these mutations in mtDNA of the corresponding plasma samples, suggesting that more sensitive methods will be needed for this approach to be of clinical utility.

Competing Interests

The Authors declare that they have no competing interests.

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3.5 Research manuscript:

Intergenomic Cross-Talk between Mitochondrial DNA Content, Mitochondrial DNA Mutations and Methylation Status of Breast Cancer Related Genes

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Summary: Proper cellular function is dependent on communication between distinct cellular compartments. In this connection mitochondrial retrograde response plays a crucial role. However, under pathophysiological conditions like cancer mitochondrial to nucleus communication has been found to be impaired. This study investigates the inter-genomic crosstalk between mtDNA alterations (mtDNA content and mutations) and methylation status of 22 breast cancer candidate genes. We try to gain new insight in how mitochondrial retrograde response might be influenced by mtDNA alterations as mtDNA mutations and/or mtDNA copy number changes in cancer.

***Author contributions:** *Corina Kohler* was involved in experimental design, performing the experiment, data analysis and writing of the manuscript.

Intergenomic Cross-Talk between Mitochondrial DNA Content, Mitochondrial DNA Mutations and Methylation Status of Breast Cancer Related Genes

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Abstract

Background: Reduced mtDNA content, increase in mtDNA mutations and alterations in tumor suppressor and oncogenes have been observed frequently in patients with breast cancer. Within this study we therefore investigated the inter-genomic crosstalk between mtDNA alterations (mtDNA content and mutations) and methylation status of 22 breast cancer candidate genes to evaluate the impact of these factors on each other.

Methods: Forty-five paired samples (cancerous/ paired normal breast tissues) were checked for mtDNA content using qPCR and for mutational status at 22 mtDNA loci using MALDI-TOF MS. Methylation status of 22 candidate genes was compared between distinct mutational status groups and correlated with mtDNA content.

Results: MtDNA content was significantly reduced in breast cancer compared to paired normal tissues (P=0.001). In total, 140 mtDNA sequence variants at six nucleotide positions were identified. No association of mtDNA content, neither with distinct mutational categories nor with clinicopathological parameters was found. Correlation analyzes revealed inverse correlation of hypermethylated *TP21* and positive correlation of hypomethylated *BRCA2* ($P<0.05$ and $P<0.05$) with mtDNA content as well as inverse correlation of hypomethylated Progesterone receptor and positive correlation of hypermethylated *GSTP1* with mtDNA mutations ($P<0.05$ and $P<0.05$).

Conclusion: This study provides closer insights into how impaired intergenomic-crosstalk might contribute to breast carcinogenesis.

Keywords: mitochondrial DNA (mtDNA), breast cancer, MALDI-TOF MS, genotyping, methylation.

Introduction

The hereditary information of human beings is primarily stored in the nuclear-, the mitochondrial- and the epigenome and alterations in each of them have been implicated with the carcinogenic process [135-137]. Nuclear DNA (nDNA) accounts for the majority of the human genetic information, represented by approximately 3 billion base pairs on 46 chromosomes. Frequent nDNA changes linked to carcinogenesis include mutations, deletions, duplications and microsatellite alterations. Human mitochondrial DNA (mtDNA), however, represents just a minor part of the genome consisting of 16569 base pairs which encode 37 genes involved in energy metabolism, aging and initiation of apoptosis [138]. In 1956 Warburg hypothesised that cancer is a disease caused by mitochondrial dysfunction [139]. Due to close proximity to reactive oxygen species (ROS), lack of protective histone proteins and less efficient DNA repair mechanisms, compared to nuclear DNA (nDNA), mtDNA is more vulnerable to oxidative injury and has a higher level of mutation rate [140-142], implying its importance in pathogenesis of cancer. In contrast to the nuclear and the mitochondrial genome the epigenome isn't encoded by - but can be defined as an add-on to the DNA sequence. Epigenetic patterns have been shown to have tremendous influence on the regulation of gene expression. Hypermethylation of cell cycle related genes like tumor suppressor genes or DNA mismatch repair genes has been shown to be a common event in many cancers leading to increased cell proliferation and impaired apoptosis and thereby driving tumorigenesis [143].

Although the nuclear and mitochondrial genomes are spatially separated, mitochondrial and nuclear functions are bidirectionally linked through *i*) anterograde regulation where the information-flow is directed from nucleus towards mitochondria, and *ii*) retrograde regulation which is defined by the reverse condition, in which nuclear gene expression is influenced by mitochondrial signals [144]. Under pathophysiological conditions like cancer mitochondrial retrograde response might be influenced by mtDNA alterations as mtDNA mutations and/or mtDNA copy number changes potentially lead to impairment of mitochondrial to nucleus communication. Indeed, mtDNA mutations found in the D-Loop and near the replication origin of the heavy-strand have been shown to decrease mtDNA copy number [78, 145] and in turn, mtDNA copy number reduction has been implicated with altered nuclear DNA methylation. Smiraglia et al demonstrated that mitochondria depleted rho^0 cell-lines exhibit a significant degree of aberrant CpG-island methylation compared to their parental cell-lines [146]. Xie et al. showed that loss or reduction of mitochondrial DNA results in the induction of DNA methyltransferase-1 and in hypermethylation of the promoters

of several nuclear genes including *endothelin B receptor*, *O^6-methylguanine-DNA methyltransferase, and E-cadherin* [147]. These findings suggest that mtDNA mutations, mitochondrial DNA copy number and nuclear DNA methylation are not independent from each other; instead it appears that they interact in a complex network.

To address this hypothesis we performed a correlation study using a cohort of 45 breast cancer patients. Correlation between mitochondrial DNA content, mitochondrial DNA mutations (located in D-Loop, 12SrRNA, 16SrRNA) and methylation status of 22 breast cancer candidate genes (*APC, BIN1, BMP6, BRCA1, BRCA2, CADHERIN1, CST6, DAPK1, EGFR, ESR2, GSTP1, NES1, Nm23-H1, P16, P21, Progesterone receptor, Prostasin, RAR-b, RASSF1, SRBC, TIMP3 and TP53*) was assessed using quantitative PCR and matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF).

Materials and Methods

Tissue Samples

The study was approved by the local ethical committee and department of pathology, Shahid Betheshi Medical University, Iran. Forty-five paired formalin-fixed paraffin-embedded samples from cancerous breast tissues and paired normal breast tissues were examined by two experienced pathologists. Clinical data of patients can be obtained from Table 1. DNA was extracted from paraffin-embedded samples (around 20 mg of tissue) using a High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's protocol and eluted into 150 μL of elution buffer. The eluted DNA was stored at -20°C until further use.

Cell culture

MDA-MB-231, MCF-7, and HS578T cells were grown in Dulbecco's modified Eagle medium (DMEM) (4.5g/l glucose L-glutamine); BT549 and T47D cells were grown in RPMI 1640 (L-glutamine); SKBR3 cells were grown in McCoy's 5A medium. All media purchased from Gibco/Invitrogen (Carlsbad, CA, USA) were supplemented 10% FCS and 1% penicillin-streptomycin. The cells were maintained in a humified incubator at 37°C 5% CO2, and harvested at 80% confluency. Before DNA extraction the cells were washed 1xPBS. DNA was extracted from $1x10^6$ cells by using the High Pure PCR Template Preparation Kit (Roche Diagnostics, Mannheim, Germany) and eluted in 100 μL of elution buffer and stored at -20°C until further use.

Quantitative analysis of mtDNA using Real-time PCR

For simultaneous quantification of nDNA and mtDNA a multiplex TaqMan real-time PCR was performed using both Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) mitochondrial ATPase subunit 8 (*MTATP 8*) reference genes. The *GAPDH* and *MTATP 8* primer and probe sequence information is shown in Supplementary Data 1 (Table 1). The PCR was performed using the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Branchburg, New Jersey, USA). 2.5 µL DNA were used as template for the PCR analysis. The real-time PCR was carried out in 25 µL of total reaction volume using a 2 minute incubation at 50°C, followed by an initial denaturation step at 95°C for 10 minutes and 40 cycles of one minute at 60°C and 15 seconds at 95°C.

The theory, practice, the amplification efficiency and results of quantitative PCR for both *GAPDH* and *MTATP 8* has been demonstrated in our previous study [148] and information about how data were processed can be retained from Kohler et al. 2009 [115].

Assay design for qualitative analyses of mtDNA variants

Based on the Cambridge Reference Sequence (CRS) of the human mitochondrial genome and the publication of Zhu et al (2005)[149], in total 45 loci were selected as input for the assay design. Due to the compromise of amplicon length, primer length and the mass range, and to reduce primer-dimer potential and false priming potential, 22 somatic mutations were selected for further analysis. Of these 22 somatic mutations, three were located in D-Loop, one in 12S ribosomal RNA, one in 16S ribosomal RNA, two in tRNA, 11 in NADH dehydrogenase subunits, one in ATPase subunit 8, one in Cytochrome C oxidase subunite III, and one in cytochrome b region. Detailed information about the location of the analyzed loci can be obtained from Figure 1 and Supplementary Data 1 (Table2).

Iplex-assay for the detection of mtDNA variants

For each variant, two primers for amplifying the product containing the variant (capture primers) and one primer for extending each variant (extension primer) were designed using MassArray Assay Design v.3.1 (Sequenom, San Diego, USA). Sequence information of SNP capture and extension primers are shown in Supplementary Data 1 (Table 2). According to the manufacture's protocol a 10-mer tag (5'-ACGTTGGATG-3') was added to the 5'end of each primer to avoid chaos in mass spectrum and improve PCR efficiency.

The SNP capture PCR reactions for amplifying 22 fragments containing the 22 sequence variants in a single well were carried out in 10 µL PCR cocktail mix containing 1µL

DNA, 1.626 mM MgCl2, 500 µM dNTP mix, 0.5 U HotStarTaq DNA polymerase and primer pairs. The amplification was performed under the following conditions: incubation at 94°C for 15 minutes, followed by 45 cycles of 94°C for 20 seconds, 56°C for 30 seconds, 72°C for one minute and final extension at 72°C for 5 min. To remove the non-incorporated dNTPs, a shrimp alkaline phosphatase (SAP) treatment was performed after the PCR reaction under the following conditions: 37°C for 20 minutes, 85°C for 5 minutes and cooling to 4°C. The iPLEX extension reaction was performed using the iPLEX gold cocktail mix (Sequenom, San Diego, USA), which contains buffer, termination mix, enzyme and extension primers, under the following conditions: 94° C for 30 seconds, followed by 40 cycles of 94°C for 5 seconds, plus 5 sub-cycles of 52°C for 5 seconds and 80°C for 5 seconds; one cycle of final extension at 72°C for 5 min, then cooling to 4°C. The iPLEX reaction products were desalted using clean resin to optimise mass spectrometric analysis and dispensed onto a 384-element SpectroCHIP bioarray by using a nanodispenser (Sequeonom, San Diego, USA) for MALDI-TOF MS analysis. The MassARRAY 4.0 software (Sequeonom, San Diego, USA) was used to process and analyze iPLEX SpectroCHIP bioarrays. The call rate, extension rate and peak area for all allele-specific analyses in any given assay were calculated using the software provided by the manufacturer. DNA from a mitochondria negative cell line, which was obtained by sub-culturing that cell line in the presence of low concentration of ethidium bromide until the cells were devoid any of mitochondria, was used as control. The negative control was run at each step and on each chip.

Methylation analysis using thymidine-specific cleavage MassArray® on MALDI-TOF MS

In a previous study we used high-throughput analysis to determine DNA methylation patterns of 22 candidate cancer gene promoters (*APC, BIN1, BMP6, BRCA1, BRCA2, CADHERIN1, CST6, DAPK1, EGFR, ESR2, GSTP1, NES1, Nm23-H1, P16, P21, Progesterone receptor, Prostasin, RAR-b, RASSF1, SRBC, TIMP3* and *TP53*) in the breast cancer and paired normal tissue samples [150]. Sequenom`s EpiTYPER™ assay in combination with matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MassCLEAVE™ reagent was performed according to the previously published protocols [150-152]. Epitect® Bisulfite Kit (QIAGEN AG, Basel, Switzerland) was used for the conversion of the target sequence, followed by PCR and in vitro transcription. For final analyzes the product was robotically dispensed (nanodispenser) onto silico chips preloaded with matrix (SpectroCHIP; SEQUENOM, San Diego) and analyzed in a mass

spectrometer (MALDI-TOF MS). Mass spectra were collected using a MassARRAY Compact MALDI-TOF (SEQUENOM) and spectra's methylation ratios were generated by the Epityper software v1.0 (SEQUENOM, San Diego).

