The biology and the genetics of Hürthle cell tumors of the thyroid

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Abstract

The biology and the genetics of Hürthle cell tumors are reviewed starting from the characterization and differential diagnosis of the numerous benign and malignant, neoplastic and nonneoplastic lesions of the thyroid in which Hürthle cell transformation is frequently observed. The clinicopathologic and molecular evidence obtained from the comparative study of the aforementioned conditions indicate that Hürthle cell appearance represents a phenotype that is superimposed on the genotypic and conventional histopathologic features of the tumors. Hürthle cell tumors differ from their non-Hürthle counterparts regarding the prevalence of large deletions of mitochondrial DNA (mtDNA), mutations of mtDNA genes coding for oxidative phosphorylation (OXPHOS) proteins (namely mutations of complex I subunit genes) and mutations of nuclear genes coding also for mitochondrial OXPHOS proteins. Such mitochondrial alterations lead to energy production defects in Hürthle cell tumors; the increased proliferation of mitochondria may reflect a compensatory mechanism for such defects and is associated with the overexpression of factors involved in mitochondrial biogenesis. The mitochondrial abnormalities are also thought to play a major role in the predisposition for necrosis instead of apoptosis which seems to be blocked in most Hürthle cell tumors. Finally, the results obtained in experimental models using cybrid cell lines and the data obtained from histopathologic and molecular studies of familial Hürthle cell tumors are used, together with the aforementioned genetic and epigenetic alterations, to progress in the understanding of the mechanisms through which mitochondrial abnormalities may be involved in the different steps of thyroid carcinogenesis, from tumor initiation to metastatization.

Endocrine-Related Cancer (2012) 19 R131–R147

Introduction

Any review on the biology of Hürthle cell tumors faces two major problems: the definition of what is and what is not (yet?) a Hürthle cell (i.e. the existence of a threshold that separates Hürthle cells from mitochondrion-rich cells) and the identification, among the numerous tumors and tumor-like lesions of the thyroid composed by Hürthle cells, those that should be diagnosed as carcinomas. Two additional problems concern the classification of the different carcinoma histotypes displaying Hürthle cells and the molecular interpretation and management of thyroid carcinomas totally or partially composed by Hürthle cells. We will start by addressing the first problem.

Hürthle cells and Hürthle cell transformation

The so-called Hürthle cells, alias oncocyctic-, alias oxyphilic-, alias eosinophilic-cells are characterized by the cytoplasmic accumulation of abundant mitochondria that frequently display abnormal morphology (Nesland *et al.* 1985, Máximo & Sobrinho-Simões 2000a).

The designation Hürthle cells is used in thyroid pathology whereas the other names are used in other settings (e.g. oncocytoma of the kidney, oxyphilic adenoma and oxyphilic carcinoma of the parathyroid; Máximo & Sobrinho-Simões 2000a). For the sake of simplicity we will use throughout the text, whenever

possible, the designation Hürthle cells regardless of the context.

Hürthle cells are frequently observed in benign and malignant tumors of several organs, as well as in hyperplastic conditions (e.g. multinodular adenomatous goiter) and in chronic inflammatory conditions (e.g. Hashimoto's thyroiditis) of the thyroid. They are also frequently observed in several endocrine organs of elderly individuals (e.g. parathyroid gland) and in lesions of patients suffering from neurodegenerative disorders caused by mitochondrial alterations (e.g. oncocyctic transformation of choroid plexus epithelium and oncocyctic cardiomyopathy patients; Silver *et al.* 1980, Kepes 1983, Ohama & Ikuta 1987, Yoneda *et al.* 1992, Tanji *et al.* 2000). In contrast with this, Hürthle cell transformation of solid cell nests is extremely rare (Cameselle-Teijeiro *et al.* 2012).

Hürthle cells are rare in 'normal' appearing thyroid glands of elderly people unless there are concurrent signs of chronic inflammation. Patients with germline mitochondrial alterations leading to neurodegenerative disorders do not display Hürthle cell transformation in the thyroid gland. There is, nevertheless, a very recent report of a case of Hürthle cell carcinoma of the thyroid in a patient with Cowden syndrome carrying both a 10q23 and a mitochondrial DNA (mtDNA) germline mutation (Pradella *et al.* 2011). The high prevalence of Hürthle cell transformation in thyroid lesions may reflect the high oxidative stress and reactive oxygen species (ROS) production in thyroid cells, during normal iodine and thyroid hormone metabolism (Wang *et al.* 2011, Xing 2012). This high ROS levels can result in mutagenic genetic events, namely in mtDNA, leading to mitochondrial dysfunction (Wallace 1999).

