Mitochondrion 39 (2018) 43–50

Contents lists available at ScienceDirect

Mitochondrion

journal homepage: www.elsevier.com/locate/mito

# Mitochondrial DNA haplogroup K as a contributor to protection against thyroid cancer in a population from southeast Europe

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ARTICLE INFO

*Keywords:*
Thyroid cancer
mtDNA haplogroups
Haplogroup K
Case-control study

ABSTRACT

We aimed to analyze the contribution of mitochondrial DNA (mtDNA) haplogroups of the mtDNA control region to thyroid cancer risk in a population from southeastern Europe consisting of 235 thyroid tumor patients, including 114 patients with thyroid follicular adenoma, 121 patients with papillary thyroid carcinoma, and 419 healthy controls. Binary logistic regression with adjustment for age and gender revealed that mtDNA haplogroup K was significantly associated with a protective role for thyroid cancer in the combined tumor group versus controls. These results indicate a potential role for mtDNA haplogroups as important candidate susceptibility markers for the patients with thyroid nodules.

## 1. Introduction

Thyroid nodules represent a very common clinical finding with a higher prevalence in women than men, and which occur more frequently in areas where iodine deficiency is common (Hegedüs, 2004). Thyroid cancer is the most common endocrine malignancy, accounting for 90% of all endocrine malignancies and up to 1.5% of all malignant disease (Pellegriti et al., 2013). Thyroid tumors can be benign adenomas or malignant lesions. Clinical follow up is recommended for thyroid nodules with benign cytology, due to a risk of malignancy of 2%–18% (Wang et al., 2011).

The most frequent pathological types of thyroid cancer originate from follicular and parafollicular thyroid cells.

Papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) are the main subtypes of histological variants of thyroid cancer clinically characterized as differentiated thyroid cancers (DTCs) in addition to poorly differentiated and anaplastic thyroid carcinomas. PTC constitutes > 80% of thyroid carcinomas (Mazzaferri, 1992), whereas FTC accounts for 10%–15% of thyroid malignancy diagnoses (Jemal et al., 2010). Follicular thyroid carcinoma displays cytological features similar to benign follicular adenoma, making diagnosis problematic. Differential diagnosis between FTC and thyroid follicular adenoma (TFA) is based on the presence of capsular invasion, vascular invasion, extrathyroidal tumor extension, and nodal or distant metastases upon postoperative histo-pathological examination (McHenry and Phitayakorn, 2011).

The incidence of thyroid cancer has increased continuously over the last three decades worldwide, at various rates in different areas of the globe (Davies and Welch, 2006). Several studies conclude that the frequent use of sensitive technologies and intensive medical surveillance are the causes of this apparent increased incidence of thyroid cancer over the last few decades (Morris et al., 2013; Hsiao and Nikiforov, 2014). On the other hand, recent reports have challenged this theory, suggesting that the increased incidence in thyroid cancer could be the result of a combination of both apparent and true increases, since a steady upward trend has occurred across all tumor sizes and stages, indicating that rising incidence has multifactorial origins (Pellegriti et al., 2013).

Mitochondria play an essential role in synthesis of ATP through the respiratory chain oxidative phosphorylation (OXPHOS), generation of reactive oxygen species (ROS), regulation of apoptosis, and intracellular signaling and metabolic pathways.

Human mitochondrial DNA (mtDNA) is a double-stranded circular DNA molecule of 16.6 kb divided into two main regions, the coding region that encodes for 37 genes (Anderson et al., 1981) and the non-coding region or control region (CR) of 1.1 kb, which controls the replication and transcription. The control region contains the displacement loop (D-loop) region and three hypervariable regions (HVS I, HVS

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http://dx.doi.org/10.1016/j.mito.2017.08.012
Received 21 December 2016; Received in revised form 16 August 2017; Accepted 23 August 2017
Available online 26 August 2017

II and HVS III), which have sequentially accumulated a great number of mutations along maternal lineages during the course of human evolution (Ingman et al., 2000; Herrnstadt et al., 2002). The majority of mtDNA single nucleotide polymorphisms (SNPs) are concentrated in the HVSI and HVSII regions.

In the European population, there are nine major haplogroups designated as H, U, J, T, K, W, I, V and X, which are characterized by unique sets of haplotypes that exhibit different combinations of specific polymorphisms (Torroni et al., 1996).

Several studies have established that the non-pathological mtDNA variability that defines these haplogroups could be involved in the pathogenesis of different diseases in specific populations because of its influence on the expression of genes related to ROS production and OXPHOS coupling efficiency (Kenney et al., 2014; Fernández-Caggiano et al., 2012).

Mitochondrial haplogroups and polymorphisms are reported as associated with various complex diseases, including metabolic syndrome (Tanaka et al., 2007), Parkinson's disease (Ghezzi et al., 2005), cardiomyopathies (Hagen et al., 2013), infectious diseases (Jiménez-Sousa et al., 2014), aging (Courtenay et al., 2012), and cancer (Theodoratou et al., 2010). Other studies reported contrasting results in different populations; for example, Govindaraj et al. did not find any association between mitochondrial haplogroups and cardiomyopathy (Govindaraj et al., 2014). Various studies have explored the predisposing or protective roles of mtDNA haplogroups and polymorphic variants (mutations and polymorphisms) in the coding and D-loop regions of mtDNA in a variety of human cancers. In particular, haplogroup K has been associated with increased risk of breast cancer (Bai et al., 2007), while haplogroup U is more common in white North Americans with prostate and renal cancer (Booker et al., 2006). Moreover, haplogroup U is reported to have a protective effect, and haplogroup H is associated with an increased risk in colorectal cancer patients (Theodoratou et al., 2010).