Statistical analysis

Data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, USA). The Shapiro-Wilk test was used for the analysis of data distribution. The data were not normally distributed. The Mann–Whitney-U test and Kruskal–Wallis test were applied to compare the mtDNA content between individuals with different mutation status/categories and tissue types. Pearson chi-square test was applied to analyze the association between mtDNA variants and clinicopathological parameters as well as the association between tissue types and clinicopathological parameters.

For gene clustering, pair-wise similarity metrics were calculated for each gene separately based on methylation ratio of cancer tissues across the paired paired normal tissues. The procedure was performed using the double dendrogram function of the Gene Expression Statistical System for Microarrays (GESS) version 7.1.19 (NCSS, Kaysville, Utah, USA)[150-152]. To evaluate whether there is a correlation between mtDNA mutations and mtDNA content with percentage of promoter methylation of the 22 candidate cancer genes a Spearman`s rho correlation was performed. For all analysis P-values ≤ 0, 05 were considered statistically significant.

Results

Comparison of mtDNA content between cancerous and paired normal tissue

MtDNA content in cancerous tissues was significantly reduced compared to paired normal tissue (Mann-Whitney: P=0.001) (Figure 2A). MtDNA contents in cell lines were 1478.58, 809.00, 3350.13, 3040.30, 910.18, and 2.89-fold high in BT549, HS578T, T47D, SKBR3, MCF-7, and MDA-MB231 cells, respectively.

Simultaneous analysis of 22 mitochondrial loci using the MALDI-TOF MS based 22-plex assay

Using the MALDI-TOF MS 22-plex assay, we examined 22 mtDNA loci in 45 cancerous and paired normal tissue samples, as well as in 6 breast cancer cell lines. In total 140 sequence variants at 6 nucleotide positions were found, thereof 130 in tissue samples and

10 in breast cancer cell lines; two were located in D-loop, one in 12S RNA, one in 16S RNA, and one in tRNA- threonine (Table 2). The location of the variants analyzed and mass spectra of detected variants and their corresponding wild-types in tissue samples are shown in Figure 1.

Association between mtDNA variants and clinicopathological parameters

Furthermore we compared clinicopathological parameters with mtDNA content between variant positive and negative individuals in cancerous and normal tissues. In this study we did not find mtDNA content in cancerous and normal tissues was associated with age, tumour type, tumour size, lymph node involvement, extent of metastasis, stage, histological grading, and ER, PR, and HER-2/neu receptors of neither variant positive nor negative individuals (Supplementary 2, Table 1).

Relation between mtDNA content and mtDNA mutations

Comparison of mtDNA content between mutation negative and mutation positive individuals within the cancerous, the paired normal group and within both groups combined showed no significant difference (Mann-Whitney: P=0.475 and Mann-Whitney: P=0.163). For analyzing the relation between mtDNA content and different mtDNA mutation categories we divided the individuals in 3 groups according to their mutational status: individuals carrying somatic mutations (mutation either present in cancerous or normal tissue); individuals carrying germline mutations (mutations present in cancerous and paired normal tissue); individuals without mutations. No significant difference could be found between the mtDNA content of individuals carrying somatic mutations, individuals carrying germline mutations and individuals without mutations (Kruskal-Wallis: P=0.311).

Correlation of mtDNA content and mtDNA mutations with methylation status of 22 candidate breast cancer genes

Two-way hierarchical cluster analysis comparing the methylation status in cancerous and paired normal tissue is shown in Figure 2B. We correlated mtDNA content and mtDNA mutations with the percentage of methylation of 22 candidate breast cancer genes. We found significant positive correlation between mtDNA content and the percentage of methylation for *BRCA2* ($P<0.05$; R=0.477), and significant negative correlation between the same parameters for *TP21* ($P<0.05$; R= -0.582) in cancerous tissues (Figure 2C). Between mtDNA mutations and percentage of methylation there was a positive correlation for *GSTP1* ($P<0.05$; R=0.489)

and a negative correlation for *Progesterone receptor* (*P<0.05*; R=-0.567) in cancerous tissues; whereas we found positive correlation for *TIMP3* (*P<0.01*; R=0.579) in the corresponding normal tissue. For comparing nDNA methylation status within different mtDNA mutation categories we divided the individuals in 3 groups as previously described. Significant difference in the methylation status was found only for the progesterone receptor gene (Kruskal-Wallis: *P<0.05*) in cancerous tissues (Figure 2D).

Discussion

Proper cellular function requires a tight interplay between distinct cellular compartments. In this regard mitochondria-to-nucleus retrograde and nucleus-to-mitochondria anterograde signaling plays an important role. Defects in this inter-genomic communication can result in altered cell metabolism and aberrant signalling, triggering carcinogenesis. Mutations in nuclear DNA are thought to lead to disruption of the normal cross-talk that regulates the integrity and quantity of mtDNA [153]. On the contrary depletion of mtDNA was shown to regulate nuclear cancer associated genes and to lead to chromosomal instability [147, 154-155]. To get better insight in the underling mechanisms that drive carcinogenesis in an intergenomic manner we investigated the correlation between mtDNA content, the mutational status of 22 mtDNA loci and methylation of 22 candidate genes in 45 breast cancer and corresponding normal tissues.

Significantly reduced mtDNA content in cancerous tissue compared to paired normal tissue has been reported in previous studies [78]. It has been hypothesized that mutations in the displacement-loop (D-Loop), a region containing the origin of replication of the H-strand and the promoters for L- and H-strand transcription, might lead to impaired transcription and replication and finally to depletion of mtDNA. Indeed, there is evidence that mtDNA depletion is correlated with the presence of somatic mtDNA mutations [70, 145]. Therefore we analyzed loci located in D-Loop, 12S rRNA, 16S rRNA and tRNA-T and correlated mutational status with mtDNA content. Although five of the six sequence variations have been reported previously as polymorphisms in Mitomap, some of the variations we only found in the patient but not in the corresponding normal sample, suggesting that in these patients the variant is of somatic character. Loci which were reported as polymorphisms but also showed somatic mutations at the same position include: 709G>A; 15924A>G; 16145G>A. As the studied population might be heterogenous and composed of a variety of haplogroups, there is the possibility that sequence variants can be highly abundant in one

haplogroup, and occur as somatic mutation in another haplogroup. Although we found significantly reduced mtDNA content in cancerous tissue compared to normal paired tissue (P=0.001), we couldn`t find a correlation with neither somatic mtDNA mutations nor with clinicopathological parameters.

Performing correlation studies we found inverse correlation between mtDNA content of variant positive individuals with hypermethylation of the *TP21* and positive correlation with hypomethylation of *BRCA2* gene. P21 is a cyclin-dependent kinase (cdk) inhibitor, known to inhibit the activity of cyclin/cdk2 complexes and thereby negatively modulate cell cycle progression. Hypermethylation of *TP21* promoter might therefore result in transcriptional repression and promotion of cell cycle progression leading to a proliferative phenotype. Although there has been no report of a direct effect of p21 loss on mtDNA depletion, there might be an indirect effect via p53, since p21 is a transcriptional target of p53 [156]. In breast cancer p53-transactivation of *TP21* is frequently impaired due to p53 inactivation [157]. In turn loss of p53 is known to mediate depletion of mtDNA by down regulating p53R2 [158-159]. On the other hand p21 is also an inhibitor of apoptosis and p21 repression may therefore have an anticancer effect [160]. Schauen et al. found that respiratory chain deficiency slows down cell-cycle progression via reduction of ROS which also was associated with downregulation of $p21^{CIP1/WAF1}$ in mitochondria deficient ρ^0-cells [161].

Although *BRCA2* gene promoter hypomethylation has been shown to correlate with elevated levels of *BRCA2* mRNA and tumor stage there is no previous report on how *BRCA2* expression might influence alteration of mtDNA content and reverse. Hypermethylation of *Glutathione S-transferase π* (*GSTP1*) was positively correlated and hypermethylation of *Progesterone receptor* (*PGR*) was inversly correlated with mtDNA mutations. It is known that Glutathione S-transferases, the enzymatic products of the *GSTP1* gene play an important role in the protection of DNA from oxidative damage [162] and that loss of *GSTP1* due to promoter hypermethylation is a common event in prostate and breast carcinogenesis [163-164]. Yet, there is no evidence that this promoter hypermethylation might be associated with mutations in mtDNA. Although hypermethylation of estrogen and progesterone receptor gene 5' CpG islands have been found to be correlated with lack of estrogen and progesterone receptor gene expression in breast tumors [165], very few studies analyzed the relationship between progesterone receptor expression and mtDNA mutations. Only Tseng et al reported that the occurrence of D-Loop mutations in mitochondria of breast cancer patients was associated with tumors that lacked expressions of estrogen receptor and progesterone receptor [70]. This is in concordance to our data and remains to be further evaluated.

To get a detailed insight into the mechanisms leading to impairment of intergenomic cross-talk between mitochondria and nucleus in breast cancer we analyzed the interplay of mtDNA content, mtDNA mutations and methylation status of 22 candidate breast cancer genes. Regarding mtDNA content in cancerous tissues our data confirm the results of previous studies which also found mtDNA depletion in cancerous compared to normal patients. Although we couldn't find a correlation between mtDNA depletion and somatic mutations we have indication that in this cohort mtDNA depletion might be associated with loss of p21 expression either due to direct inhibition via *TP21* hypermethylation or as consequence of impaired p53-transactivation due to loss of p53. However, we cannot exclude the possibility that mtDNA depletion is caused by a completely different mechanism. In total, our data provide some insight in how and by which factors intergenomic cross-talk might be deregulated in breast cancer patients. Decrypting these complex intergenomic mechanisms between nucleus and mitochondria in larger cohorts should be the aim of further studies.

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Tables and table headings

Table 1. Clinical data of patients according to 2 different breast cancer types.

Breast cancer type	Total no. of patients	Age (years) (mean ± s.d. [range])	Tumor size (cm) (mean ± s.d. [range])	No. of patients with lymph node involvement	No. of patients with metastasis	ER status		PR status	
						pos	neg	pos	Neg
Infiltrating ductal carcinoma	37	49± 11.45 [32-78]	3.0 ± 2.6 [0.8-12]	31	8	11	26	14	23
Infiltrating lobular carcinoma	8	46 ± 11.9 [32-65]	2.6 ± 1.7 [1.5-4.5]	5	1	1	7	1	7

Table 2. Mutation frequencies of variant positive mtDNA loci. mtDNA sequence variants were present at six out of 22 nucleotide positions in 45 paired breast/normal tissues and six breast cancer cell lines. Variation positive mtDNA loci have been found in D-loop (displacement-loop); 12S (12S ribosomal RNA); 16S (16S ribosomal RNA); tRNA-T (tRNA threonine) genes.