Hürthle cell transformation is thought to be caused by disequilibrium between mitochondrial proliferation, on one side, and mitochondrial destruction and/or cell division, on the other, leading to the accumulation of mitochondria (Katoh *et al.* 1998, Máximo & Sobrinho-Simões 2000a). Time factor is crucial for the acquisition of Hürthle cell features since it takes a lot of time to increase from 100/150/200 mitochondria to the 4000/5000 mitochondria per thyroid cell that characterizes Hürthle cells (Sobrinho-Simões *et al.* 1985, Máximo & Sobrinho-Simões 2000a). That is the reason why autoimmune thyroiditis in young, <20 years patients, rarely has Hürthle cells, in contrast to similar conditions in adult or elderly patients. The existence of many lesions in which the cells have an increased number of mitochondria without displaying the full-blown characteristics of Hürthle cells, the so-called 'mitochondrion-rich' cells (Sobrinho-Simões *et al.* 1985), reinforces the importance of time and

demonstrates that Hürthle cell transformation is a continuous process rather than a black-and-white phenomenon. Time and the recently disclosed knowledge on the spatial organization of the mitochondria in interphase cells – mitochondria appear to fuse together in some interphase periods allowing the intermitochondrial transfer and the dispersion of mutations among different mitochondria (Takai *et al.* 1999, Nakada *et al.* 2001, Meeusen *et al.* 2004, Chan 2006, Chen & Chan 2010) – contribute to explain the trend toward homoplasmy of mitochondrial gene mutations that are frequently observed in neoplastic lesions composed of Hürthle cells (Polyak *et al.* 1998, Chan 2006). The trend toward homoplasmy after a starting point characterized by different levels of heteroplasmy highlights also the importance of some mitochondrial alterations in terms of growth advantage (Polyak *et al.* 1998, Gasparre *et al.* 2008).

Hürthle cell tumors

It is now generally acknowledged that almost all histological types of benign and malignant epithelial thyroid tumors have a Hürthle cell counterpart (Sobrinho-Simões *et al.* 2004). There are Hürthle cell variants of follicular thyroid adenoma (FTA), papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC), medullary thyroid carcinoma (MTC), and poorly differentiated thyroid carcinoma (PDTC; Sobrinho-Simões *et al.* 2004, Garcia-Rostan & Sobrinho-Simões 2011). Undifferentiated (anaplastic) carcinomas represent the only exception to the aforementioned rule – there is no Hürthle cell counterpart of undifferentiated carcinoma – probably because the neoplastic cells divide too often to allow the accumulation of mitochondria.

The criteria used in the diagnosis of the Hürthle cell variants of PTC, FTC, and PDTC are those used in the diagnosis of their non-Hürthle cell counterparts: nuclear characteristics of the PTC-type, signs of capsular, and/or vascular invasion, and the morphologic features of the so-called Turin algorithm respectively (Volante *et al.* 2007). In all these instances, the Hürthle cell appearance is thought to represent a phenotype that is superimposed on the genotypic and conventional histopathologic features of the tumors; this assumption is confirmed by the similar prevalences of *RET/PTC* rearrangements and BRAF V600E mutation in Hürthle and non-Hürthle cell variants of PTC and by the existence of a Hürthle cell variant of *RET* mutated MTC (Dominguez-Malagon *et al.* 1989, Cheung *et al.* 2001, Trovisco *et al.* 2004).

The search for signs of invasion is crucial to achieve the differential diagnosis between Hürthle cell FTA and Hürthle cell FTC. Invasion is also very important to diagnose Hürthle cell PTC because the typical features of PTC nuclei are more difficult to evaluate in Hürthle cells than in normal-looking cells (Sobrinho-Simões *et al.* 2004). Immunohistochemistry and molecular genetics are diagnostically useless in concrete, difficult cases (Couto *et al.* 2009).

Hürthle cell carcinomas, regardless of being papillary or follicular, tend to be encapsulated or at least to display pushing rather than infiltrative borders. These expansive lesions, that may have follicular, trabecular, solid, or papillary architecture, are observed in Hürthle cell FTC and Hürthle cell PTC, the latter displaying usually a follicular or solid growth pattern. Prominent infiltrative borders are rarely seen in any type of carcinoma composed by Hürthle cells, in contrast to classic, poorly circumscribed PTCs (M Sobrinho-Simões 2011, unpublished observations).

