NEOPLASIA
www.neoplasia.com

Volume 20 Number 7 July 2018 pp. 687–696 687

Tumor-Specific Mitochondrial DNA Variants Are Rarely Detected in Cell-Free DNA[1,2]

M.J.A. Weerts[*], E.C. Timmermans[†], A. van de Stolpe[†], R.H.A.M. Vossen[‡], S.Y. Anvar[‡,§,¶], J.A. Foekens[*], S. Sleijfer[*] and J.W.M. Martens[*]

[*]Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands; [†]Philips Research Laboratories, High Tech Campus 11, Eindhoven, The Netherlands; [‡]Leiden Genome Technology Center (LGTC), Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; [§]Department of Human Genetics, Leiden University Medical Center, Leiden, The Netherlands; [¶]Department of Clinical Pharmacy and Toxicology, Leiden University Medical Center, Leiden, The Netherlands

Abstract

The use of blood-circulating cell-free DNA (cfDNA) as a "liquid biopsy" in oncology is being explored for its potential as a cancer biomarker. Mitochondria contain their own circular genomic entity (mitochondrial DNA, mtDNA), up to even thousands of copies per cell. The mutation rate of mtDNA is several orders of magnitude higher than that of the nuclear DNA. Tumor-specific variants have been identified in tumors along the entire mtDNA, and their number varies among and within tumors. The high mtDNA copy number per cell and the high mtDNA mutation rate make it worthwhile to explore the potential of tumor-specific cf-mtDNA variants as cancer marker in the blood of cancer patients. We used single-molecule real-time (SMRT) sequencing to profile the entire mtDNA of 19 tissue specimens (primary tumor and/or metastatic sites, and tumor-adjacent normal tissue) and 9 cfDNA samples, originating from 8 cancer patients (5 breast, 3 colon). For each patient, tumor-specific mtDNA variants were detected and traced in cfDNA by SMRT sequencing and/or digital PCR to explore their feasibility as cancer biomarker. As a reference, we measured other blood-circulating biomarkers for these patients, including driver mutations in nuclear-encoded cfDNA and cancer-antigen levels or circulating tumor cells. Four of the 24 (17%) tumor-specific mtDNA variants were detected in cfDNA, however at much lower allele frequencies compared to mutations in nuclear-encoded driver genes in the same samples. Also, extensive heterogeneity was observed among the heteroplasmic mtDNA variants present in an individual. We conclude that there is limited value in tracing tumor-specific mtDNA variants in blood-circulating cfDNA with the current methods available.

Neoplasia (2018) 20, 687–696

Introduction

Mitochondria are organelles within our cells responsible for a variety of functions, including energy production and initiating apoptosis. Their small circular genome (mitochondrial DNA, mtDNA) encodes for proteins essential in the oxidative phosphorylation system and the tRNA and rRNA molecules of the mitochondrial translation apparatus. Within a single cell, multiple copies of mtDNA exist (mtDNA content), but due to its small size, the mtDNA represents only a minor fraction of the total cellular DNA (<0.1%). In general, cells with high energy demand (e.g., muscle cells) have a higher mtDNA content than cells with lower energy demand (e.g., blood

Abbreviations: mtDNA, mitochondrial DNA; nDNA, nuclear DNA; cfDNA, cell-free DNA; SMRT, single-molecule real-time sequencing; dPCR, digital PCR

Address all correspondence to: M.J.A. Weerts, Department of Medical Oncology and Cancer Genomics Netherlands, Erasmus MC Cancer Institute, Erasmus University Medical Center, Rotterdam, The Netherlands. E-mail: m.weerts@erasmusmc.nl

[1] Competing Interests: The authors declare no conflicts of interest.

[2] Funding: This work was supported by a grant from Philips Research (Eindhoven, The Netherlands).

Received 6 February 2018; Revised 7 May 2018; Accepted 8 May 2018

1476-5586
https://doi.org/10.1016/j.neo.2018.05.003

cells) [1]. In human cancer, changes in mtDNA content have been reported when tumor specimens are compared to their normal counterparts [2]. The polyploid nature of mtDNA invokes the concept of only a single (homoplasmy) or two or more mitochondrial genotypes (heteroplasmy) within a cell. It has been shown that heteroplasmy patterns within an individual can differ between tissues, even in an allele-specific manner [3–6]. Also within cancer, tumors harbor mtDNA that is genetically different to their normal counterparts, either at a homo- or heteroplasmic level, and their number and position vary among tumors [7–9]. Interestingly, since the mutation rate of mtDNA is several orders of magnitude higher than that of nuclear DNA (nDNA) [10], it is very informative to assess phylogenetic distance not only intra- and interspecies but also interindividual.