Gene	Gene ID	Nucleotide position/ modification	Number of times detected			Frequency (%)		
			Germline	Somatic (Cancer/Normal)	Cell line	Germline	Somatic	Cellline
D-Loop	–	311-315 ->Cins	44	0	6	97.7	0.0	100.0
12S	4549	709G>A	6	(1/0)	1	13.3	(2.2/0)	2.2
16S	4550	2706A>G	4	0	3	8.8	0.0	50.0
tRNAT	4576	15924A>G	2	(2/0)	0	4.4	(4.4/0)	0.0
D-Loop	–	16145G>AG	0	(0/3)	0	0.0	(0/6.7)	0.0
D-Loop	–	16145G>A	6	(2/2)	0	13.3	(4.4/4.4)	0.0

Figures and figure legends

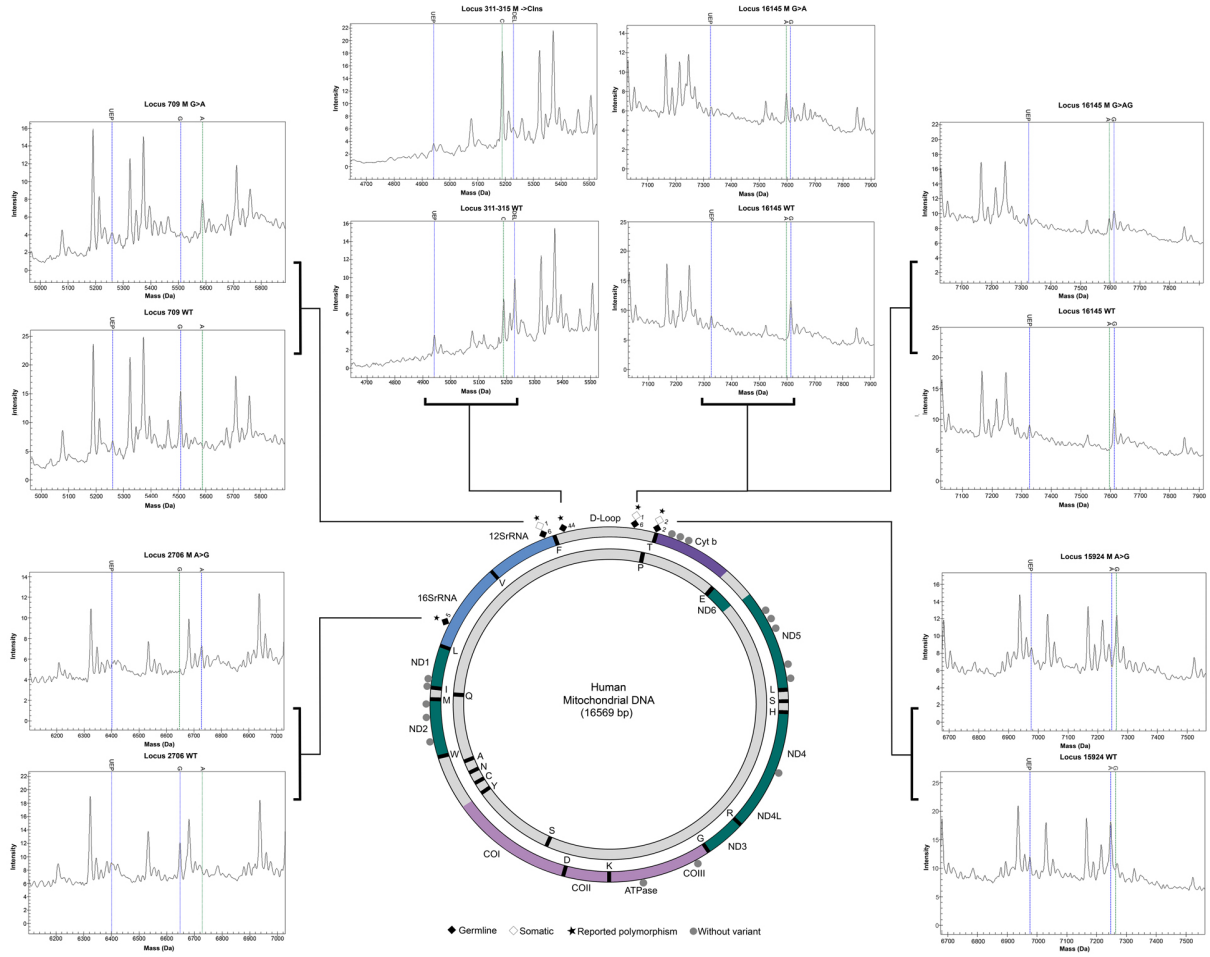

Figure1. Schematic illustration of mtDNA structure with positions of 22 analyzed mtDNA loci and MALDI-TOF MS spectrograms of the six variants detected. The mtDNA structure shows the 37 genes including 13 protein encoding genes, 22 tRNAs (black bars) and 2 rRNAs. Grey filled circles represent loci where no variant was found; Black filled rhombs represent germline mutations; white filled rhombs represent somatic mutations. Numbers on the right side of symbols indicate frequency of occurrence. MALDI-TOF spectrograms for each detected mutation (M) and the corresponding wild-type (WT) are depicted. Similar colored dashed lines represent the unextended primers (UEP) and the respective wild-type alleles.

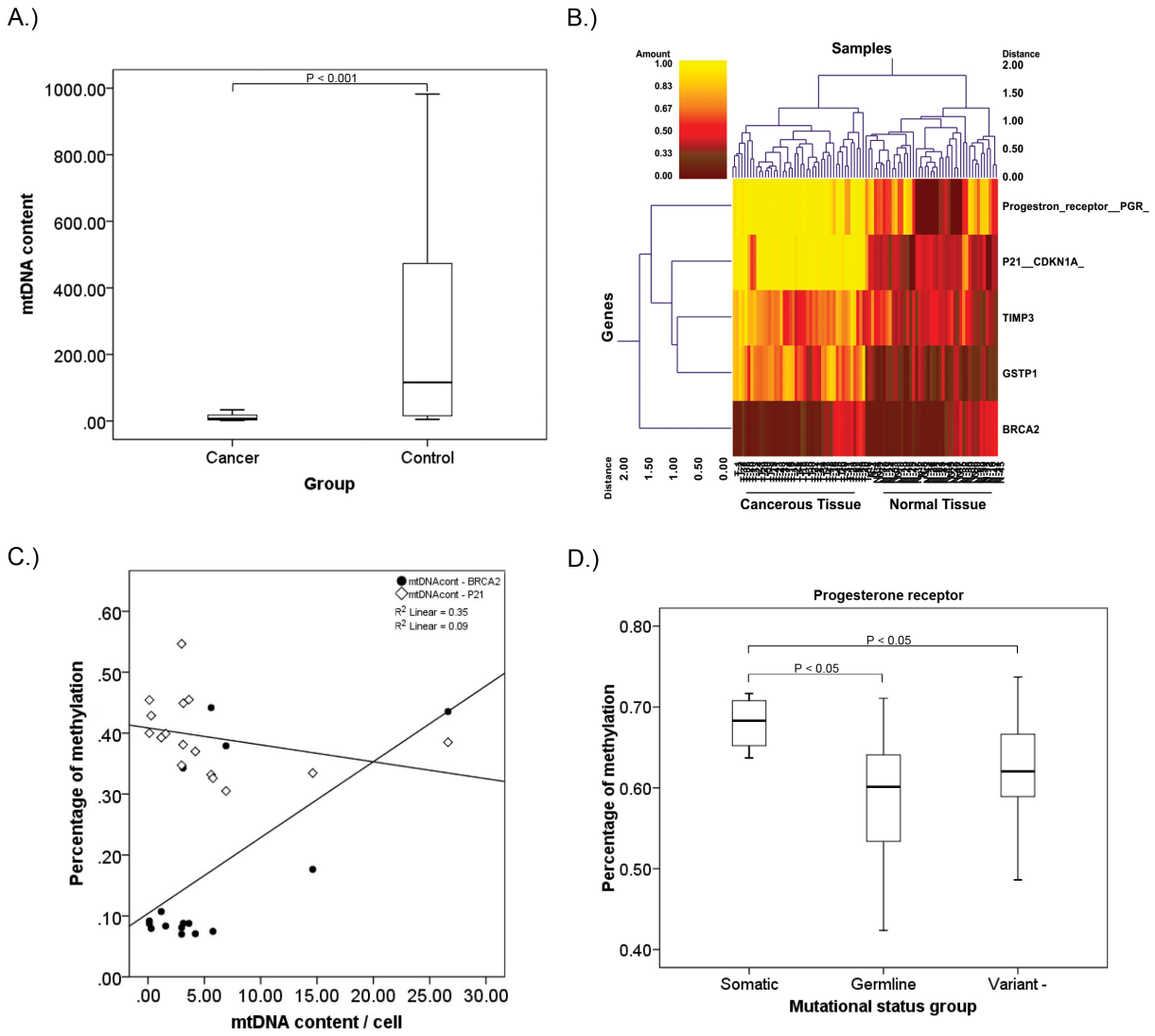

Figure2. A.) Boxplot for comparison of mtDNA content between paired cancer and normal breast tissues. (significant: Mann-Whitney-U; $P < 0.001$). B.) Methylation analysis of informative CpG sites for the five genes that showed correlation of percentage of methylation with either mtDNA content or mutational status. Two-way hierarchical cluster analysis of 45 cancerous breast and corresponding normal tissues (red clusters indicate 0% methylated; yellow clusters indicate 100% methylated; color gradient between red and yellow indicates methylation ranging from 0 to 100). C.) Correlation of mtDNA content with percentage of methylation for significant genes (*BRCA2*/*P21*). D.) Boxplot for comparison of percentage of methylation within different mutational status groups for *Progesterone receptor* (significant: Kruskal-Wallis: *P<0.05*).

Supplementary Data 1

Supplementary Table 1. Primer and probe sequence information for *GAPDH* and *MTATP 8* reference genes used for simultaneous quantification of nDNA and mtDNA by multiplex TaqMan real-time PCR.

Gene	Gene ID	Primer and Probe Sequences (5`→3`)		Length of primer/ probe	Amplicon lengths (bp)
GAPDH	2597	Forward	CCC CAC ACA CAT GCA CTT ACC	21	97
		Reverse	CCT AGT CCC AGG GCT TTG ATT	21	
		Probe	(MGB) TAG GAA GGA CAG GCA AC (VIC)	17	
MTATP8	4509	Forward	AAT ATT AAA CAC AAA CTA CCA CCT ACC	27	78
		Reverse	TGG TTC TCA GGG TTT GTT ATA	21	
		Probe	(MGB) CCT CAC CAA AGC CCA TA (FAM)	17	

Supplementary Table 2. Gene, gene ID, nucleotide positions of analyzed loci and sequence information of SNP capture and extension primers used for MALDI-TOF MS 22-plex mtDNA mutation assay.

Gene	Gene ID	Nucleotide position (np)	Mutation capture primer	Extension primer
D-Loop	-	207	ACGTTGGATGTTACAGGCGAACATACTTAC ACGTTGGATGAAGTGGCTGTGCAGACATTC	TACATGCGAACATACTTACTAAAGTGT
D-Loop	-	311-315	ACGTTGGATGAAATTTCCACCAAACCCCCC ACGTTGGATGGCTGGTGTTAGGGTTCTTTG	CAAACCCCCCCTCCCCC
12S	4549	709	ACGTTGGATGAGGTTTGGTCCTAGCCTTTC ACGTTGGATGTCGTGGTGATTTAGAGGGTG	GGGTGAACTCACTGGAA
16S	4550	2706	ACGTTGGATGAGGGTTCAGCTGTCTCTTAC ACGTTGGATGCATAGGGTCTTCTCGTCTTG	GTTATTCTCGTCTTGCTGTGT
ND1	4535	3849	ACGTTGGATGACACCTCTGATTACTCCTGC ACGTTGGATGTCGGTTGGTCTCTGCTAGTG	TCCTGCCATCATGACCCTT
tRNA-I	4565	4323	ACGTTGGATGCCCTCAAACCTAAGAAATATG ACGTTGGATGGGTTCGATTCTCATAGTCCT	CCCCATAGGAGCTTAAACCCCC
ND2	4536	4499	ACGTTGGATGTGTTGGTTATACCCTTCCCG ACGTTGGATGTGATGAGTGTGCCTGCAAAG	GCCCAACCCGTCATCTA
ND2	4536	4665	ACGTTGGATGCATCAAGTATTTCCTCACGC ACGTTGGATGATGGTTCATTGTCCGGAGAG	TCCTCACGCAAGCAACC
ND2	4536	5240	ACGTTGGATGCTTAATTCCATCCACCCTCC ACGTTGGATGCTTCGATAATGGCCCATTTG	GATTTGGGCAAAAAGCCGGT
ATP8	4509	8498	ACGTTGGATGCACAAACTACCACCTACCTC ACGTTGGATGCGTTCATTTTGGTTCTCAGG	GGTTCTCAGGGTTTGTTATAATTTT
COXIII	4514	9885	ACGTTGGATGTATCTGCTTCATCCGCCAAC	TCCGCCAACTAATATTTCAC