The frequent occurrence, either spontaneously or after fine needle aspiration biopsy, of large areas of necrosis in benign and malignant Hürthle cell tumors contrasts with the rare images of apoptotic cell death. These findings support the assumption that apoptosis is blocked in Hürthle cell tumors probably as a consequence of mitochondrial abnormalities (see below; Muller-Hocker (1999), Máximo & Sobrinho-Simões (2000a), Volante *et al.* (2001), and Allia *et al.* (2003)).

At variance with the balanced distribution of Hürthle cell variants in every FTC category, there is a clear asymmetry of the prevalence of Hürthle cells in different PTC variants. This holds particularly true regarding the Warthin-like, hobnail and tall-cell variant PTC which are, by definition, composed by Hürthle cells or at least by mitochondrion-rich cells (Sobrinho-Simões *et al.* 1985, LiVolsi *et al.* 2004, Albores-Saavedra 2010, Asioli *et al.* 2010). The designation mitochondrion-rich cells appears to be more adequate than Hürthle cells in tumors in which there is a well-organized papillary growth pattern and the neoplastic cells keep a well-defined polarity. In our experience, such characteristics are not met by full-blown Hürthle cells that lose polarity and tend to form trabeculae, sheets, or solid clusters (M Sobrinho-Simões 2011, unpublished observations).

The nomenclature decision on concrete cases is not always easy. For instance, whenever dealing with a follicular-patterned PTC composed by Hürthle cells some authors use the designation ‘Hürthle cell variant of follicular-patterned PTC’, whereas others prefer to use ‘Follicular variant of PTC composed by Hürthle cells’.

The individualization of the Hürthle cell variant of PTC is relatively recent (DeLellis *et al.* 2004). This turns difficult to evaluate with precision some of the old studies on the so-called Hürthle cell carcinomas that most likely encompass both Hürthle cell variants of FTC and PTC. In the last years, namely after the acknowledgment of the clinicopathologic and genetic differences between PTC and FTC composed by Hürthle cells, it has been generally accepted that the Hürthle cell variant of PTC should be referred to in this way, whereas the Hürthle cell variant of FTC may be designated like this, or just by Hürthle cell carcinoma. Herein, we will assume that whenever the designation Hürthle cell carcinoma is used, the authors are referring to Hürthle cell variant of FTC.

The prognosis of patients with Hürthle cell variants of FTC and PTC is similar to that of patients with the respective conventional, non-Hürthle cell carcinomas, provided the completeness of the surgery, the age of the patients, and the staging of the tumors are comparable (Rosai *et al.* 1992, Evans & Vassilopoulou-Sellin 1998, LiVolsi *et al.* 2004, Sobrinho-Simões *et al.* 2011). From a clinical standpoint, the negative aspect of Hürthle cell FTC and Hürthle cell PTC is their lesser ability to trap iodine, thus rendering them less responsive to radioactive iodine (Rosai *et al.* 1992, LiVolsi *et al.* 2004, Sobrinho-Simões *et al.* 2011).

A last point to refer that despite the ‘old’ idea that Hürthle cell FTCs tended to carry a guarded prognosis, there is consistent evidence showing that encapsulated, nonangioinvasive Hürthle cell FTC and Hürthle cell PTC carry, like their non-Hürthle cell counterparts, an excellent prognosis even after being treated conservatively (Widder *et al.* 2008, Piana *et al.* 2010, Woodford *et al.* 2010, Sobrinho-Simões *et al.* 2011).

Genetics, Hürthle cell transformation and tumorigenesis

The genetics of Hürthle cell FTC has to be divided for the sake of simplicity into two categories: genetic alterations that are linked with the three main histotypes of follicular cell-derived thyroid carcinomas (FTC, PTC, and PDTC) and the genetic alterations that are linked to the acquisition of Hürthle cell features. We know this subdivision represents an oversimplification since it is likely that both groups of genetic alterations interact with each other. Moreover, as stated above, we ignore the precise mechanism(s) through which mitochondrial alterations may be involved in tumorigenesis.

The prevalence of aneuploidy and mutations in oncogenes and tumor suppressor genes does not differ substantially in Hürthle cell FTCs from their non-Hürthle cell counterparts (Máximo & Sobrinho-Simões 2000a). In Table 1, we have summarized the data concerning the frequency of alterations in oncogenes and tumor suppressor genes in Hürthle cell tumors (Table 1).