Within oncology, the use of blood-circulating cell-free DNA (cfDNA) as a "liquid biopsy" is being explored for its potential as a screening tool, to establish prognosis, or as a marker for response to treatment. The origin of cfDNA is mainly from apoptotic cells, hence its typical fragmentation pattern representing DNA cleavage between nucleosomes or chromatosomes (~146-166 base pairs and multiples thereof) [11]. The physical characteristics of cf-mtDNA have not been studied as extensively as its nuclear counterpart. Since mtDNA is packed into nucleoids [12], which are not fragmented during apoptosis [13], the fragmentation pattern as seen for nDNA does not apply to mtDNA. Indeed, the majority of the cf-mtDNA in human plasma appears associated with particles of at least 0.45 μm in diameter [14], and a fraction of it is severely fragmented down to at least 30 base pairs [15–18]. If not fragmented, the circular nature of mtDNA might render it less susceptible to enzymatic cleavage and thus more stable within the circulation. The total amount of cfDNA is often increased in cancer patients compared to healthy individuals, for both DNA from the nucleus as well as mtDNA [16,19–23]. The detection of tumor-specific cfDNA is aided by the aberrations present in the cancer's genome and thus by the detection of tumor-specific mutations within the cfDNA. A few studies have attempted to detect mtDNA variants in blood-derived cfDNA [4,24–29] or other bodily fluids [24,30–33]. However, in the studies on blood-derived cf-mtDNA, used methods were either not very sensitive (i.e., conventional Sanger sequencing), or the variants were not truly tumor-derived (i.e., already present in matched normal specimens). Also, quantitative variant allele frequencies were not reported in all assessed samples, making interpretation of these results difficult. Nevertheless, the combination of a high copy number per cell, a high mutation rate, and potentially high stability within the circulation makes it worthwhile to explore the potential of tumor-specific variants in cf-mtDNA as a cancer biomarker.

In this study, we used a targeted single-molecule real-time (SMRT) sequencing approach to profile the entire mtDNA of the primary tumor and/or metastatic sites, tumor-adjacent normal tissue, and cfDNA of eight cancer patients. We have recently shown that the SMRT sequencing approach is able to reliably detect unknown variants ≥1.0% allele frequency and to trace known low-frequent variants down to at least 0.1% allele frequency [34]. In our cohort, we observed tumor-specific mtDNA variants for each patient and explored the feasibility to trace these tumor-specific variants in cf-mtDNA as a cancer biomarker.

Materials and Methods

Patient Selection and Sampling

We used material from our biobank at the department of Medical Oncology of the Erasmus MC Cancer Institute, Rotterdam, the Netherlands. Patient selection was based on availability of a frozen blood derivate (plasma or serum) to obtain cfDNA and fresh-frozen resection material of tumor tissue (primary or metastasis). For all except one case, fresh-frozen material of normal tissue originating from the same resection material was available. Blood sampling was done in either serum separation tubes according to routine procedures in our hospital or in EDTA tubes followed by cell separation within 24 hours after blood draw (10 minutes at 800×*g*). Obtained serum or plasma samples had been stored at −80°C until use. After thawing, plasma samples underwent additional sedimentation at 1020×*g* for 10 minutes at 4°C, of which the supernatant was used. Use of the patient material was approved by the medical ethics committee of the Erasmus MC (MEC 02.953 and MEC 06.089) and conducted in accordance to the Code of Conduct of the Federation of Medical Scientific Societies in the Netherlands.

DNA Extraction

For fresh-frozen tissue specimens, a DNA extraction method that enriches for mtDNA was performed as described before [34]. Briefly, 20 cryosections of 30 μm (average input of 30 mg tissue, range of 16-59 mg) per specimen were lysed to solubilize cellular membrane and release all cellular compartments (10 minutes, 1 ml of 0.5× TBE containing 0.5% (v/v) Triton X-100). Cell nuclei were removed (10 minutes 1020×*g*), and DNA was extracted from the remaining supernatant using the QIAamp Circulating Nucleic Acid Kit (*Qiagen*) according to the suppliers' protocol. For four specimens, an additional sample was obtained by an independent DNA extraction (as described above) with subsequent enzymatic degradation for linear DNA as described before [34]. Briefly, those DNA extracts (max. 250 ng) were incubated with ATP-dependent exonuclease PlasmidSafe (*Epicenter*) (40 U, 3 hours at 37°C), heat-inactivated (30 minutes 70°C), and purified (ethanol precipitation, 70% ethanol). For some frozen tissue specimens, DNA extracts were already available in our biobank and had been obtained by either the PureLink Genomic DNA kit (*Invitrogen*) or the DNeasy Tissue Kit (*Qiagen*) as described by the supplier. For each tissue sample, 5-μm sections were obtained on microscopy slides and hematoxylin and eosin (HE) stained to estimate the percentage of tumor cells within the sections used for DNA extraction. For the blood derivates, after thawing at 4°C, DNA was extracted using the QIAamp Circulating Nucleic Acid Kit (*Qiagen*) according to the suppliers' protocol. Serum input ranged from 450 to 500 μl, and plasma supernatant input was 1000 μl. Specifications for each sample are provided in Supplementary Table 1.

DNA Quantification and mtDNA Purity Assessment

All DNA extracts were quantified using the Qubit dsDNA HS assay kit (*Life Technologies*) according to the suppliers' protocol. Purity of mtDNA was measured in duplicate runs of a multiplex qPCR assay targeting a nuclear and a mitochondrial encoded gene to calculate the ratio of mtDNA molecules opposed to nDNA molecules by the relative quantitation method (2^ΔCq) as described before [35].

SMRT Sequencing

SMRT sequencing was performed as described before [34]. Briefly, amplicons covering the complete mtDNA were generated by singleplex (tissue DNA) or multiplex (cfDNA) PCR with initial denaturation for 3 minutes at 98°C, 15 or 18 cycles of a three-step PCR with 10-second denaturation (98°C), 30-second annealing (67°C) and 90-second extension (72°C), followed by a final extension (72°C) for 5 minutes. DNA input was set to contain at least 100,000