			ACGTTGGATGAAAATGCCAGTATCAGGCGG	
ND4	4538	11768	ACGTTGGATGCGGGCTTACATCCTCATTAC ACGTTGGATGTGAGAGAGGATTATGATGCG	GGGCACTCAAACTACGAACGC
ND5	4540	12642	ACGTTGGATGCATCCCTGTAGCATTGTTCG ACGTTGGATGGATTAATGTTTGGGTCTGAG	GAGTTACATGGTCCATCATAGA
ND5	4540	12852	ACGTTGGATGATCAGTTGATGATACGCCCG ACGTTGGATGGAAACCGATATCGCCGATAC	TTCTCAGCAGCCATTCAAGCAAT
ND5	4540	13263	ACGTTGGATGATCGTAGCCTTCTCCACTTC ACGTTGGATGAGGAATGCTAGGTGTGGTTG	TTATTGTAACTATTATGAGTCCTAG
ND5	4540	13398	ACGTTGGATGCTATTTATGTGCTCCGGGTC ACGTTGGATGGGTTGAAGTGAGAGGTATGG	ACAACCTTAACAATGAACA
ND5	4540	13674	ACGTTGGATGGCTTCCCCACCCTTACTAAC ACGTTGGATGAATCCTGCGAATAGGCTTCC	CCCCCCTTACTAACATTAACGAAAA
ND5	4540	15700	ACGTTGGATGTCCCCATCCTCCATATATCC ACGTTGGATGGCGGCTAGGAGTCAATAAAG	GAGTGATTGGCTTAGTGG
CYTB	4519	15783	ACGTTGGATGGCAGACCTCCTCATTCTAAC ACGTTGGATGCGGATGCTACTTGTCCAATG	GACAACCAGTAAGCTACC
CYTB	4519	15824	ACGTTGGATGCATTGGACAAGTAGCATCCG ACGTTGGATGGGGAGATAGTTGGTATTAGG	CCTCAGCATCCGTACTATACTTC
tRNA-T	4576	15924	ACGTTGGATGAAATGGGCCTGTCCTTGTAG ACGTTGGATGTTTCTCTGATTTGTCCTTGG	ACACTAATACACCAGTCTTGTAA
D-Loop	□	16145	ACGTTGGATGGCCAGCCACCATGAATATTG ACGTTGGATGGGGTTTTGATGTGGATTGGG	TATTGTACGGTACCATAAATACTT

Supplementary Data 2

Table 1. Comparison of clinicopathological parameters with mtDNA content between variant positive and negative individuals in cancerous and paired normal tissues.

Clinico-pathological parameters	Sub-classification	n (paired)	mtDNA content (Cancerous) Median		*P*-value	mtDNA content (Normal) Median		*P*-value
			With variant	Without variant		With variant	Without variant	
Age	< 50	27	15.77	5.8	0.548	119.44	50.39	0.576
	≥ 50	18	4.98	3.38		188.5	60.38	
Histological type	Ductal	38	9.88	3.63	0.782	30.91	50.39	0.791
	Lobular	7	5.76	5.7		216.02	352.46	
Primary tumor	T1	20	19.23	6.32	0.372	21.86	66.95	0.39
	T2	14	6.92	2.98		30.91	53.82	
	T3	7	5.76	7.9		823.14	37.67	
Lymph node involment	Negative	7	17.87	1.53	0.684	108.04	106.15	0.828
	Positive	37	5.76	5.8		30.91	53.82	
Metastasis	M0	37	12.45	5.8	0.747	123.46	66.95	0.81
	M1	7	3.6	3.11		211.82	53.82	
Stage	I	5	17.87	1.19	0.87	108.04	9.48	0.394
	II	24	6.43	5.6		18.96	66.95	
	III	6	10.19	-		584.61	-	
	IV	6	6.92	3.11		420.22	53.82	
Nuclear grading	1	3	24.25	3.75	0.18	1406.29	7.3	0.159
	2	11	3.59	10.08		184.47	96.37	
	3	15	1.59	2.98		10.48	49.35	
ER	Negative	12	2.98	3.4	0.575	16.06	49.35	0.067
	Positive	17	9.88	15.13		823.14	572.56	
PR	Negative	12	1.56	3.11	0.208	7.31	49.35	0.235
	Positive	17	9.88	16.43		823.14	79.98	
Her2	Negative	12	12.45	3.11	0.171	665.8	106.15	0.398
	Positive	17	2.98	16.43		16.06	52.1	
P53	Negative	26	6.34	3.63	0.52	26.38	50.39	0.54
	Positive	3	15.03	1.55		2304.13	106.15	
PS2	Negative	18	8.4	3.51	0.93	26.38	52.1	0.723
	Positive	10	10.39	26.63		665.8	106.15	

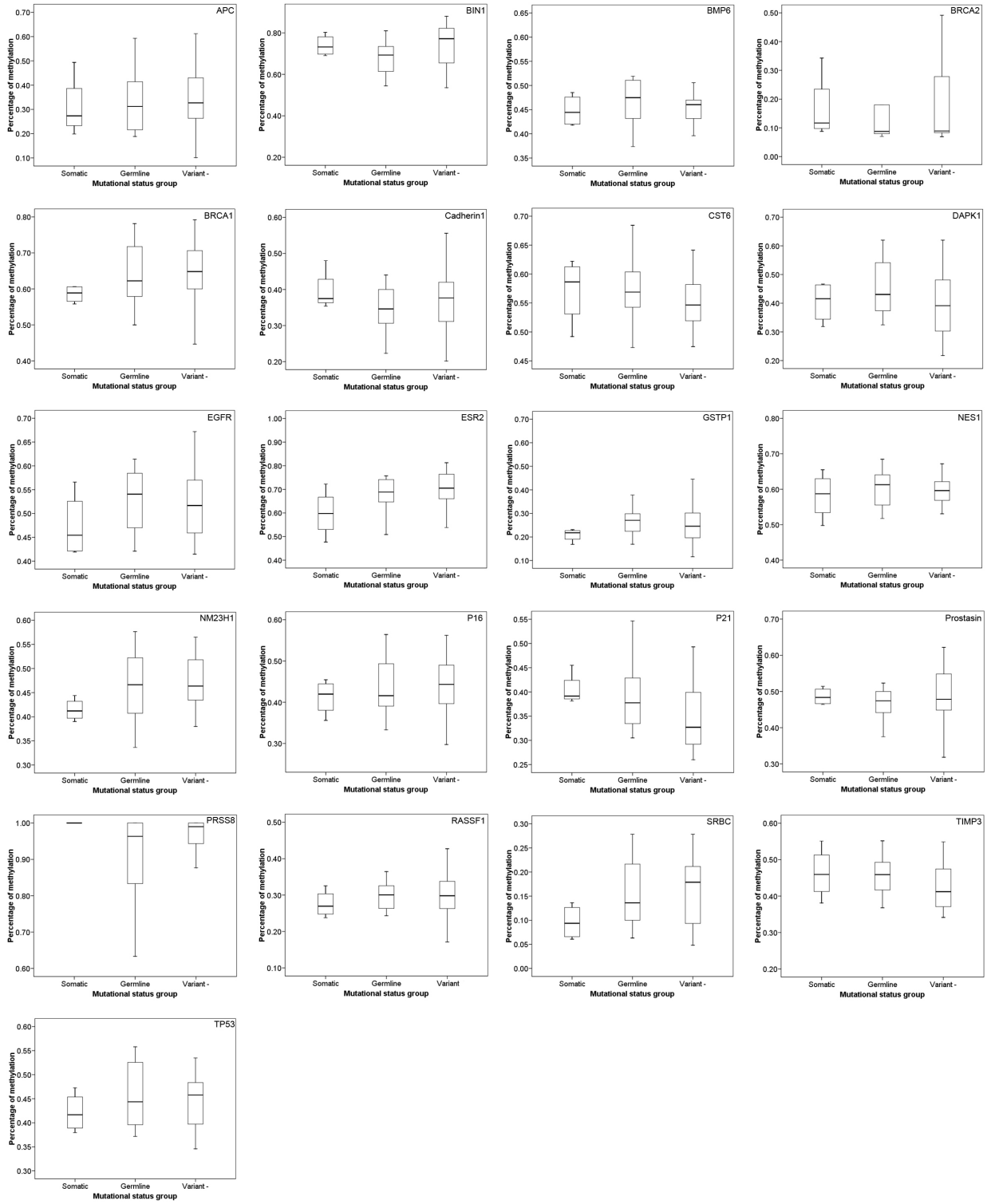

Figure 1. Comparison of nDNA methylation status within 3 different mtDNA mutation categories (somatic; germline; variant-) for non-significant genes.

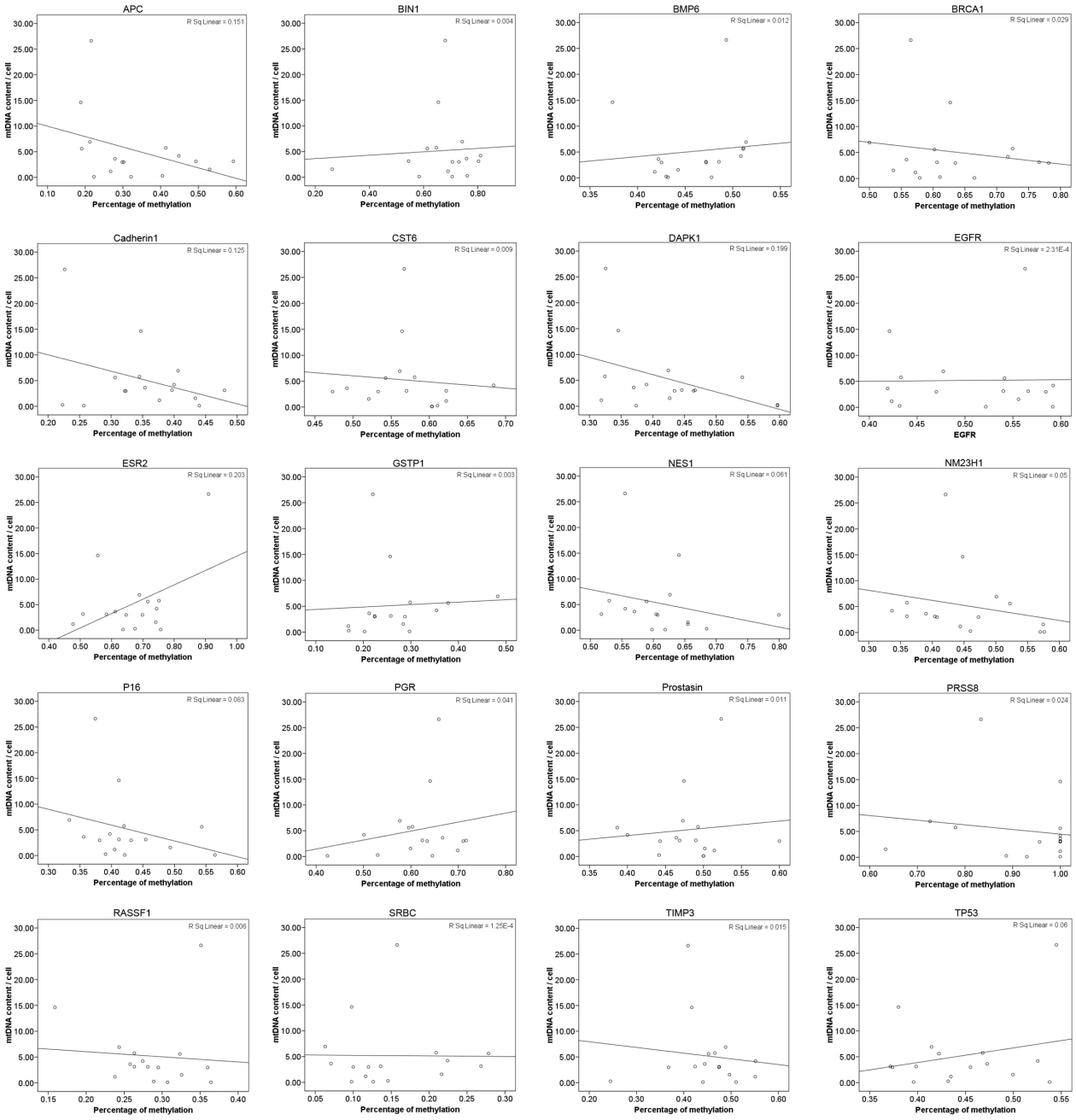

Figure 2 . Correlation of nDNA methylation status with mtDNA content per cell for non-significant genes.