Furthermore, in Asian populations, sub-haplogroup F1 confers genetic susceptibility to nasopharyngeal carcinoma (Hu et al., 2014), while haplogroup D4a is associated with an increased risk of thyroid cancer (Fang et al., 2010).

In addition to germline polymorphisms, many somatic mutations in the D-loop region are identified as predicting cancer risk for various tumor types (Navaglia et al., 2006). An improved characterization of the susceptibility background markers by haplogroup association studies in different geographic populations could have a pivotal role for assessing the differential risk of thyroid cancer, with an effective impact on the evaluation of preoperative risk for patients with low-risk thyroid cancer (Yip, 2015). Given the growing body of evidence providing important insights into the low or high risk of cancer associated with mtDNA variability (including mtDNA haplogroups and various SNPs) in the human population, here we assessed, for the first time in a European population, the potential contribution of mtDNA haplogroups to thyroid cancer risk. To test our hypothesis, we analyzed in a case-control study the distribution of different haplogroups in a southeast European population of 114 patients with TFA, 121 patients with PTC, and 419 healthy controls. In addition, we investigated the frequency of occurrence of polymorphisms in the mitochondrial DNA control region between the patient groups and controls.

## 2. Materials and methods

### 2.1. Patients and control subjects

Peripheral blood samples from 114 patients with TFA, 121 patients with PTC and 419 control subjects with no genetic relations were obtained at “C. I. Parhon” National Institute of Endocrinology between 2013 and 2014. Informed written consent was obtained in accordance with protocols that complied with the Declaration of Helsinki and were approved by C. I. Parhon National Institute of Endocrinology's ethical committee (Bucharest, Romania). Experiments were carried out in accordance with these approved guidelines.

The healthy subjects were randomly selected from the general population who participated in a health screening program and had no family or personal history of thyroid disorders. The ages of the study subjects ranged from 6 to 79 years (mean age, 45.21 ± 17.04 years) in the control group, 17 to 70 years (mean age: 45.27 ± 12.76 years) in the TFA group, and 11 to 81 years (mean age, 46.63 ± 14.75 years) in the PTC group. The female to male ratio was approximately 3:1 in both cancer groups and 2:1 in the control group.

Genealogical information was recorded for patients and healthy controls for a minimum of two generations. All samples included in the present study were from ethnic Romanians, and no individuals from ethnic minorities were included in the study.

For all cases included in the study, histological assessment and classification of cancer type were carried out immediately following surgery. All thyroid lesions were classified as follicular adenoma or papillary thyroid carcinoma, according to the most recent WHO diagnostic histological guidelines (DeLellis et al., 2004), by two experienced certified pathologists, who were blinded to the clinicopathological features of the patients. PTCs were staged according to the American Joint Committee on Cancer's TNM classification system for differentiated thyroid carcinoma (Edge et al., 2010).

### 2.2. MtDNA genotyping

Genomic DNA was extracted from 200 μL of whole blood in EDTA using a PureLink Genomic DNA Mini kit (Invitrogen, USA).

The sequences of the mtDNA control regions of all samples were determined by sequencing the hypervariable segments HVS I (nps 16024-16416) and HVS II (nps 1-410) using two specific pairs of primers covering the HVS-I and HVS-II segments, respectively (Hervella et al., 2012).

PCRs were conducted in 25 μL of reaction mixture using 1 × PCR buffer (15 mM Tris–HCl pH 8.0 and 50 mM KCl), 200 μM of each dNTP, 2.5 mM MgCl2, 0.2 μM of each primer, 0.2 U of AmpliTaq Gold DNA polymerase (Life Technologies, USA), and approximately 30 ng of DNA.

PCR cycling parameters were as follows: 95 °C for 10 min, followed by 36 cycles of 95 °C for 20 s, 60 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min for amplification of the HVS I sequence and 95 °C for 10 min, followed by 36 cycles of 95 °C for 20 s, 58 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min for the HVS II sequence. Amplicons were purified using a QIAquick PCR purification Kit (Qiagen, Germany) and sequenced using the Big Dye Terminator v3.1 Cycle Sequencing kit on ABI 3130XL Genetic Analyzers (Applied Biosystems, USA).

A hierarchical system for mtDNA haplogrouping that combined sequencing of the HVS I and HVS II regions and PCR restriction fragment length polymorphism (PCR-RFLP) was used to assess the prevalent European mtDNA haplogroups. Thus, samples ambiguously classified using mtDNA control region sequence variations were further assessed by PCR-RFLP, using RFLP markers for haplogroup assignment, as described elsewhere (Santos et al., 2004).

Sequences were edited using ABI PRISM SeqScape v.2.5 software. The mtDNA HVS I and HVS II sequences of each individual were aligned using MEGA 6 software and compared with the revised Cambridge Reference Sequence rCRS (Anderson et al., 1981; Andrews et al., 1999). The recorded variants in each mtDNA sequence were also analyzed using MitoTool (www.mitotool.org) (Fan and Yao, 2011). All the haplogroups analyzed in this study were assigned using online programs, including HaploGrep (Kloss-Brandstätter et al., 2011) and Phylotree (mtDNA tree Build 16) (Van Oven and Kayser, 2009).

### 2.3. Accession numbers

The control region sequences of the 654 subjects generated in this