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REVIEW ARTICLE

MtDNA As a Cancer Marker: A Finally Closed Chapter?

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Abstract: Sequence alterations of the mitochondrial DNA (mtDNA) have been identified in many tumor types. Their nature is not entirely clear. Somatic mutation or shifts of heteroplasmic mtDNA variants may play a role. These sequence alterations exhibit a sufficient frequency in all tumor types investigated thus far to justify their use as a tumor marker. This statement is supported by the high copy number of mtDNA, which facilitates the detection of aberrant tumor-derived DNA in bodily fluids. This will be of special interest in tumors, which release a relatively high number of cells into bodily fluids, which are easily accessible, most strikingly in urinary bladder carcinoma. Due to the wide distribution of the observed base substitutions, deletions or insertions within the mitochondrial genome, high efforts for whole mtDNA sequencing (16.5 kb) from bodily fluids would be required, if the method would be intended for initial tumor screening. However, the usage of mtDNA for sensitive surveillance of known tumor diseases is a meaningful option, which may allow an improved non-invasive follow-up for the urinary bladder carcinoma, as compared to the currently existing cytological or molecular methods. Following a short general introduction into mtDNA, this review demonstrates that the scenario of a sensitive cancer follow-up by mtDNA-analysis deserves more attention. It would be most important to investigate precisely in the most relevant tumor types, if sequencing approaches in combination with simple PCR-assays for deletions/insertions in homopolymeric tracts has sufficient sensitivity to find most tumor-derived mtDNAs in bodily fluids.

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1. MtDNA: A SMALL EXTRANUCLEAR GENOME

Half a century ago Margit and Sylvan Nass reported for the first time that mitochondria contain fibrous material with physicochemical features of DNA [1]. These authors recognized that the appearance of these intramitochondrial fibers, as visible in electron micrographs after different fixation procedures, resembled one of prokaryotic nucleoplasm, suggesting for the first time that the organelles may contain their own genome. A few years earlier some reports had already described traces of DNA in other non-nuclear fractions of eukaryotic cells, but they had been usually interpreted as contaminations, although plant plastids belonged to those suspicious cell fractions, which are meanwhile known to contain their own small genome, just like mitochondria. Nearly in parallel with that electron microscopic study, Schatz *et al*. confirmed the presence of DNA in yeast mitochondria with biochemical methods [2].

It still took a long time, until full sequencing of this small circular genome (Fig. **1**) generated the first complete sequence database of all mtDNA encoded proteins, rRNAS and tRNAs [3, 4]. At the end of 1980s, the first reports on neuromuscular disorders due to large scale deletions of mtDNA [5-7] and of a retinal disease [8] due to a missense mutation were published. They were immediately followed by two reports describing neuromuscular disorders elicited by single nucleotide exchanges in two tRNA genes within the mitochondrial genome [9, 10]. The late 1980s were thus the starting point of mitochondrial medicine, which became a rapidly growing field with many confirmed pathogenic mtDNA mutations. Mitochondrial syndromes were clinically described with an increasing awareness of the fact that often no sharp borders exist between the initially defined syndromes. MtDNA mutations can also cause some isolated and less severe neurologic, muscular or metabolic symptoms (*e.g.* muscle weakness, ataxia, deafness, diabetes), which may be overlooked or not attributed to mitochondrial dysfunction. For a review of the historical perspective on mitochondrial disorders see DiMauro and Garone [11].

The mtDNA mutations in these classical mitochondrial disorders occur either newly in the diseased individual, such as large scale deletions, or are maternally inherited along with the general maternal inheritance of mtDNA. Since the mutations either affect a subunit of the mitochondrial electron transport chain (ETC), a subunit of the F_oF_1-ATPase or their protein biosynthesis on mitochondrial ribosomes, they obviously affect oxidative ATP-production (OXPHOS) and may cause oxidative stress in addition. The latter can be caused by an increased generation of reactive oxygen species (ROS).

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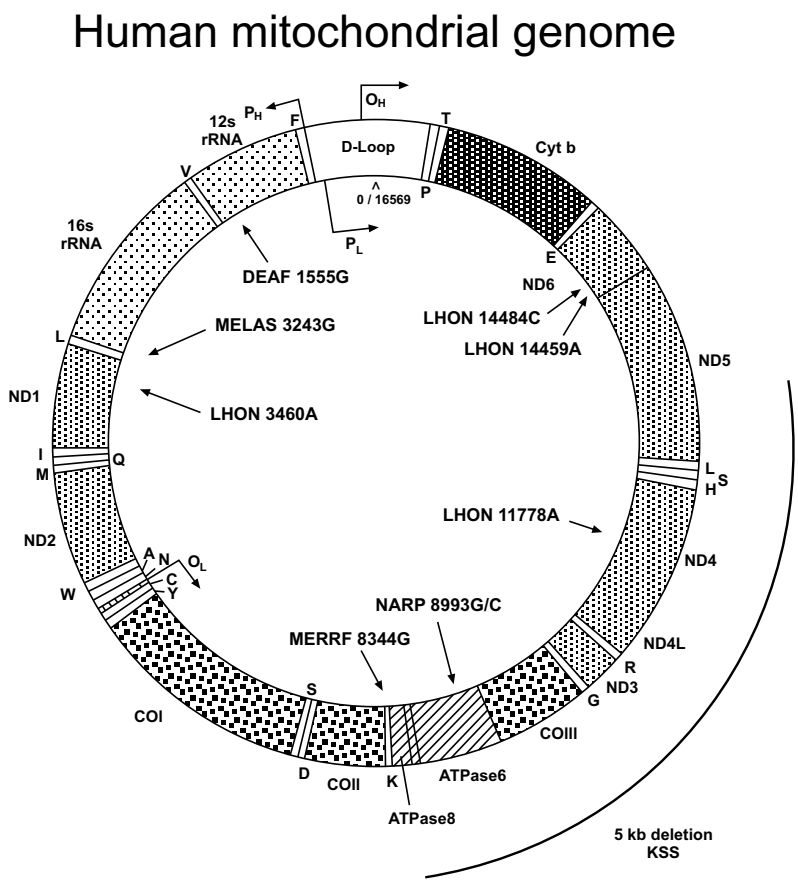

Fig. (1). Schematic drawing of the human mitochondrial genome (mtDNA), modified according to Mitomap-databse (http://www.mitomap.org). The control region (designated as D-loop) contains several short sequence elements, which are essential for transcription and replication, but in parallel three hypervariable regions (all listed in Mitomap-database in detail). The rest of the circular DNA molecule is completely filled with intron-less mitochondrial genes, which leave nearly no non-coding junctions between them (polycistronic transcripts). Genes coding for several subunits of ETC-complex I (NADH-Ubichinon-Oxidoreductase, ND), ETC-complex IV (Cytochrom Oxidase, CO), F_oF_1-ATP-synthetase (ATPase) and for Cytochrome B (Cyt b) are separated by single or clustered tRNA-genes, designated according to the single-letter code of the corresponding amino acids. These tRNAs differ from cytoplasmic ones, thereby generating a genetic code, which deviates from the universal code. Two genes for ribosomal RNAs are designated as 12S- or 16S-rRNA. The drawing also depicts the positions of some major mutations implicated in mitochondrial disorders (LHON, MELAS, MERRF, NARP, DEAF, KSS).