3.6 Preliminary data:

The PGC Regulatory Network and its Implication in Decreased MtDNA Content in Breast Cancer

Summary: In our previous study analyzing the inter-genomic cross-talk between mtDNA alterations and methylation of nuclear encoded genes, we could not find a meaningful correlation between mitochondrial DNA content, mitochondrial DNA mutations and methylation status of breast cancer related genes. In cancer, reduction of mtDNA content and mitochondrial dysfunction has also been associated with oxidative stress, deregulation of PGC-1 co-activators and their target genes. Within this study we investigated the PGC-1 regulatory network of to evaluate its role in the regulation of mtDNA content in breast cancer.

***Author contributions:** *Corina Kohler* was involved in experimental design, performing the experiment, data analysis and writing of the manuscript

Preliminary data:
The PGC Regulatory Network and its Implication in Decreased MtDNA Content in Breast Cancer

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Abstract

Background: In cancer, reduction of mtDNA content and mitochondrial dysfunction has been associated with oxidative stress, deregulation of PGC-1 co-activators and their target genes. Within this study we investigated the PGC-1 regulatory network of to evaluate its role in the regulation of mtDNA content in breast cancer.

Methods: Thirty paired samples (breast cancer and adjacent normal tissues) were checked for mtDNA content, mRNA expression of PGC-1α and PGC-1β and their target genes using Taqman quantitative real time PCR. Hypoxic status and oxidative DNA damage were assessed by HIF-1α expression and ARP-assay, respectively. Methylation analysis of PGC-1β promoter region will be performed using pyrosequencing.

Results: MtDNA content in cancer tissues was significantly lower in comparison with paired normal tissues ($P < 0.001$). PGC-1β was significantly down-regulated ($P<0.05$). While NRF1, TFB1M and TFB2M ($P<0.05$; respectively) showed significant up-regulation, POLRMT was, also if not significantly, down-regulated in cancer tissues. HIF-1α mRNA expression and oxidative DNA damage was higher in cancer than in normal tissues. No correlation was found between PGC-1β expression status neither with number of AP sites nor with expression of VHL/HIF-1/C-MYC. Analysis of PGC-1β promoter methylation is ongoing.

Discussion: Reduced mRNA expression of PGC-1α and PGC-1β was not associated with down-regulation of target genes, such as NRF1, TFB1M and TFB2M. POLRMT was down-regulated in breast cancer tissues what might be implicated in decrease in mtDNA content. PGC-1β down-regulation was neither correlated with oxidative DNA damage status nor with the expression of genes of the VHL/HIF-1/C-MYC-pathway. We suspect that PGC-1β promoter methylation might be causal for its down-regulation.

Introduction

Metabolic reprogramming, also known as the Warburg effect, is based on the shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis and has been suggested as an emerging hallmark of cancer [8]. Although the cellular and molecular mechanisms that promote this metabolic switch have not yet been fully elucidated, it is evident that impairment of OXPHOS and mitochondrial dysfunction are prominent features of tumor cells *[166]*. In this context, reduced mitochondrial DNA content has been observed in a variety of cancers such as breast [70], colorectal [75], gastric [76] cancers and renal cell carcinoma [77]. There is evidence that impairment of mitochondrial biogenesis, through the deregulation of genes involved in mitochondrial transcription and translation, might be crucial for reduction in mtDNA content in these cancers.

Peroxisome proliferator-activated receptor - γ coactivators PGC-1α and PGC-1β are members of a family of transcription co-activators that are involved in variety of mitochondria-related processes, including fatty acid oxidation, heme- and mitochondrial biogenesis [79]. Both co-activators are known to interact directly or via mediators with a wide range of transcription factors, such as ERR α, β, γ and nuclear respiratory factors 1 and 2 (NRF-1/-2). Downstream targets of NRF-1 and NRF-2 include the genes which code for key components involved in transcription initiation such as mitochondrial RNA polymerase (POLRMT), the mitochondrial transcription factor A (TFAM) and the dimethyladenosine transferases 1 and 2 (TFB1M, TFB2M)[167]. Thus it has been suggested that reduced expression of the PGC-1α and/or PGC-1β could lead to a repression or target genes and of mitochondrial biogenesis. Indeed, a decrease of PGC-1a expression has been found in several cancers including breast [80-81], colon [168] and ovarian cancer [169]. PGC-1β was also shown to be down-regulated in breast cancer cells [82].

Various mechanisms have been implicated in the regulation of PGC-1α and PGC-1β. PGC-1α and PGC-1β are involved in the coordination of oxidative stress defence. While overexpression of these co-activators has been linked to decreased ROS generation [170], it is suspected that down-regulation of PGC-1 co-activators might lead to increased ROS production leading to oxidative DNA damage. Barrès et al reported that non-CpG hypermethylation of the PGC-1α promoter through DNMT3B leads to reduced expression of PGC-1α what might implicate a mechanism for decreased mitochondrial content in Type 2 diabetes mellitus (T2DM) [171]. Additionally, PGC-1α and PGC-1β have been shown to be regulated in a hypoxia-dependent manner. HIF-1 mediated down-regulation of PGC-1α has been observed in epithelial cells under hypoxic conditions [172]. Zhang et al. found evidence

that HIF-1 also negatively regulates mitochondrial biogenesis via the VHL/HIF-1/C-MYC/ PGC-1β-pathway [173]. To better understand the mechanisms that lead to decreased mtDNA content in breast cancer we evaluated the regulatory network of PGC-1 co-activators and their target genes in breast cancer and adjacent normal tissue.

Material and Methods

Study cohort

The tissue samples used in this study were obtained from the UMASS Cancer Center Tissue Bank with approval of the Institutional Review Board (IRB) of UMASS Medical School. In total 60 samples (paired cancerous and normal breast tissues) of 30 patients were included in the study. Patients' data (age, tumor size, lymph node involvement, extent of metastasis, estrogen receptor, progesterone receptor and Her2/neu - status) were obtained from the pathological reports. The study cohort (n = 30) was divided into 3 groups according to pathological stage: 1.) Stage 1 (n = 10); 2.) Stage 2 (n = 10) and 3.) Stage 3 (n = 10). Clinical characteristics are of patients are summarized in Table 1.

DNA and RNA extraction

Tissues were homogenized using a tissue homogenizer (MICCRA D-3, ART Prozess - & Labortechnik GmbH & Co. KG, Müllheim, Germany). DNA and RNA were extracted using the "High Pure PCR Template Preparation Kit" (Roche) and the RNeasy Kit (Qiagen) according to the manufacturer's protocol. DNA and RNA were quantified using a Nanodrop spectrophotometer (Thermo scientific). DNA samples were stored at -20 ºC, RNA samples at -80 ºC until further use.

Multiplex real-time qPCR for quantification of nDNA and mtDNA

Simultaneous quantification of nDNA and mtDNA from tissues was performed using multiplex real-time qPCR and Glyceraldehyd-3-phosphat-dehydrogenase (*GAPDH*) and the mtDNA encoded ATPase 8 (*MTATP 8*) reference genes. The threshold cycle (Ct) values were obtained by the ABI Prism 7000 software. Each sample was analyzed in duplicate and one negative control was included in every run. The efficiency of the multiplex assay for amplifying both nDNA and mtDNA simultaneously was measured in our previous study using standard curves generated by dilution series. The content of mtDNA was calculated using the delta Ct (ΔCt) of an average Ct of mtDNA and nDNA (ΔCt = CtnDNA - CtmtDNA) in the same well as an exponent of 2 ($2^{\Delta Ct}$) [174]. The sequences and additional information of

primers and probes for the *GAPDH* and the *MTATP 8* reference genes as well as detailed reaction protocol is shown in Supplementary 1 (Table 1).

Expression analysis

Reverse Transcription of RNA to cDNA

"High Capacity cDNA Reverse Transcription Kit" (Applied Biosystems) was used for reverse transcription of RNA to single-stranded DNA. The reverse transcription reaction was carried out in 20µl of total reaction volume. For reverse transcription of RNA 10µl of a 2× RT Mastermix was prepared as follows: 2µl 10× RT Buffer, 0.8µl 25× dNTP Mix (100mM), 2µl RT Random Primers, 1µl MultiScribe^{TM} Reverse Transcriptase, 1µl RNase Inhibitor und 3.2µl H_2O. 10µl RNA template was added. The reaction was performed using a Mastercycler (Eppendorf) under the following conditions: a first step at 25ºC for 10 min, a second step at 37ºC for 2 h and a third step at 85ºC for 5 min, followed by cooling to 4 ºC.

Quantitative real-time PCR for analysis of mRNA expression

mRNA expression of selected genes was evaluated by qRT PCR. Data were normalized in relation to the geometric mean of two reference genes: Beta-2-microglobulin (B2M) and Actin-beta (ACTB). Fold-changes in gene expression were calculated using $2^{-\Delta\Delta CT}$ cycle threshold method [175]. The sequences and additional information of primers as well as detailed reaction protocol is shown in Supplementary 1 (Table 2).

ARP-Assay

Measurement of oxidative stress induced DNA damage was done using the OxiSelect™ Oxidative DNA Damage Quantitation Kit (AP sites); (Cell Biolabs, CA, US). The assay was performed according to the manufacturer's protocol. In brief, a known concentration of DNA from tissue samples was dissolved at 100µg/mL in TE-buffer. Incubation with ARP solution was performed for 1h at 37ºC. ARP-labeled DNA was obtained using ethanol precipitation. The DNA pellet was washed and dissolved in TE buffer at a concentration of 1 µg/mL. DNA was bound to a microwell plate, incubated with streptavidin-enzyme conjugate and absorbance was measured using ELISA reader (SpectraMAX 250; Molecular Devices, LLC US) at 450nm. Quantities of AP sites in unknown samples were determined by comparison with a standard curve generated from supplied DNA standard containing predetermined AP-sites (4-40 AP sites / 10^5 bp).

Methylation analysis

For bisulfite conversion of the target sequences, the Epitect Bisulfite Kit (QIAGEN AG, Basel, Switzerland) was used according to the manufacturer's protocol. Detailed protocol for PCR on bisulfite converted genomic DNA and additional information of primers is shown in Supplementary 1 (Table 3 / Figure 1). Procedure for pyrosequencing assay for DNA methylation analysis has been described previously [176]. Pyrosequencing was performed on the PyroMark Q96 MD (QIAGEN AG, Basel, Switzerland).

Statistical Analysis

Statistical analyses were performed using SPSS 19.0 (SPSS Inc., Chicago, USA). Normality distribution of the data was determined using the Shapiro-Wilk-Test. The data were not normally distributed. Mann-Whitney-U-Test was applied for comparing ccf nDNA and mtDNA levels between the three groups (Stage 1, Stage 2 and Stage 3). Wilcoxon signed rank test was performed for comparing expression data. *P*-values ≤ 0, 05 were considered statistically significant.

Results

Mitochondrial DNA content in paired cancer and normal tissues

The mtDNA content of the paired cancer and normal tissues was measured using multiplex real-time PCR. MtDNA content in cancer tissues was significantly lower in comparison with paired normal tissues (Mann-Whitney-U; $P < 0.001$). The comparison of mtDNA content between paired cancer and normal breast tissues within different stages showed no significant difference in mtDNA content for stage 1 breast cancer tissues (Mann-Whitney-U; n.s.); however stage 2 and 3 breast cancer tissues had significantly decreased mtDNA content compared to adjacent normal tissues (Mann-Whitney-U; $P <0.05$ and $P < 0.05$) (Figure1).

Comparison of mtDNA content with clinicopathological parameters

For the cancerous tissues the association between mtDNA content and established clinical parameters, including age, tumor size, lymph node involvement, extent of metastasis and the status of estrogen receptor (ER), progesterone receptor (PR) and Her2/neu were analyzed. No statistical significance between mtDNA content and any of the clinicopathological parameters could be found.