Studies on gene expression profiles and microarray data repeatedly demonstrated the similarity of most genetic alterations of Hürthle cell carcinomas and their non-Hürthle cell counterparts. Hoos et al. (2002) studied 18 normal thyroids and 69 Hürthle cell benign and malignant tumors, using tissue microarrays of paraffin-embedded tissues. The molecular phenotype of Hürthle cell tumors, independently of the histopathologic subtype, was characterized by p53(–), mdm2(+), p21(±), cyclin D1(–), and Bcl-2(±). High Ki-67 proliferative index was associated with significantly reduced relapse-free and disease-specific survival, being clinically evident only in aggressive, widely invasive Hürthle cell FTC, and the Bcl-2(+) phenotype was associated with improved relapse-free survival and disease-specific survival in widely invasive Hürthle cell FTC (Hoos et al. 2002). Although quite interesting these data do not indicate whether benign and malignant Hürthle cell tumors have distinct expression profiles from FTA, FTC, and PTC without Hürthle cells. The same holds true regarding the occurrence of the PAX8–PPAR γ (PPARG) fusion gene, caveolin-1 (CAVI) gene, CBP/p300-interacting transactivator with GLU/ASP-rich C-terminal domain, 1 (CITED1) gene, and Aplasia ras homolog I (ARHI (DIRAS3)) gene (Aldred et al. 2003a,b, Prasad et al. 2004, Weber et al. 2005).

mtDNA encoded mitochondrial genes alterations in Hürthle cell tumors

Hürthle cell tumors differ from their non-Hürthle cell counterparts regarding the prevalence of alterations in mtDNA genes and in nuclear genes (nuclear

DNA (nDNA)) coding for mitochondrial proteins (Máximo et al. 2002, 2005a, Abu-Amero et al. 2005, Bonora et al. 2006b, Gasparre et al. 2007, Zimmermann et al. 2009).

Such genetic alterations encompass large deletions of mtDNA (Máximo et al. 1998, Máximo & Sobrinho-Simões 2000b), mutations of mitochondrial genes coding for oxidative phosphorylation (OXPHOS) proteins, namely mutations in complex I subunits (Máximo et al. 2002, Gasparre et al. 2007, 2008), and mutations in nuclear genes coding for mitochondrial proteins, namely OXPHOS genes (e.g. NDUF13 or GRIM-19 gene; Máximo et al. 2005a).

Numerous mutations in all genes that encode OXPHOS proteins, as well as three mutations in three tRNAs, were detected in a series of 66 tumors (Máximo et al. 2002). Combining the results obtained in this study with those reported by Yeh et al. (2000) it was suggested that alterations in mtDNA genes affecting complex I may increase susceptibility to thyroid tumorigenesis (Yeh et al. 2000, Máximo et al. 2002). This assumption was later on confirmed by other groups (Abu-Amero et al. 2005, Bonora et al. 2006b, Gasparre et al. 2007).

Mitochondria are responsible for producing most of the cellular ATP by OXPHOS in an oxygen-dependent process (Wallace 1986, 1999). In addition to OXPHOS, cells can also produce ATP through glycolysis which takes place in the cytosol and does not require O₂. Since the discovery of Otto Warburg (Warburg 1956a,b), who showed that cancer cells prefer to metabolize glucose by glycolysis even in the presence of O₂ (Warburg effect or aerobic glycolysis), a lot of studies have proved that aerobic glycolysis is a hallmark of cancer. Despite the abundant evidence on record on this cancer characteristic, both the genetic basis of the Warburg effect and the role in cancer progression remain to be clarified. One hypothesis to explain the abovementioned metabolic shift in cancer cells is related to OXPHOS defects caused by mutations in mtDNA – encoded OXPHOS genes that

Table 1 Summary of alterations in oncogenes and tumor suppressor genes in Hürthle cell tumors

Diagnosis	Rearrangements		Mutations	
	RET/PTC	PAX8/PPAR γ	BRAF(V600E)	N-, H-, K-RAS
HCFTA	27/89 (30%)	0/33 (0%)	0/46 (0%)	2/52 (4%)
HCFTC	34/98 (35%)	5/94 (5%)	0/74 (0%)	17/100 (17%)
HCPTC	27/28 (96%)	0/3 (0%)	6/28 (21%)	0/3 (0%)
Total	88/215 (41%)	5/130 (4%)	6/148 (7%)	19/155 (13%)

HCFTA, Hürthle cell follicular thyroid adenoma; HCFTC, Hürthle cell follicular thyroid carcinoma; HCPTC, Hürthle cell papillary thyroid carcinoma. Adapted from De Vries et al. (2012).

push cancer cells toward glycolysis (Brandon *et al.* 2006, Lu *et al.* 2009, Máximo *et al.* 2009, Czarnecka *et al.* 2010, Chandra & Singh 2011).