Since oxidative energy metabolism is essential for the nervous system and large scale deletions of mtDNA were early shown to accumulate in most parts of the brain during aging [12], the field of mitochondrial medicine became extended also to more prevalent neurodegenerative disorders, which often exhibit mitochondrial defects. The true impact of mitochondrial dysfunction and especially of mtDNA mutations in neurodegenerative disorders still remains unclear (for review see [13, 14]).

While the above mentioned fields of mitochondrial medicine are focused on disturbed biochemical function of mitochondria, it should be mentioned that forensic genetics utilizes harmless polymorphisms of mtDNA for individualization of DNA-traces. The viewpoint of forensic research is of some interest in tumor studies, because it highlights the potential role of so-called heteroplasmy, *i.e.* the co-existence of mutant and wild-type mtDNA in the same tissue. The phenomenon of heteroplasmy leads to some theoretical considerations regarding the potential origin of mtDNA alterations in tumors (see below), which were not discussed by most tumor studies. In addition, forensic science takes advantage of the high copy number of mtDNA, which supports analysis of degraded traces of blood or skin or bodily fluids. This high sensitivity is also of importance for cancer surveillance.

2. IS HOMOPLASMY A COMMON STATE?

2.1. Lessons from Forensic and Aging Studies and From Colonic Crypts

Our first idea regarding the suitability of mtDNA in tumor research resulted from an in-house cooperation with the forensics department of the University of Magdeburg (Germany) in the late 1990s. We had learned that mtDNA is of special worth in forensic casework due to its high copy number per cell, which makes a polymorphic region of this DNA a reliable identity marker, even if only traces of DNA are available, *e.g.* attached to hair shafts without bulbs [15], found at a crime scene, or if DNA is heavily degraded. The power of mtDNA for 'individualization' of DNA remains is mostly due to the fact that it contains a 1.2 kb non-coding region (control region) with many polymorphic sites, especially concentrated in three hypervariable segments (HVR-

1,-2 and -3, Fig. **2**). HVR-2 contains a cytosine tract (C_nTC_6), which exhibits a moderate degree of length variability, despite its essential role for mtDNA maintenance. The tract is largely identical with CSB-II, one out of three adjacent conserved sequence blocks, which are required for mtDNA replication [16, 17]. Since the thymine residue occupies nucleotide position 310 according to the revised Cambridge reference sequence [3, 4], this tract was later often designated as 'D310' (D-loop, np 310) in the tumor literature.

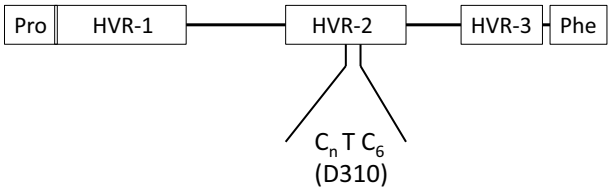

Fig. (2). Schematic drawing of the conrol region, which is flanked by the genes for proline- and phenylalanine-tRNAs. It contains three hypervariable regions(HVR), one of which contains a homopolymeric cytosine tract of variable length, often designated in the tumor literature as D310. In blood the first half of the c-tract contains usually 7,8 or 9 cytosine residues, while the second half nearly invariably contains 6 (in contrast to 5 in the HeLa-derived Cambridge reference sequence).

Forensic scientists are highly interested in avoiding confusion during 'individualization' of DNA remains, which may be due to a heteroplasmic state. Heteroplasmy bears the risk that such a mixture may not be quantitatively constant between tissues of an individual or even within a tissue over time. Besides the well-known heterogeneity of mutation loads among tissues of a patient with a classical mitochondrial disorder, forensic and aging studies shed a light on intra-individual heterogeneity of heteroplasmic mitochondrial genomes [18-21]. Because these studies were devoted to non-coding sites of the control region, they demonstrated some risk of intra-individual heterogeneity and inconstant heteroplasmy of regions, which were seemingly not affected by selective pressure. Random shifts may sometimes occur over time by a somatic bottleneck of mtDNA propagation, *e.g.* in hair bulbs. An underestimated risk during usage of mtDNA in tumor-studies is the current uncertainty, if a somatic bottleneck may also occur in some tumor-affected organs, *e.g.* in an epithelium consisting of numerous, but small regenerative islands. Such somatic bottlenecks can help to push minor mtDNA variants in normal cells above the detection limit without an underlying neoplastic process. A good example is the freely competing stem cells at the bottom of colonic crypts, which give rise to several small clonal cell lineages, which are usually restricted to a single crypt, as long as no crypt division occurs (a rather rare event). The small number of stem cells can lead frequently to a take-over of a crypt by a mutant mtDNA-clone. Spontaneous mtDNA mutations can thus even be exploited to track the cellular dynamics in colonic crypts [22]. In times of increasing sensitivity of analytical techniques, it cannot be excluded that potential hot-spot mutations, occurring in a small subset of crypts, become detectable in the stool, without any colorectal malignancy. In addition, restricted cell numbers driving some stage of tumor spread may constitute a bottleneck within the tumor, able to change heteroplasmic patterns. Both aspects potentially compromise the application of mtDNA-analysis.

They have largely been neglected so far. For example, we found some degree of instability of heteroplasmic mtDNA-patterns during long-term cultures of glioma cells including series of strong dilutions, artificially constructing such bottleneck (unpublished data). On the other hand, we were able to demonstrate sufficient stability of a heteroplasmic mtDNA-pattern *in vivo* as a clonal marker of tumor growth in gliomatosis cerebri [23], an extremely rare disease, in which a slowly growing glioma had infiltrated wide regions of both brain hemispheres.