Expression Analysis of genes involved in mitochondrial biogenesis

Real-time qPCR was performed to analyze mRNA expression of 7 transcription factors (PGC-1α, PGC-1β, NRF1, NRF2, TFAM, TFB1M, TFB2M) and mitochondrial RNA polymerase (POLRMT). While PGC-1β was significantly down regulated (-1.0-fold), NRF1, TFB1M and TFB2M showed significant up-regulation (1.4-fold; 1-fold; 1.8-fold) in cancer tissues. POLRMT, also if not significantly, was down-regulated (-0.3-fold) (Figure 2).

Assessment of hypoxic status and oxidative DNA damage

As it has been shown that PGC-1β expression can be regulated in a hypoxia dependent manner and mtDNA depleted cells enhance expression of mitochondrial biogenesis genes such as NRF-1 and Tfam with increasing amounts of ROS, we assessed the hypoxic status and the status of oxidative DNA damage of cancer and normal tissue samples. HIF-1α was up-regulated (4.7-fold) in cancer tissues. Number of AP-sites were significantly higher in cancer than in normal tissues (9.56 vs. 8.69; Wilcoxon: $p<0.05$); what can be mainly attributed to stage 2 samples, the only stage exhibiting a significantly elevated number of AP-sites in cancer in comparison to normal tissues (11.88 vs. 9.67 ; Wilcoxon: $p<0.05$) (Figure 3).

Expression analysis of the VHL/HIF-1/C-MYC/ PGC-1β pathway

PGC-1β down-regulation was shown to be associated with HIF-1 gain of function and loss of C-MYC expression in VHL deficient in renal carcinomas. In our cohort, VHL as well as HIF-1α and C-MYC were up-regulated in cancer tissues. Inverse correlation between the expression levels of VHL and HIF-1α with increasing stage could be observed.

Methylation analysis

Since we found decreased expression of PGC-1β, we will perform methylation analysis using Pyrosequencing. Two amplicons within the promoter region are currently analyzed. Amplicon 1 (-378 until -157) containing 20 CpGs ; amplicon 2 (-179 until +85) containing 35 CpGs.

Discussion

To get better insight in the mechanisms that lead to reduction of mtDNA content in breast cancer patients, we investigated the the PGC-1 regulatory network. It is believed that the transcriptional co-activators PGC-1α and PGC-1β are involved in the regulation of mitochondrial biogenesis, mainly through interaction with their target genes NRF1 and NRF2; which in turn are known to induce downstream targets such as POLRMT, TFAM and TFB1M

and TFB2M. Depletion of PGC-1α and/or PGC-1β has therefore been suspected to lead to impairment of mitochondrial biogenesis.

We observed a significant decrease in mtDNA content in breast cancer tissue in comparison to adjacent normal tissue. Comparison of mtDNA content between different stages showed no significant difference between breast cancer and adjacent normal tissues for stage one, however significant decrease in cancer tissues could be observed for stage 2 and 3. Decreased mtDNA content has been found in patients suffering from various cancers such as renal carcinomas [77], gastric cancers [76], HCCs [177] and breast cancer [115, 178] and has been associated with mtDNA mutations [70, 145] and altered mRNA expression of the genes involved in mitochondrial biogenesis [179].

To check whether deregulation of genes involved in mitochondrial biogenesis might be implicated in decrease of mtDNA content in our cohort, we firstly evaluated the expression status of PGC-1α and PGC-1β co-activators and their target genes. Both co-activators were down-regulated in breast cancer tissues, however only for PGC-1β significant down-regulation of mRNA expression could be observed. In contrast, PGC-1β target genes NRF-1, TFB1M and TFB2M were significantly up-regulated. This seems quite contradictory, since it has been reported that PGC-1α and PGC-1β co-activate directly or via NRF1/NRF2 target genes like TFAM, TFB1M and TFB2M. Yet, Miranda et al. reported that in mtDNA depleted HeLa cells, increased concentration of reactive oxygen species (ROS) enhance the expression of nuclear encoded mitochondrial biogenesis genes such as NRF-1 and Tfam [180]. Correia et al. got similar results; they reported correlation of mtDNA depletion with up-regulation TFAM, TFB1M in astrocytomas and hypothesized that up-regulation of genes involved in mitochondrial biogenesis could be a compensatory mechanism for the decrease in mtDNA content [181]. Analyzing the hypoxic status and the oxidative DNA damage we found a significant increase in HIF-1α mRNA expression and significantly increased amount of AP sites in cancer tissues, suggesting that ROS levels might be higher in cancer tissues, supporting the aforementioned theory. However, although important target genes of PGC co-activators were up-regulated in this cohort, POLRMT, also if not significantly, was found to be down-regulated in breast cancer tissues what might be implicated in decrease in mtDNA content in our cohort.

With the aim to get closer insight which mechanisms lead to the decrease of PGC-1β mRNA expression and of mtDNA content, we assessed VHL/HIF-1/C-MYC-pathway. Zhang et al found evidence that PGC-1β expression is regulated via VHL/HIF-1/C-MYC-pathway in renal carcinoma cell lines deficient for VHL [173]. They showed that VHL loss of function

can lead to HIF-1 gain of function, which leads to the inhibition of C-MYC transcriptional activity by MXI-1 expression, resulting in loss of C-MYC-dependent PGC-1β expression and in reduced mitochondrial mass. Our cohort showed no VHL deficiency. We instead found significant up-regulation of mRNA expression of VHL and HIF-1α and C-MYC. However, there was an inverse correlation between the expression levels of VHL and HIF-1α with increasing stage, suggesting that HIF-1α might be negatively regulated by VHL. We conclude that in our cohort PGC-1β is not regulated in a VHL/HIF-1/C-MYC dependent manner.

In conclusion, in this cohort except for POLRMT, expression of all PGC target genes was up-regulated. The decrease in mtDNA content in the analyzed breast cancer tissues seems therefore not due to reduced expression of NRF-1, NRF2, TFAM or TFB1M and TFB2M. Yet, POLRMT is a central factor in mtDNA transcription and down-regulation of POLRMT might be implicated in decrease in mtDNA content. Since down-regulation of PGC-1β seems not to be regulated via the VHL/HIF-1/C-MYC-pathway, we are currently evaluating the methylation status of the PGC-1β promoter region using pyrosequencing to assess whether promoter methylation might play a role in the down-regulation of PGC-1β.

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Tables and table legends

Table 1. Clinical data of patients. Patient data divided into three subgroups according to stage (stage I, stage II, stage III). ER (Estrogen receptor); PR (Progesterone receptor); Her2/neu (Herceptin receptor).

Stage	Total no. of patients	Age (years) mean ± s.d. [range]	Grade			ER positive	PR positive	Her2/neu positive
			1	2	3			
1	10	59.8 ± 18.4 [36-94]	2	6	2	10	6	0
2	10	62.6 ± 10.5 [51-82]	0	6	4	9	7	0
3	10	59.7 ± 12.6 [43-82]	1	4	5	7	4	1

Figures and figure legends

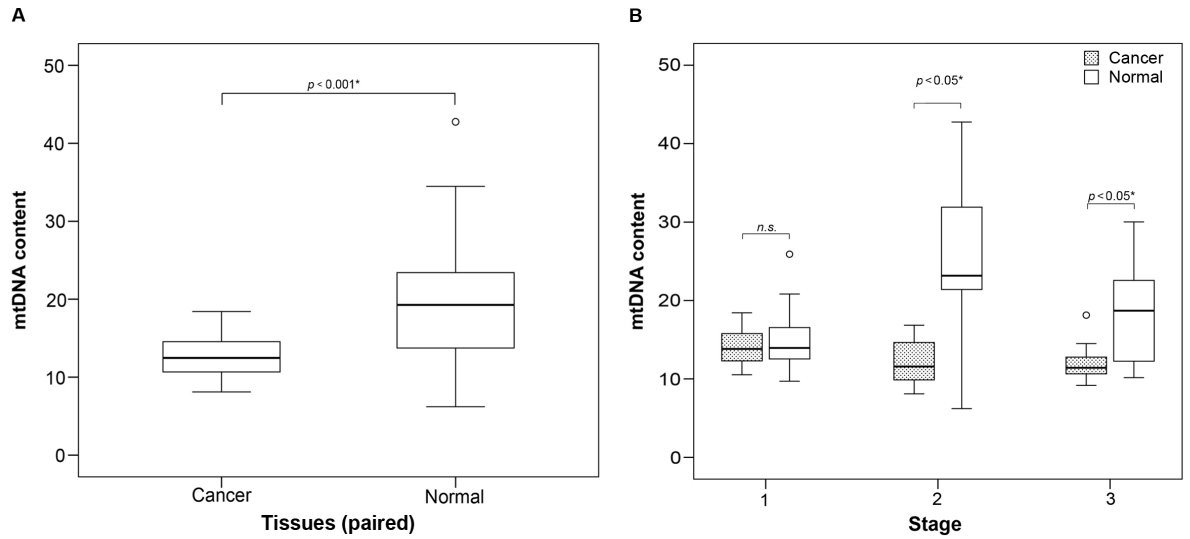

Figure 1. Boxplots for comparison of mtDNA content for IDC tissues (n=30) and adjacent normal tissues (n=30). **A.** Comparison of mtDNA content between cancer and normal breast tissues. **B.** Comparison of mtDNA content between cancer and normal breast tissues within different stages. (*significant correlation: Mann-Whitney-U).

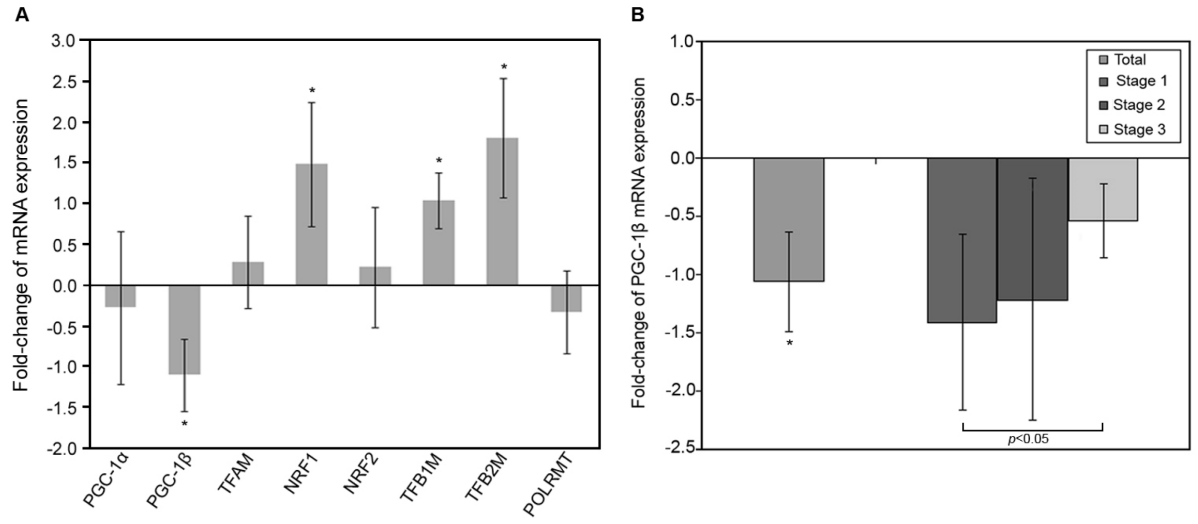

Figure 2. A. Real-time PCR quantification of mRNA expression for genes involved in mitochondrial biogenesis (PGC-1α, PGC-1β, TFAM, NRF1, NRF2, TFB1M, TFB2M, POLRMT) for IDC tissues (n=30) and adjacent normal tissues (n=30); **B.** Real-time PCR quantification of total and stage related (stages1-3) PGC-1β mRNA expression for IDC tissues (n=30) and adjacent normal tissues (n=30). mRNA expression normalized to B2M/ACTB; given as fold-change ± SEM; * indicates significant difference from adjacent normal tissues; $P<0.05$ (Wilcoxon signed rank test).