Although this review is not the right place to deal with this aspect in detail, the reader may have in mind that mitochondrial genetics generally can be viewed as a 'quantitative genetics', in which heteroplasmic mtDNA molecules can account for a variable percentage of total mtDNA in different tissues of an individual. It is thus not surprising that forensic researchers, who rely on the long-lasting stability of their markers, had a close look at potential heteroplasmy of mtDNA in normal individuals, not affected by mitochondrial disorders. It was found for example that individuals with more than 7 cytosine residues in the first half of the HVR-2 cytosine tract often exhibited a length-heteroplasmy. The forensics department of our university developed some PCR-based techniques to easily identify heteroplasmy in HVR-2 and other non-coding regions of mtDNA, such as heteroduplex analysis [24]. Based on this technique, on fluorescent PCR of HVR-2 with subsequent capillary electrophoresis and by cloning and sequencing with proofreading polymerase, we detected low-level length-heteroplasmy of HVR-2 and sometimes synonymous base exchanges in HVR-1 and HVR-2 of various normal tissue samples.

Moreover, small percentages (<1%) of a 5 kb mtDNA deletion–actually designated as 'common deletion'-had been shown by several authors, including ourselves [25], to accumulate in most brain regions of aged individuals, confirming the original observation made by the group of Douglas Wallace [12], thus again rejecting the point of view that mtDNA is commonly homoplasmic outside mitochondrial disorders. Heteroplasmy may usually be characterized by one dominant sequence, with minor sequences accounting for a few percent of total DNA, which remain invisible during Sanger-sequencing.

2.2. Can Shifts Among Hetereplasmic mtDNAs be Expected to Occur in Cancer?

For mitochondrial DNA every clonal cell expansion may act as a somatic bottleneck. This bottleneck should even be narrowed in clonal expansions of tumor cells, which contain a limited number of mitochondria due to a partial switch to non-aerobic energy metabolism. MtDNA is still over-represented as compared to nuclear single-copy genes, but the copy number is limited. We thus recognized early the likelihood that especially length-heteroplasmy in the HVR-2 cytosine tract may often change during tumor development, simply by random drift during mitochondrial fission and cell divisions. Clonal tumor growth may implicate the possibility of shifting undetected minor mtDNA sequences in the tissue

of origin across the detection limit. Eventually these minor variants may even become homoplasmic, being by chance trapped in the homoplasmic state by the clonal cell proliferation. This can happen without any need of positive selection, as modeled by Coller and colleagues in computer simulations [26]. If that had happened, tumor-derived mtDNA may be a quite stable tumor marker for the individual patient.

We were *e.g.* able to proof by cloning and sequencing of mtDNA from paired samples of tumor and normal tissue of a glioma-patient [27] that quantitative shifts had occurred between preexisting heteroplasmic mtDNA molecules during tumor development. This was made very clear in one case by the identification of mtDNA clones differing in three nucleotide positions of the control region, including the length of the HVR-2 cytosine tract. This ruled out quite well the possibility that somatic mutations in tumor tissue had caused these sequence changes, because three independent events would have been necessary in that case, while a quantitative shift between two different preexisting mtDNA clones (heteroplasmy-shift) could easily explain the result. The likelihood of such changes in quantitative patterns, especially in regions with balanced heteroplasmy in normal tissues, such as D310, originally fed our interest in mtDNA for purposes of cancer research. To the best of our knowledge, Habano and colleagues [28] were the first to propose the idea of heteroplasmy-shifts in their work about colorectal cancer (Fig. **3**). Since then this concept as a major basis of ntDNA alterations in solid human tumors seems to have been largely forgotten or at least it was not mentioned in the many publications, which arose since that time. This may be especially astonishing, since D310 was later found to be a major hotspot of 'mtDNA mutations' in tumors [29]. Although having in mind the likely participation of simple shifts among preexisting mtDNA molecules in some hot-spots, this point will not be further stressed in the review. Irrespective of the nature of the observed sequence changes, the review will refer to them as 'somatic mutations', like most of the literature. However, abundant heteroplasmy-shifts may at least partially explain the high frequency of sequence alterations found in mtDNA of solid human tumors, which by the literature were usually attributed to a higher mutation rate of this extrachromosomal genome due to less efficient repair mechanisms.

3. MtDNA MUTATIONS IN CANCER

3.1. Are They Common in Solid Tumors?

MtDNA mutations have been meanwhile detected in a large percentage of colorectal cancers [28, 30], lung cancers [31, 32], head and neck cancers [31, 33], urinary bladder carcinomas [31, 32, 34] renal cell carcinomas [32, 34], pancreatic cancers [35], ovarian carcionomas [36], breast cancers [37], gastric cancers [38], gliomas [39] and several other solid tumors. Several early studies were focused on the control region, probably because the hypervariable segments suggested a higher frequency of non-functional mutations, which provoked no selective pressure tending to eliminate mutated mtDNA. While it is difficult to extrapolate the mutation frequencies of the whole mitochondrial genome for a given tumor entity from such restricted studies, later studies performed a whole mt-genome sequencing by means of MitoChip technology [32, 33, 40]. From these results, it could be reasonably deduced that in some entities two thirds or even all of the patients harbor an mtDNA mutation in their tumor, which is detectable at the sensitivity level of sequencing. Maybe the frequency is even somewhat higher, if one considers that MitoChip technology is more sensitive to the detection of single base substitutions, but less sensitive to detection of length variation in the marker D310, found to be a hot spot in several tumor entities [29]. A supplementation of whole mt-genome sequencing by a specific PCR-approach for D310 may have yielded even higher percentages of tumors, which exhibit at least one mutation in the mitochondrial genome, as compared to the paired normal tissue of the same individual.

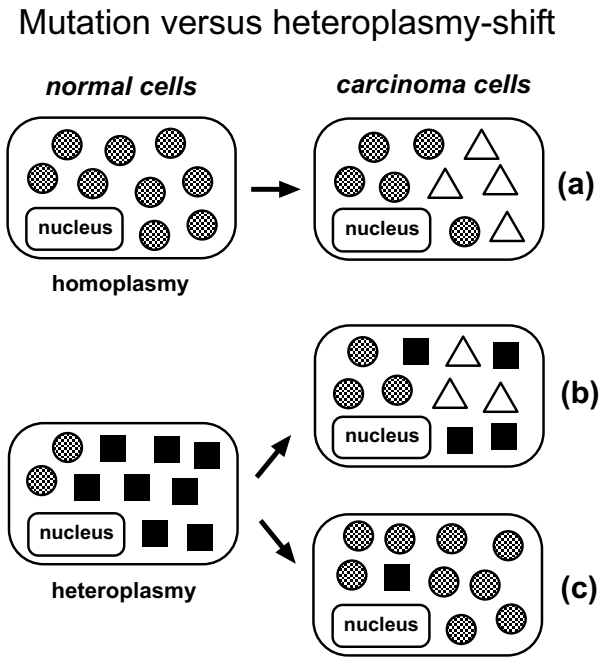

Fig. (3). Figure demonstrating that a heteroplasmic mixture of mtDNA molecules in a carcinoma cell (circles, squares, triangles) cannot unequivocally be attributed to a somatic mutation in the tumor (triangles). Even a minor mtDNA-clone of the corresponding normal tissue can become the dominating one in the tumor by heteroplasmy-shift, as depicted in c. Modified according to [28].

It must be emphasized at this point that the claim of a tumor-specific mtDNA alteration absolutely requires a sequence comparison between the tumor and the constitutive mtDNA of the same individual, ideally derived from adjacent tumor-free tissue or from blood. Due to the high degree of sequence variability within the human population, it is necessary to use this individualized reference instead of just referring to the revised Cambridge sequence. This huge variability is partly caused by the above mentioned control region, but also by many variable nucleotide positions defining the so-called mitochondrial haplogroup, *i.e.* the evolutionary branch of the human mtDNA tree. As has been commented in depth by Bandelt and colleagues, the occurrence of several nucleotide substitutions perfectly matching a haplogroup switch, is always suggestive for sample contamination or sample mix-up. It is therefore mandatory to apply computer-aided haplogroup-analysis [41, 42] to identify tumor samples at risk of contamination, which should better be re-analyzed.

This would be an important and easy issue in case of a practical application of mtDNA in clinical settings.

4. MtDNA MAY NOT BE USEFUL FOR CANCER SCREENING

The knowledge regarding the overall frequency of mtDNA mutations in solid tumors is still too incomplete to allow a faithful estimation of their suitability for screening of bodily fluids from patients at risk. Even in especially relevant entities, such as bladder cancer and head and neck cancer, the coverage of cases cannot be sufficiently estimated due to the small group sizes of available studies [31, 32].

Sensitivity near 100% in bodily fluids is not an indispensable demand for screening. Even established molecular tumor markers in bladder carcinoma (see paragraph 6.1) have limited sensitivity and are currently used solely as an additive to conventional techniques. However, the molecular alteration to be detected in a bodily fluid should at least be present in nearly 100% of analyzed tissue samples of the tumor entity.

Besides incomplete coverage of cases (tumors with true wild-type mtDNA), which automatically yields false negative results, the limited sensitivity of fast sequencing approaches required to analyze the whole 16.5 kb mtDNA (MitoChip) is a further obstacle for screening of urine and saliva. Any statement regarding an applicability of Chip or next generation sequencing (NGS) technology for urine or saliva screening would be premature at this point. It can at least not be excluded that MitoChips and software with enhanced sensitivity to heteroplasmic sites [33] or NGS may become fruitful in this field (see paragraph 6.2).

Already, now the rapid MitoChip technology is certainly a reliable tool for the tissue-based tumor analysis. It is based on a single hybridization of tumor DNA to a preformed chip, on which all nucleotide positions of human mtDNA are represented by four spots of oligonucleotides, differing solely in one nucleotide in the middle of the sequence –representing all four possible DNA-bases- and allowing optimal hybridization of tumor-derived DNA only to the perfectly matching spot. The chip, *i.e.* the position of all spots with perfect matches, is read by a fluorescence-based high resolution reading device, thus allowing high standardization and reproducibility [40]. On the other hand it cannot reach the sensitivity of specific PCR-based techniques targeting a single mtDNA mutation [31].

However, the requirements of non-invasive follow-up differ from those of screening of patients at risk. In the setting of cancer surveillance the analysis can be restricted to mutations already detected by tissue-based methods. This brings mtDNA-analysis close to applicability with currently existing methods.

5. MtDNA IN CANCER FOLLOW-UP: ARE THERE STILL UNEXPLOITED CHANCES?

First of all, a nearly complete coverage of all cases is no principal requirement for an mtDNA-based test of tumor reoccurrence. It would already be a helpful assay, if applicable to a large fraction, *e.g.* 50%, of patients with a given type of carcinoma, provided that it brings any advantage over existing non-invasive methods. This advantage could easily be higher sensitivity, especially as compared to existing cytologic techniques, based on microscopic detection of tumor cells.

Secondly, the surgically resected tumor tissue or biopsy sample provides relatively pure tumor DNA, easily allowing the identification of patients, which are suitable for an mtDNA-based follow-up. MitoChip sequencing [32, 33, 43-45] of pure tumor DNA and reference DNA from the same individual on two chips, enriched by a specific assay targeting D310, would identify the mutated site(s) within a few days. This would allow the choice of a corresponding specific assay for a highly sensitive detection of tumor cells in bodily fluids. A tumor entity with a good chance for a potential benefit from an inclusion of mtDNA in non-invasive follow-up should be the urinary bladder carcinoma. First, this carcinoma is known to seed tumor cells relatively early into the urine and cytologic identification of these cells is already a well-established tool, which supplements the direct visual control of the urinary bladder by endoscopy. This makes it likely that tumor-derived mtDNA usually arises early in the urine. If specific mutations are at all present, it is very likely to detect them with higher sensitivity as compared to cytologic identification of tumor cells. Secondly, there is still a need for a further improvement of non-invasive early detection of recurrences due to the limited sensitivity of routine cytology.

6. MOLECULAR FOLLOW-UP IN URINARY BLADDER CARCINOMA

6.1. Best Validated Current Urine Markers

Bladder cancer (BC) represents the fourth most common cancer in men and the ninth most commen in women [46]. The requirement of a life-long surveillance of patients with non-muscle invasive bladder cancer (NMIBC) is a relevant source of inconvenience for the patients and of costs for the public health systems. These patients resemble about 75% to 85% of BC cases [46] and must be strictly controlled in order to avoid progression to a life-threatening muscle invasive cancer. Most patients suffering from NMIBC will develop a recurrence within the first 5 years after initial diagnosis [47]. If the urinary bladder is not removed and local chemotherapy of the organ is applied instead to cure the disease, frequent controls of potential recurrence are required, usually every three months. Currently endoscopic cystoscopy, eventually enhanced by fluorescence techniques [48, 49], is the gold standard for this surveillance, but a large fraction of patients does not sufficiently adhere to cystoscopic follow-up. Although it is regarded as the most reliable surveillance strategy, it has its limits *e.g.* in case of very small lesions, in the setting of bladder inflammation obscuring a cancer and due to its inability to detect carcinomas of the upper urinary tract. The latter are often diagnosed only by the occurrence of symptoms despite additional application of sonography [50].