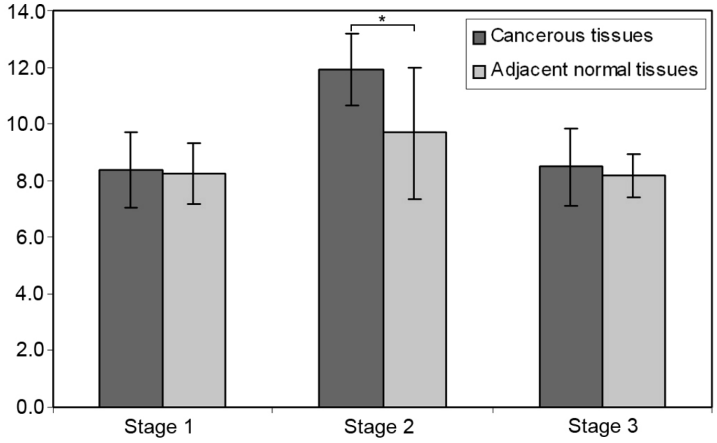

Figure 3. Oxidative DNA damage quantitation by ARP-Assay for IDC tissues (n=30) and adjacent normal tissues (n=30). Number of AP sites in cancerous and adjacent normal tissues per 10^6 bp. * indicates significant difference from adjacent normal tissues; $P<0.05$ (Wilcoxon signed rank test).

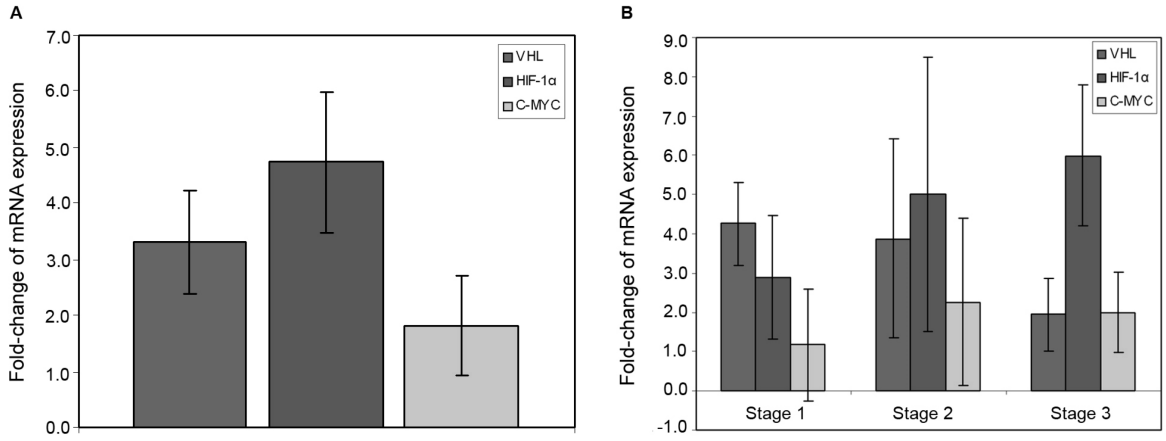

Figure 4. Real-time PCR quantification of mRNA expression for HIF-1α, VHL and C-MYC for IDC tissues (n=30) and adjacent normal tissues (n=30); normalized to B2M/ACTB; given as fold-change ± SEM. **A.** Total fold-change. **B.** Fold-change according to different stages. * indicates significant difference from adjacent normal tissues; $P<0.05$ (Wilcoxon signed rank test).

Supplementary

Table 1. Real-time PCR primers for nDNA/mtDNA quantification.

Gene	Gene ID	Primer ID	Primer sequence 5`→3`	Length (bp)	T_m	Amplicon lenght (bp)
GAPDH	2597	GAPDH_F	CCCCACACACATGCACTTACC	21	60	97
		GAPDH_R	CCTAGTCCCAGGGCTTTGATT	21		
		Probe	MGB–TAGGAAGGACAGGCAAC–FAM	17		
MTATP8	4509	MTATP8_F	AATATTAAACACAAACTACCACCTACC	27	60	79
		MTATP8_R	TGGTTCTCAGGGTTTGTTATA	21		
		Probe	FAM-CCTCACCAAAGCCCATA-MGB	17		

For the simultaneous quantification of nDNA and mtDNA the mulitplex real-time PCR was carried out in 25 µl of total reaction volume containing 7 µl H2O, 12.5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems, Branchburg, New Jersey, USA), 0.75 µl of each of the above mentioned 10 µM primers (Microsynth, Balgach, Switzerland), 1 µl of a 5 µM FAM-labeled MTATP 8-probe and 0.5 µl of a 5 µM VIC-labeled GAPDH-probe (both probes from Applied Biosystems, Rotkreuz, Switzerland). For each reaction 1 µl of template was added. The real-time PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems) under the following conditions: an initiation step for 2 minutes at 50°C is followed by a first denaturation for 10 minutes at 95°C and a further step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Table 2. Real-time PCR primers for mRNA expression.

Gene	Gene ID	Primer ID	Primer sequence 5`→3`	Primer length (bp)	T_m	Amplicon lenght (bp)
HIF-1α	3091	HIF1_F	TGCTCATCAGTTGCCACTTC	20	60	178
		HIF1_R	AAAACCATCCAAGGCTTTCA	20		
C-MYC	4609	C-MYC_F	TCCTCGGATTCTCTGCTCTC	20	60	180
		C-MYC_R	CTCTGACCTTTTGCCAGGAG	20		
NRF-1	4899	NRF1_F	AGAAAGCTGCAAGCCCATCT	20	60	193
		NRF1_R	CTGTGTTTGCGTTTGCTGAT	20		
NRF-2	2551	NRF-2_F	AAGTGACAAGATGGGCTGCT	20	60	171
		NRF-2_R	CCGAAATGTTGAGTGTGGTG	20		
PGC-1α (PPARGC1A)	10891	PGC-1α_F	CACCAGCCAACACTCAGCTA	20	60	220
		PGC-1α_R	GTGTGAGGAGGGTCATCGTT	20		
PGC-1β (PPARGC1A)	133522	PGC-1β_F	ATGACTCCGAGCTCTTCCAG	20	60	152
		PGC-1β_R	CGAAGCTGAGGTGCATGATA	20		
POLRMT	5442	POLRMT_F	CTGCAGTGCCTCTTTGAGAA	20	60	150
		POLRMT_R	CAGTGCTTTCTCCCATTGGT	20		
TFAM	7019	TFAM_F	TACCGAGGTGGTTTTCATCTG	21	60	150
		TFAM_R	AACGCTGGGCAATTCTTCTA	20		
TFB1M	51106	TFB1M_F	AGCACCTGGGAAACTGAGAA	20	60	206
		TFB1M_R	TGCCATAAACAAAAGGTCCA	20		
TFB2M	64216	TFB2M_F	CGAGTGATCCACTGTGACTTCT	22	60	182
		TFB2M_R	CAAAGTGCCCTTTTCTCACC	20		
VHL	7428	VHL_F	TCTCAATGTTGACGGACAGC	20	60	165
		VHL_R	ACATTTGGGTGGTCTTCCAG	20		
B2M *	567	B2M_F	GTGCTCGCGCTACTCTCTCT	20	60	150
		B2M_R	GTCAACTTCAATGTCGGATGG	21		
ACTB *	60	ACTB_F	CGTCTTCCCCTCCATCGT	18	60	181
		ACTB_R	GGTGTGGTGCCAGATTTTCT	20		

The real-time PCR was carried out in a 12.5 µl total volume, containing 4.5µl H_2O, 6.2µl PCR SybrGreen Mastermix (Applied Biosystems, Branchburg, New Jersey, USA), 0.4µl of each primer (10µM) and 1µl of cDNA template. The real-time PCR was performed using ABI PRISM 7000 sequence detection system (Applied Biosystems) under the following conditions: an initiation step for 2 minutes at 50°C is followed by a first denaturation for 10 minutes at 95°C and a further step consisting of 40 cycles of 15 seconds at 95°C and 1 minute at 60°C.

Table 3. PGC-1β primers for PCR of the two amplicons on bisulfite-converted genomic DNA.

Gene	Primer	Sequence (5`→3`)	Length (bp)	Tm	Product size (bp)	Start/End
PPARGC1B	PPARGC1B_A1_F	AGTTGTGTAGAAGAAGTAGGTTGT	24	59	221	-378 / -157
	PPARGC1B_A1_R	RAAAATCRCTCACCCACT	19			
	PPARGC1B_A2_F	GAAAGTGGGTGAGYGATTT	19	61	264	-179 / +85
	PPARGC1B_A2_R	AAAAAAAAAAAAAACTCTTCRTCC	24			

The PCR on bisulfite-converted genomic DNA was carried out in 50µl total reaction volume containing 20 ng DNA, 1pmol of each primer, 200µM dNTP, 0.2 U Hot Start Taq DNA polymerase, 1.5 mM MgCl2 and 10* PCR buffer.The reaction was performed under the following conditions: an initial denaturation at 94ºC for 10 min, followed by 40 cycles of denaturation at 94ºC for 20s, annealing at 56 ºC for 30s and primer extension at 72°C for 1 min.

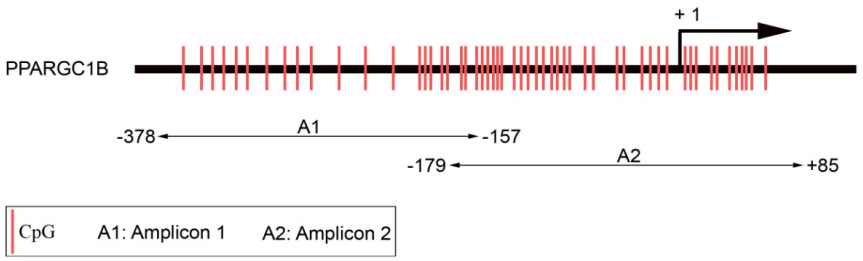

Figure 1. Schematic representation of the PPARGC1B gene promoter region. The transcription start side is indicated by one-sided arrow (+1); CpG sites are shown as red lines.; two-sided arrows represent the two amplicons (A1, A2) analyzed by pyrosequencing.

4. SUMMARY AND FINAL CONCLUSION

Breast cancer remains the most common cancer among women worldwide. Yet, during the past decades a reduction in breast cancer mortality could be observed in westernized countries, mainly attributable to earlier diagnosis due to regular screening and frequent use of systemic adjuvant treatment [1-3]. Although standard screening methods such as clinical breast examination (CBE) and Full-Field Digital Mammography (FFDM) are non-invasive itself, their sensitivity and specificity is limited and they still require biopsy proof after suspicion. Circulating cell-free nucleic acids represents a new generation of markers with the advantage that both, quantitative as well as qualitative alterations of ccf-nucleic acids are potentially applicable as non-invasive biomarker.

In the first part of this study, we investigated quantitative alterations of ccf-nDNA and ccf-mtDNA in the plasma of breast cancer patients and healthy controls to evaluate the applicability of ccf-nuclear and mitochondrial DNA as biomarker for distinguishing between different study groups (malignant, benign, and healthy). We found increased levels of ccf-nDNA in the cancer group in comparison with the benign tumor and the healthy control groups. In contrast ccf-mtDNA was found to be significantly lower in the two tumor-groups (benign and malignant) in comparison with the healthy control group. This phenomenon has been reported frequently, however the underlying mechanisms leading to an increase of ccf-nDNA and to a decrease of ccf-mtDNA in cancer patients have not been elucidated. It is speculated that high cellular turn-over at tumor site might lead to an increased shedding of tumor-derived ccf-nucleic acids into the circulation [4]. However, this would not explain the decreased levels in ccf-mtDNA. A decrease in mtDNA has not only been observed in the circulation but also in cancer cells themselves. It has been suspected that mtDNA mutations and deregulation of genes involved in mitochondrial biogenesis might be causal [5-7]. While using nDNA levels we could distinguish between breast cancer patients and healthy controls with a sensititvity of 81% and a specificity of 69%, mtDNA levels enabled a discrimination between breast tumor patients (cancerous and benign) and healthy controls with a sensititvity of 53% and a specificity of 87%. Altogether this suggests that although ccf-nDNA as well as mtDNA levels could be used for discrimination between the study groups, the limited sensitivity and specificity of the approach doesn't allow for clinical application. Limited sensitivity and specificity is a well known problem using quantitative alterations of ccf-nucleic acids as biomarker. Other groups, using ccf-nDNA levels as potential

diagnostic/predictive marker for different cancer types, got similar results. In non-small cell carcinoma of the lung a differentiation between 79 healthy individuals and 151 patients could be performed with a sensitivity of 85.8 % and a specificity of 46.8 % using 2 ng /mL as cut-off value [8]. Recently, Kamat et al. determined the role of pre-operative total plasma ccf-DNA levels in predicting clinical outcome in patients with ovarian cancer. Diagnosis of invasive ovarian cancer yielded a sensitivity of 87% and specificity of 87% with a cut-off value of 4,500 GE/ml. Interestingly, the sensitivity and specificity of predicting malignancy in their cohort using CA125 (cutoff of ≥ 35 IU/mL) was comparable with 89% and 77%, respectively [9]. Although recently researchers tried to develop new state of the art approaches for the quantification of ccf-nucleic acids no successful improvement towards increased sensitivity and specificity could be achieved. We showed that ccf-nDNA and mtDNA has limitations as a biomarker for early diagnosis of breast cancer. Further studies might identify specific patient cohorts in which such alterations can serve as a prognostic and predictiv biormarker for breast cancer. To this end, it is necessary to find cancer-specific qualitative alterations which provide high enough sensitivity and specificity. Such alterations could be applied primarily as diagnostic biomarker to non-invasively screen ccf-DNA.