A routinely used supplement to cystoscopy is urine cytology, aiming to detect cancer cells by microscopic evaluation. It has been shown to be a fruitful supplement, especially for early detection of high-grade BC, while having a tendency to overlook recurrences of low-grade tumors. For the latter the sensitivity is in the range between 4 and 31%

only [51, 52]. Most importantly, even the combination of cystoscopy and urine cytology exhibits no completely satisfying specificity and sensitivity for early detection of NMIBC.

Therefore, huge efforts have been undertaken and are still in progress to develop molecular tumor markers, which are detectable in the urine [52-57]. Especially due to the relatively high rate of false-positive results of several already available urine tests [55-57], they have a limited value as additives to standard care. Among the non-invasive techniques, conventional urine cytology has still the lowest rate of false-positive results, *i.e.* a mean specificity of usually more than 93% in various studies. Unluckily this welcome feature is combined with a relatively low sensitivity [55-57]. However, there is hope that molecular assays will build a third column of surveillance in the future, which is able to fulfill two major demands: 1) earlier detection of recurrences in problematic settings and 2) extending the intervals for cystoscopy. At present, only a few assays are commercially available and only a few of them have been approved by the FDA for diagnosis or surveillance of BC. These relatively well validated essays rely either on the detection of specific proteins by ELISA or partially by bed-side applicable methods, or rely on the detection of specific DNA alterations. They all have their short-comings.

The bladder tumor antigen assays (BTA stat and BTA TRAK) detect complement factor H and complement factor H related protein, which are also present in blood at high concentrations. The essays are thus hampered by minimal bleeding, which must not necessarily indicate BC and can thus provoke false positive results [55, 56]. The sensitivity of these assays for detection of tumors is also limited. For screening of risk groups, it seems to be in the range of 70% (66%) for BTA stat (BTA TRAK) according to the meta-analysis of large numbers of cases (1160 and 892, respectively). For the other FDA approved application, *i.e.* as an additive to cystoscopy during surveillance of patients with known BC, the sensitivity seems to be around 58% (71%) for BTA stat (BTA TRAK), as deduced from analysis of large cohorts [58].

Assays relying on nuclear matrix protein 22 (NMP22, NMP22 Bladder Check) are based on the observation that this nuclear protein is much more abundant in urothelial cancer cells as compared to the normal endothelium [59] and is released by apoptosis into the urine. Sensitivity and specifity of NMP22 assays are also limited and depend on the chosen cut-off value [59-66].

The ImmunoCyt/uCyt assay relies on immunofluorescence detection of a panel of antigens, which are enriched in exfoliated bladder cancer cells released into the urine. It is thus a cell-based essay, requiring microscopic evaluation by trained personnel. Although its overall sensitivity and specificity are limited [67-69], it is superior for the detection of low-grade BC as compared to conventional cytology [70].

While all the above mentioned assays are finally based on the detection of proteins with limited specificity for BC cells, the last well validated assay (UroVysion) relies on the detection of aneuploidy (chromosomes 3,7,17) and deletions of the 9p21 locus of the tumor suppressor p16 by FISH. These chromosomal alterations again are not completely specific for BC. Moreover these gross chromosomal alterations occur more frequently in high-grade tumors, thereby reducing the sensitivity of the essay for early detection or close surveillance of low-grade NMIBC. A large meta-analyses revealed a rather good sensitivity (70-80%) and specificity (80%) only, if very small and low-grade lesions were excluded, but suggested overall superiority over conventional cytology [71].

In summary, the above mentioned markers already allowed some improvement in diagnostics and follow-up of BC, but are currently of limited value due to a tendency of some assays to generate false positive results, while other assays do not sufficiently close the gap of missing sensitivity of conventional cytology for low burdens of cells exfoliated into urine from a recurring low-grade NMIBC.

6.2. Potential Improvement by mtDNA-analysis?

One potential benefit of mtDNA is simply its higher abundance as compared to single-copy nuclear genes. This relies on the fact that a cell contains dozens or hundreds of mitochondria, each of which harbors several copies of the circular genome. Extremely mitochondria-rich human tissues can thus contain several thousand mtDNA circles per diploid nuclear genome, *e.g.* about 3650 in skeletal muscle or even 7000 in heart muscle [72]. Although cancer cells contain less mitochondria, they may still harbor several hundred mtDNA circles, *e.g.* about 500 in the human ovarian carcinoma cell line A2780 [73]. In this cell line mtDNA-sequences are thus about 250-fold more abundant than nuclear single-copy sequences. Correspondingly, it has been shown as a proof of principle that mtDNA mutations sequenced from pure tumor DNA are represented in matched bodily fluids at much higher abundance than mutations of the classical tumor suppressor p53, previously sequenced from the tumor tissue [31].

Secondly, mtDNA represents a relatively short stretch of DNA (16.5 kb), accumulating a relatively high burden of mutations in solid human tumors. This highly mutated region offers the possibility to find a tumor-specific marker for most -if not all- individual BC patients by fast MitoChip mediated sequencing.

In the nearly 20-year old study of Fliss and colleagues, which still used conventional sequencing of only 80% of mtDNA, already 64% of bladder cancer tissues were found to harbor a somatic mutation by comparison with the constitutive mitochondrial genotype of the same patient [31]. In addition, 46% of head and neck cancers and 43% of lung cancers contained a tumor-specific somatic mtDNA mutation [31]. This early study already suggested that solid human tumors of various origins were likely to harbor detectable mtDNA mutations in every second case. This estimation was supported by many other studies, which focused solely on the control region (1.2 kb) and also detected base substitutions or length alterations of the D310 marker in every third or fourth case of various solid tumors. However, more recent MitoChip based studies suggest that the frequencies were underestimated originally. Jakupciak and colleagues analysed a small series of patients with early cancers of bladder (n=3), lung (n=8) and kidney (n=13). By MitoChip technol-

ogy the authors compared tumor and blood to identify true tumor-specific events and analyzed corresponding bodily fluids of most patients with the same method. Mutations were identified in all BC cases, in 79% of lung cancers and 69% of renal cell cancers [32]. Simply by applying the same array, the authors were able to detect these altered mtDNA sequences in 1 out of 3 urine samples from BC, in 4 out of 12 kidney cancer urine specimens and in 3 out of 7 BAL specimens, *i.e.* bronchoalveolar lavage fluid from the lung cancer patients. Although MitoChp arrays may only be intended for the tissue-based analysis, which then guides a more sensitive analysis of a patient-specific marker in urine (Fig. **4**), the publication demonstrated that tumor-specific mutations can be principally found in corresponding urine specimens by a whole mtDNA approach.

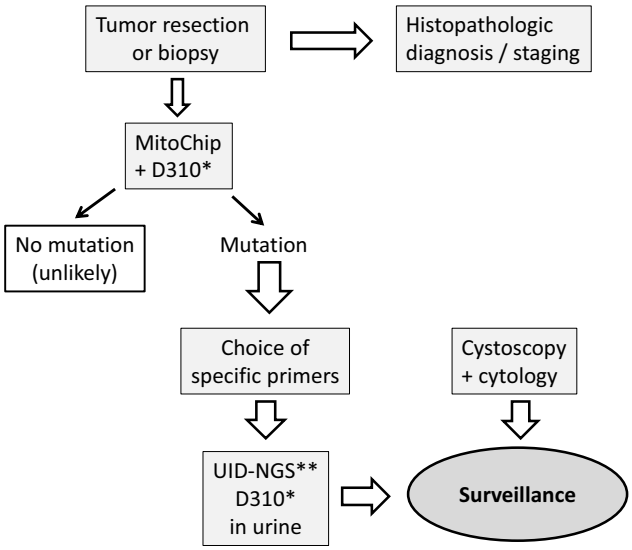

Fig. (4). Scheme illustrating the suggested two steps of tissue-based and urine-based mtDNA-analysis for a potential improvement of BC follow-up. *Analysis of D310 length variation by fluorescent PCR, which may eventually include also a second cytosine tract and a CA-repeat of mtDNA, although they have not been reported to be as variable in tumors as D310. **Besides UID-NGS (barcoded NGS) other strategies may be developed to reduce error rate, *e.g.* comparison of two libraries.

At the stage of tissue-based analysis the achievable sensitivity of Chip hybridization seems already to be sufficient to detect mutations in up to100% of BC cases [32]. The group size analyzed (n=3) was much too small for a precise estimation of coverage of cases, but by comparison with the old results of Fliss and colleagues [31] it can at least be assumed that more than two thirds of BC patients will harbor a specific marker in their tumor-derived mtDNA, which can be exploited for surveillance. The efficacy of MitoChip technology for tissue-based detection of minor mtDNA clones (heteroplasmy) -and correspondingly for subsequent urine-based examination of mtDNA mixtures from exfoliated tumor and normal cells- may be further enhanced by developing other or modified Chip-based sequencing approaches or even by software optimization, dedicated to a more facile detection of heteroplasmic sites [33]. In their study Mithani and colleagues were able to detect known mtDNA mutations of head and neck cancers in the corresponding salivary rinses in 76.9% (10 out of 13) of cases [33]. Tumor-specific mtDNA sequences could still be found at a dilution of 1:200. The authors used the normal MitoChip v2.0, which is commercially available from Affymetrix (Gene Chip Human Mitochondrial Resequencing Array 2.0). Instead of solely using the single probe intensity of the dominant peak, the software was modified to exploit in addition the ratio of single probe intensities of dominant *versus* second most dominant peak, termed 'secondary base intensity'. As expected, this new measure was more suitable for sensitive detection of heteroplasmic sites and accordingly the 'secondary base intensity algorithm' detected a higher percentage of previously known mtDNA mutations in the corresponding bodily fluid.

Sensitivity may be further increased by the addition of a simple PCR-based D310 assay [23]. Chances should be good to find mutations in much more than two thirds of analyzed tumor tissues in BC. Although the distribution of mutations along the mtDNA is not simply stochastic, it remains a challenge to translate the already successful tissue-based assays into future urine analysis.

It seems reasonable to assume, that next generation deep sequencing strategies can be developed, which detect minor sub-clones of cancer cells by mutations of low abundance [74]. It is a realistic assumption that next generation (NGS) deep sequencing [74-79] of a short mtDNA region would thus be able to detect mtDNA mutations in urine samples. In January 2016 Kou and colleagues published a study, which basically established a 'bar-coded' NGS approach on an Illumina platform, suitable for extremely sensitive detection of BC mutations in urine specimens. Using artificial mixtures of mutated and wild-type DNA, the authors delivered the proof of principle for their approach [74]. It is certainly applicable to mtDNA. The study took advantage of the still relatively young technique of 'bar-coded NGS' with unique molecular identifiers (UID). The idea is that the likelihood of PCR and sequencing artifacts limits the power of ultra-deep sequencing to reach the highest possible sensitivity. The use of UIDs enables the software to distinguish artifacts from true mutations, thus increasing the achievable sensitivity. To reach this goal, hybrid 5'-extensions were attached to the gene-specific primers, which were used in the initial two rounds of PCR. These hybrid extensions consisted of a constant tag and a highly variable 'random' sequence (UID). Sequence variability of UIDs was calculated to exceed by far the number of primer molecules in the reaction. This ensures that all individual template molecules (more precisely: all early copies) are labeled by a unique sequence stretch. After the first two rounds the UID-labeled gene-specific primers are replaced by tag-primers. As a result, sequence reads with base substitutions or indels, which share the same UID with the wild-type sequence are very likely to represent artifacts and can be excluded by the software. Other NGS strategies have also been successfully tested recently for their potency of enhanced detection of minor tumor cell clones, *e.g.* by comparison of two independent libraries to separate true mutations from sequencing errors [80].

Last but not least, it has to be mentioned that mtDNA mutations can be expected to occur also in low-grade lesions.

Actually mtDNA mutations have been found in pre-cancerous and low-grade lesions. This early occurrence of mtDNA mutations during carcinogenesis is best documented for cancers of the gastrointestinal tract [81-83]. In which phase of carcinogenesis mutations occur, may vary strongly among tumor entities. Their occurrence seems to be rather late *e.g.* in head and neck cancers [84]. For BC no comparable data of significant series of patients are available, which would allow a precise analysis of this aspect. However, at least a high percentage (about 80%) of length-mutations of HVR-2 and other homopolymeric tracts or CA-repeats had been observed by PCR-assays in early stage BC as compared to corresponding blood samples [85]. This study contained a significant fraction of low-grade tumors, most of which exhibited such somatic length-mutations. Even an earlier study had already observed length-mutations solely of HVR-2 in 25% of BC cases [86]. These results suggest that mtDNA mutations should be present in most low-grade NMIBC lesions, if search is extended to the whole mitochondrial genome. Larger series with reported histology would be required to finally clarify this important point.