To identify cancer-linked genes, Sjöblom et al. and Wood et al. performed a genome-wide mutation screening in human breast and colorectal cancer. Within their study they tested more than 13,032 human protein-coding genes in human breast and colorectal cancer. By using very stringent criteria, 189 genes were found to be mutated at a significant frequency in those cancers, which they called candidate cancer genes (CAN-genes) [10, 11]. We evaluated the value of these CAN-gene mutations as biomarkers for breast cancer. Using MALDI-TOF MS 40-plex assay, we checked 21 CAN-genes and 40 loci in 6 breast cancer cell lines and in tissue samples of 19 breast cancer patients and 55 healthy controls .The mutation rate of the analyzed loci in our sample cohort was very low. Only a single breast cancer tissue sample showed heterozygosity at locus c.5834GA within the *ZFYVE26* gene (Zinc finger FYVE domain-containing gene 26). Sjöblom et al./Wood et al. already showed that the vast majority of genes are mutated at very low frequency and therefore rarely detectable. We suppose that a low mutation frequency of our loci analyzed in this study might be one reason we couldn't detect a huge amount of mutations in our cohort. A study by Balakrishnan et al., evaluated whether the CAN-genes are universal for all cancer types or cancer type specific. They analyzed the mutational profiles of 19 CAN-genes in the highly aggressive tumors: glioblastoma, melanoma, and pancreatic carcinoma. They mainly found novel somatic

mutations in a subset of genes. None of the somatic mutations described by Sjöblom et al. were found in their analyses of glioblastoma multiforme, melanoma, and PDAC samples [12]. These results implicate that each type of tumor might have its own CAN-gene signature and only a few of the CAN-genes are maybe shared by different tumor types. The value of CAN-gene mutations found by Sjöblom et al. / Wood et al. could therefore be restricted to the cancer type they were detected in and might therefore only usable as cancer type specific marker. In general, the interpretation of genome-wide mutation screenings and the search for cancer specific qualitative nuclear DNA alterations seems to be a tricky undertaking. First of all screening approaches usually yield an enormous amount of candidates, making it difficult to select key alterations for detailed evaluation. In addition, it is known that besides inter-variations between distinct cancer types there are enormous intra-variation within the same cancer type, implying that carcinogenesis is also partly be individually determined, what suggests a probable need for personalized approaches.

Compared to nDNA, the analysis of mtDNA mutations has some advantages such as higher mtDNA copy numbers and higher mutation rate. We used sanger sequencing with the aim to determine somatic mtDNA mutations within the D-loop region in breast cancer tissues and to evaluate whether it is possible to detect these mutations in the matched plasma samples. We therefore sequenced the two hypervariable regions HVR1 and HVR2, which are located in the D-Loop and are known as mutational hotspots in ten paired tissues and plasma samples from breast cancer patients. MtDNA mutations were found in all patients' samples. A total of 85 germline mutations and of 42 somatic mutations in the D-loop region were found. MtDNa mutations seem to be a more promising biomarker than nDNA mutations for several reasons. Firstly, mtDNA is highly abundant in every cell making it easily accessible. The mutation frequency is known to be much higher what was also evident in our study and the homoplasmic nature of mtDNA makes it an ideal molecular marker [13, 14]. Using sanger sequencing we could not find mutant mtDNA in plasma samples. This might be due to the fact that the amount of circulating mutant mtDNA is tiny in comparison to background wild-type mtDNA. At the moment, highly sensitive applications that enable a more sensitive discrimination between circulating mutant gene fragments and normal circulating DNA fragments are on the way [15, 16].

In general, limited sensitivity and specificity can be considered as the bottleneck of using quantitative as well as qualitative alterations as marker. In the past few years, researchers tried to overcome that problem working on methodological improvements and applying state of the

art technologies for detecting and the analyzing quantitative and qualitative alterations of ccf-nucleic acids [15, 17-19]. However, even these measures could not achieve a designated outcome. A first step towards overcoming the problem of limited sensitivity and specificity should be therefore the use of multi-marker approaches. Flamini et al. recently evaluated a combined use of cell-free DNA and carcinoembryonic antigen (CEA) serum levels for diagnosis of colorectal cancer. ROC curve analysis using a combination of CEA and cell-free DNA levels showed a higher diagnostic capacity than that of markers considered singly [20]. In another multi-marker study, Sunami et al. assessed serum prostate specific antigen (PSA) levels in combination with ccf-DNA for allelic imbalance of 6 genome microsatellites and methylation status of *RASSF1*, *RARB2*, and *GSTP1*. By combining the 2 assays, the number of prostate cancer patients positive for methylated or LOH marker increased to 63%. Combination of DNA and PSA assays gave 89% sensitivity.

In our first study, we found decreased mtDNA content in tumor patients in comparison with healthy controls. A phenomenon that has been frequently reported in breast cancer [7, 21, 22]. Warburg found that malignant transformation is often accompanied by a shift from oxidative phosphorylation (OXPHOS) to aerobic glycolysis and he hypothesized that this could be the result of mitochondrial dysfunction [23]. Events that have been suspected to lead to mitochondrial dysfunction include among others mtDNA mutations and altered expression of genes involved in mitochondrial biogenesis.

In a first part we therefore investigated the inter-genomic crosstalk between mtDNA alterations (mtDNA content and mutations) and methylation status of 22 breast cancer candidate genes. Although we found significantly reduced mtDNA content in cancerous tissues compared to normal paired tissues, we couldn`t find a correlation with neither somatic mtDNA mutations nor with clinicopathological parameters. Correlation analyzes revealed inverse correlation of hypermethylated *TP21* and positive correlation of hypomethylated *BRCA2* with mtDNA content as well as inverse correlation of hypomethylated progesterone receptor and positive correlation of hypermethylated *GSTP1* with mtDNA mutations. A correlation between mtDNA mutations and a decrease in mtDNA content has been reported in several studies. Yu et al. observed that esophageal tumors harboring mutations in displacement (D)-loop region, particularly at the polycytidine stretch or close to the replication origins of the heavy-strand, had a significantly lower copy number of mtDNA than the ones without D-loop alterations. Lee et al. on the one hand showed that mtDNA copy number of HCC was significantly decreased in 60.5% of the patients with hepatoma,

especially in those with somatic mutation(s) in the D-loop of mtDNA; but interestingly, they found that 42.9% (6/14) of the HCCs without mutation in the D-loop had a reduced copy number of mtDNA, indicating that other unidentified factors involved in mitochondrial biogenesis might be defective in the tumor. Yet, various other groups couldn't find an association between somatic mtDNA mutations and reduced mtDNA content [24]. Since mtDNA has been shown to be highly mutated in cancer, it is difficult to assess which mutations are the causing mutations, leading to a decrease in mtDNA content. Unfortunately until now there are very few studies that analyzed the intergenomic cross-talk between nDNA methylation and mtDNA alterations, making it difficult to see our results in a general context.

There is evidence that impairment of mitochondrial biogenesis, through the deregulation of genes involved in mitochondrial transcription and translation, might be causal for reduction in mtDNA content. Analysis of the regulatory network of PGC-1 co-activators and their target genes in 30 paired breast cancer and adjacent normal tissue samples revealed down-regulation of PGC-1α and PGC-1β. Reduced mRNA expression of PGC-1α and PGC-1β was not associated with down-regulation of target genes, such as NRF1, TFB1M and TFB2M. POLRMT was down-regulated in breast cancer tissues what might be implicated in decrease in mtDNA content. PGC-1β down-regulation was neither correlated with oxidative DNA damage status nor with the expression of genes of the VHL/HIF-1/C-MYC-pathway. Future analyzes of the methlytion status of the PGC-1β promoter region might clarify if methylation is involved in down-regulation of PGC-1β in cancer patients. Until this point, we could confirm the results of other groups, observing reduced mRNA expression of PGC-1α and PGC-1β in breast cancer tissues. Although we found several PGC-1 target genes upregulated, downregulation of POLRMT might be implicated in decrease of mtDNA in our cohort. Our ongoing studies will provide answers to these open questions.

To conclude, although during the last decade a boost for research on ccf-nucleic acids has led to increased knowledge about their release mechanisms, biological characteristics, physiological and pathophysiological behaviour, the conclusions drawn from the tremendous amount of studies are rather vague and many questions are still unanswered. Firstly, one can not ignore that the varying results obtained by different studies - for quantitative as well as for qualitative alterations - are a general problem in the research on ccf-nucleic acids. Therefore a major issue that should be tackled within the coming years is the standardization of assays in regard to sampling procedures (blood collection, processing, storage), isolation methods (commercial kits, laboratory protocols), the use of assay platforms (PCR, BEAMing, massive

parallel sequencing) and data analyzes [25]. Another major problem concerning quantitative as well as qualitative ccf-nucleic acid alterations is their limited specificity and sensitivity. The lack of suitable qualitative alterations as marker might be solved in recent years considering the technological advances in the development of high-throughput technologies. However, until the detection of more sensitive qualitative markers, one of the primary aims of future research should be the development of multi-marker assays to overcome the obstacle of limited sensitivity and specificity. In addition there is a need for basic research to improve the understanding of molecular mechanisms that underlie quantitative and qualitative alterations in physiological as well as in pathophysiological conditions. Summing up, investment in basic research in combination with standardization, use of state of the art technologies and the application of multi-marker approaches could lead to the development of more sensitive and specific methods and finally to the detection and clinical application of new non-invasive biomarkers for cancer management.

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Corina Kohler
University of Basel
September 2011

6. APPENDIX

6.1 Short Curriculum Vitae

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04/2007 – 03/2008:	Diploma thesis at the Institute for Human Genetics, University of Ulm, Germany. Thesis title: Analyses of the Nuclear Localisation and Chromatin Structure of Active and Inactive Genes in Murine Cells.
09/2001 – 03/2007:	Studies of Biology, University of Ulm, Germany. Main focus: genetics, virology, ecology
08/1998 – 07/2001:	Abitur (University entrance qualification), Commercial high school of Öhringen, Germany.

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ORAL PRESENTATIONS

a.) ICPH-Meeting (Vevey; Switzerland on April 26-28, 2009): Presentation “Developing a novel MALDI-TOF MS-based method to detect CAN-gene mutations in the circulation for early diagnosis, monitoring and determination of the prognosis of breast cancer.”

b.) EuCC-Meeting (Freiburg; Germany on May 15, 2009): Presentation: “Developing a novel MALDI-TOF MS-based method to detect CAN-gene mutations in the circulation for early diagnosis, monitoring and determination of the prognosis of breast cancer.”

c.) Symposium: Mass Spectrometry: The future technology in Forensic, Medical, and Microbiological Genetics? (Strasbourg; France on June 24/25. 2010); Presentation: “Mass spectrometry for genotyping of human platelet-specific antigens.”