7. CHANCES FOR A PERSONALIZED SURVEILLANCE?

All above described BC marker assays (paragraph 6.1) and several new assays in the pipeline aim to detect quantitative or qualitative changes in urine, which are generally observed in BC. In contrast, mtDNA-based assays require some kind of 'personalized surveillance'. This can be imagined as a two-step procedure (Fig. **4**), in which a general and fast tissue-based assay is followed by a periodically applied urine-based assay, which is chosen from a validated set for each individual patient. Due to recent progress, validation of step 1 would be quite easy, limited only by the number of available patients. The true challenge would be to establish the best strategy for mutation detection in a very small fragment of mtDNA in urine samples (or saliva for head and neck cancer) with high sensitivity. In larger cohorts, researchers must exclude the appearance of too many false positive results, *e.g.* due to technical errors (as in ultra-deep sequencing) or due to biological reasons, *e.g.* low abundant variants of mtDNA in exfoliated normal cells. A promising method may be the above described 'bar-coded' deep sequencing [74] with primer sets chosen for the individual patient according to results of step 1.

We are living in times of an upcoming personalization not only of cancer therapy, but also of molecular cancer surveillance. In oncologic areas, where large sets of preformed molecular tools or even true 'personalization' are indispensable, such complex approaches are being developed, despite the huge efforts necessary. A good example is acute myeloic leukemia (AML), where huge numbers of possible mutations or fusion-transcripts indeed required the development of a large panel of assays, from which the clinicians can choose for small subsets of patients. Only the availability of a large number of molecular assays (often based on quantitative PCR) allows the highly sensitive surveillance of minimal residual disease (MRD) in all individual AML patients and the early prediction of relapse, indicated by a quantitative increase of these DNA markers [87]. This control of MRD is essential for planning of therapy.

In a first step the mutations or translocations are assessed and then a specific test is chosen for most sensitive recognition of a reappearance of leukemic blasts in blood samples. These individual assays are designed to find less than 1% of leukemic cells with mutant DNA in the blood. It is not impossible to transfer such a two-step strategy to mtDNA mutations in cancer surveillance. Actually, a limited number of primer sets for overlapping amplicons of the 16.5 kb circle would be sufficient for the tool box.

An important and eventually neglected aspect, to be considered in cancer, is the careful exclusion of a quantitatively inconstant pattern of heteroplasmic mtDNA in a tumor over time. For non-functional polymorphisms such shifts over time in various normal tissues have been nicely shown in systematic studies of transgenic mice [88]. It seems thus reasonable to have a close look on the stability of tumor-specific mtDNA mutations over time. Theoretically, all somatic alterations are originally heteroplasmic, due to the multiple copies of mtDNA per cell. It is likely that a growing sensitivity of analytical techniques for heteroplasmic positions, will find an increasing frequency of mtDNA mutations in many cancer entities. On the other hand, it must be kept in mind that this growing sensitivity will at the same time increase the risk of detecting minor sub-clones, which have not yet reached a stable equilibrium and have not been trapped in the homoplasmic state, respectively. This aspect of a somewhat variable transmission of heteroplasmic patterns to daughter cells forces cancer-researchers to be cautious. This inconstant behavior of mtDNA is a prerequisite for the accumulation of mutations in the tumors, but at the same time determines the risk of instability over time. This potential obstacle must be considered, when surveillance techniques are tested in depth.

8. A SHORT NOTE: DO mtDNA MUTATIONS DRIVE TUMOR GROWTH ?

Due to the dominance of non-functional mutations of the control region it seems likely that most mutations are no relevant drivers in human tumors. Only occasionally mutations have been observed, which should per se have biochemical consequences in OXPHOS and mitochondrial generation of ROS (*e.g.* deletions in conserved OXPHOS polypeptides) or which had been shown in mouse xenograft experiments to support tumor growth *in vivo*. One better characterized example is a 21 bp deletion in the cytochrome B (*cyt b*) gene, which had been found actually in BC by one of the earliest studies aiming to establish mtDNA as a tumor marker in bodily fluids [31]. Later this *cyt b* deletion was cloned and introduced into mouse BC cells, which not only exhibited an altered biochemical phenotype of mitochondria, including increased ROS production, but also faster proliferation, increased colony formation in soft-agar assays and increased matrigel invasion [89]. Most important, these cells initiated a dramatically faster subcutaneous tumor growth in mice, as compared to their counterparts transfected with wild-type *cyt b* [89]. Since the cells could be implanted into syngenous mice, the tumor-promoting effect was independent of artificial shutdown of the immune system, which is necessary in most other xenograft models. The tumors showed a dramatically increased density of blood vessels in addition. The authors presented some evidence that the ob-

served phenotype may in part be explained by increased production of ROS and may be mediated by the redox-sensitive transcription factor NFkappa-B2, which promoted cell cycle and restricted apoptosis. Activation of transcription factors by ROS is generally a thinkable mechanism, how some mtDNA mutations may promote tumor growth and has also been suggested in other cases, *e.g.* for a missense mutation in a mitochondrial gene for a subunit of the F_oF_1-ATP-synthetase. That particular mutation had long been known from classical mitochondrial disorders, when it was detected by the group of Douglas Wallace in a prostate carcinoma. The mutation turned out to be tumorigenic in mice [90].

Recent NGS approaches combined with sophisticated data analysis to select mutations of likely functional relevance, may shed a much more systematic light on the potential role of mtDNA mutations for tumor biology in the future. A group of researchers from Italy recently implemented a multistep bioinformatics analysis of NGS-based sequence data, which detected many, mostly low abundant heteroplasmic mtDNA mutations in glioblastomas and neuroblastomas, the vast majority of which was likely to represent merely passengers of the clonal cell expansion without any role for tumor growth [91, 92]. The bioinformatics tool used, called MToolBox, had been recently developed [93] and consists of several layers of subsequent filters, intended to exclude irrelevant polymorphisms and to select or assign higher priority to mutations, which may deserve further analysis of a potential clinical role. First, all base substitutions were excluded, which occurred in nucleotide positions defining haplogroups. This is certainly a meaningful criterion, because haplogroups simply define branches of the evolutionary mtDNA-tree. Although haplogroups are at least discussed as moderate susceptibility or modifying factors for some classical mitochondrial or neurodegenerative diseases, all these variable nucleotide positions are not likely to support tumor growth essentially. Further filters included the variability score of the affected nucleotide position and of the corresponding amino acid in case of translated regions and also the available direct information for a pathogenic role of a specific missense or tRNA/rRNA mutation, as available in disease-related mitochondrial databases. Only a limited number of mtDNA mutations survived the filter process in glioblastomas and neuroblastomas and may thus more likely play a pathogenic role in these tumors. The results were consistent with the hypothesis that most mutations were merely passengers. NGS is by far more sensitive for detection of low abundant heteroplasmy as compared to Sanger sequencing [94]. The number of detected heteroplasmic sites can be expected to grow with increasing sequencing coverage. It is clear now, that based on deep NGS sequencing, individuals can generally be expected to exhibit heteroplasmic mitochondrial genomes. It is thus highly important to use bioinformatics tools to reconstruct a collection of 'allowed heteroplasmic variants', as a basis to screen for clinically relevant private mutations, which do not occur in this collection, may drive tumor growth and could be eventually of prognostic value. It will be necessary to analyze NGS data of more tumor entities with bioinformatics tools for functional prioritization of mutations.

However, the functional relevance of mtDNA mutations plays no role for its potential suitability in surveillance of patients. Nor does the question play a role, if the frequently observed sequence alterations can really be called 'somatic mutations' or may in part rely on heteroplasmy-shifts. It will just be important to check the specificity, sensitivity and long-term stability of these alterations for analysis of bodily fluids of cancer patients.

9. IS mtDNA COPY NUMBER ASSOCIATED WITH PROGNOSIS OR GENERAL CANCER RISK ?

Above, the potential usefulness of mtDNA for tumor screening and -more likely- for surveillance of known cancer patients has been discussed. However, there is also growing evidence connecting the copy number of mtDNA in the tumor tissue with prognosis of cancer patients. Systematic approaches to investigate this issue will be a potential field for data mining in existing NGS data, because the number of sequence reads aligning to mtDNA and nuclear genome (as a reference) contain the desired copy number information. Moreover, an individual's constitutive copy number, usually determined from blood by Real-Time-PCR, became increasingly associated with cancer risk. These two aspects certainly warrant further research, because currently available results do not deliver a homogenous and easily interpretable picture.

With respect to the potential prognostic relevance, it makes sense to have a look on studies comparing normal and cancerous tissues or advanced cancers with early lesions of the same entity for the first estimate of the role of mtDNA copy number in tumor diseases.

It was observed that mtDNA copy number is indeed often altered in cancer. Primarily a decrease of mtDNA may be expected to support cancer growth, having in mind the switch of energy metabolism from OXPHOS to aerobic glycolysis in many (but not all) tumor cell types and Warburg's old hypothesis, that this switch drives tumor development. But it is known meanwhile that mtDNA content and OXPHOS activity do not always correspond well in tumor tissues and -more importantly- copy number studies sometimes did not even deliver homogenous results within a single entity. For example, in colorectal cancer one recent study observed a lower mtDNA copy number in colorectal carcinomas as compared to adenomas [95], while another publication described higher copy numbers in colorectal cancer tissues as compared to adjacent normal tissues and a positive association of high copy number with poor prognosis [96]. An increased mtDNA-level in gliomas was observed as compared to (not matched) normal brain tissues, high copy number being associated with better prognosis [97]. In a cohort of lymph-node negative breast cancers, the patients with the lowest quartile of mtDNA-levels exhibited a significantly lower 10-year survival [98].

In 2016, Reznik and colleagues exploited the comparison of the number of mitochondrial and nuclear sequence reads to determine mtDNA copy number changes between tumor and adjacent normal tissues in 15 tumor types, for which NGS data of at least 10 paired samples were available [99]. Thereby the authors provided a more comprehensive overview. Only about half of the analyzed tumor types exhibited a significant decrease of mtDNA abundance as compared to the paired normal tissues, *i.e.* cancers of bladder, breast, kidney, head and neck, esophagus and liver, especially pro-

nounced in bladder cancer. Irrespective of the issue of copy number change, mtDNA abundance in the tumor was prognostic in five cancers. While high copy number was associated with better survival in adrenocortical carcinoma, chromophobe renal cell carcinoma and low grade glioma, the opposite occurred in clear cell renal carcinoma and melanoma. In summary, tumor development is often associated with changes of mtDNA-levels, but direction and especially prognostic significance of this change seem to vary between entities.

It may thus not be surprising that studies trying to asses an individual's tumor risk based on its constitutive mtDNA copy number in blood led to interesting, but currently still inhomogenous results, demanding more generalized meta-analyses, as recently performed for the first time [100].

For example, in a large study of 1108 cases and 1099 controls Lemnrau and colleagues had detected a positive association between a high mtDNA-content in peripheral blood cells and the risk for breast cancer [101]. An OR of 1.37 was found for the comparison of breast cancer risk between the highest and lowest quartiles of mtDNA copy number in blood samples taken prior to cancer diagnosis (mean time interval 6 years). Although age of patients, as expected, also had a significant influence on mtDNA-levels, the comparison of these large cohorts established mtDNA-levels as a promising factor for risk analysis in breast cancer. Similar results were communicated by another publication [102]. A comparison of 500 melanoma patients with 500 controls [103] also revealed a positive association between high mtDNA content and melanonma risk. Although in a relatively small case-control study (139 cases and 139 age-matched controls), an enhanced risk for endometrial cancer was obtained for a lower mtDNA content of peripheral blood cells, in contrast to the situation in the aforementioned entities [104].

CONCLUSION

A meta-analysis of risk studies [100] compared and critically interpreted data for all so far analyzed tumor entities from 26 qualified English publications available at that time, *i.e.* published between 2008 and 2014. The meta-analysis did thus not contain the above mentioned recent risk studies. All 26 included publications had used DNA from peripheral blood cells, except for one lung cancer study, which used sputum. One of the most important results of this approach may be the absence of significant unidirectional effects across all tumor entities. Looking for specific tumor types, the positive association between high mtDNA copy number and increased risk for lymphoma (OR 1.76, p=0.023), but reduced risk for skeleton cancers (OR 0.39, p = 0.001) were important. The authors critically remark that these results must be interpreted with care. For skeleton tumors only a single underpowered primary study had been available. However, most important is the remark of the authors that, due to the general use of blood cell DNA, the convincing results for lymphomas represented the only collection of studies, in which a large fraction of the analyzed mtDNA is isolated from cells, which are derived from the relevant cell type of origin for the malignant process. If a more generalized relation between mtDNA-level and cancer risk should exist, this may be better detectable in sufficiently large studies analyzing the relevant normal tissue of origin for each tumor type. Of course this will be difficult.

While the usage of mtDNA as biomarker for tumor risk and prognosis will require further broad NGS and bioinformatics approaches, its potential use for cancer surveillance depends mainly on focused studies in a few eligible entities, especially in bladder cancer.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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