The most common mtDNA mutation found in Hürthle cell tumors is a large deletion, encompassing 4977 bp of mtDNA, known as the mtDNA common deletion (CD; Máximo & Sobrinho-Simões 2000b). This deletion removes several mtDNA genes, thus leading to severe impairment of the OXPHOS system. It is believed that this impairment induces a positive feedback mechanism resulting in an increase in mitochondrial mass and accumulation of dysfunctional mitochondria (Attardi *et al.* 1995, Heddi *et al.* 1996, Sobrinho-Simões *et al.* 2005). The mtDNA CD is often found in FTA, FTC, and PTC composed by Hürthle cells and was proposed as a hallmark of such tumors (Máximo *et al.* 2002).

Smaller mtDNA mutations are also frequently detected both in benign and malignant Hürthle cell tumors (Máximo *et al.* 2002, Gasparre *et al.* 2007). They occur both in the 'C' tract of the D-loop region and in all the 13 genes that encode OXPHOS proteins, being more frequent in complex I genes than in the genes of the other complexes (Máximo *et al.* 2002, 2005b, Gasparre *et al.* 2007, Zimmermann *et al.* 2009). Occasionally, mutations in some tRNAs have also been detected (Máximo *et al.* 2002). The OXPHOS gene mutations include frameshift and both silent and missense point mutations. Disruptive mutations (either frameshift or nonsense) tend to be concentrated in complex I genes, without any apparent concentration in a single gene (Máximo *et al.* 2002, Gasparre *et al.* 2007, Zimmermann *et al.* 2009). Immunohistochemical analyses of Hürthle cell FTCs have confirmed a specific lack of complex I, which was expressed at <5% of the level in surrounding nontumoral tissue (Zimmermann *et al.* 2009). Gasparre *et al.* (2007) found that the only breast carcinoma of their series that presented an mtDNA complex I gene mutation was a mitochondrion-rich carcinoma, thus reinforcing the association between disruptive mtDNA complex I mutations and the occurrence of Hürthle cell phenotype. Mutations in complex I genes, as well as mutations in other genes of the mitochondrial respiratory chain (MRC) complexes, may also result in OXPHOS system impairment and, subsequently, lead to accumulation of abnormal mitochondria (Máximo *et al.* 2002, Gasparre *et al.* 2007). Using bioinformatic tools, we have recently demonstrated that high pathogenicity mtDNA mutations, namely in complex I genes, lead to the oncogenic phenotype (Pereira *et al.* 2012).

mtDNA germline variants affecting genes of complex V (ATPase6 and ATPase8) were more frequently detected in Hürthle cell FTC and Hürthle cell PTC than in their non-Hürthle cell counterparts, and were more frequently detected in malignant Hürthle cell tumors than in Hürthle cell FTA (Máximo *et al.* 2002, Gasparre *et al.* 2007). These findings do not indicate that such mtDNA variants are associated with an increased risk for tumor development, but they suggest that, in case the patient harboring such variants will develop a thyroid tumor, it is more likely that the tumor will have Hürthle cell features and fall in the malignant category (Máximo *et al.* 2002).

mtDNA encoded mitochondrial genes alterations in Hürthle cell tumors

Additional evidence for the involvement of OXPHOS complex I in thyroid tumorigenesis has come from the identification of three *GRIM-19* missense somatic mutations and one missense germline mutation in four Hürthle cell thyroid tumors (Máximo *et al.* 2005a). *GRIM-19* is a complex I nuclear gene associated with retinoid-IFN-Induced Mortality – also known as NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 13 (*NDUFA13*; Huang *et al.* 2004, Máximo *et al.* 2008). No mutations were detected in any of the 20 non-Hürthle cell tumors tested, nor in any of the 96 blood donor samples (Máximo *et al.* 2005a). *GRIM-19* mutations are the only nuclear gene mutations specific of Hürthle cell tumors reported to date. It was proposed that such mutations may be involved in the genesis of sporadic or familial Hürthle cell thyroid tumors through the dual function of *GRIM-19* in mitochondrial metabolism and cell death (Angell *et al.* 2000, Máximo *et al.* 2005a, Moreira *et al.* 2011). Downregulation of *GRIM-19* confers growth advantage apparently via reduced apoptosis (Angell *et al.* 2000). In one of the sporadic Hürthle cell PTC positive for the *GRIM-19* mutation, an *RET/PTC-1* rearrangement was detected, suggesting that *GRIM-19* mutation may serve as a predisposing alteration for the occurrence of tumors with Hürthle cell transformation (Máximo *et al.* 2005a). These results also indicate that alterations other than *GRIM-19* mutation, such as *RET/PTC* rearrangement or *BRAF* mutation, may be necessary for the acquisition of the malignant phenotype. If the mitochondrial alterations and the Hürthle cell phenotype precede the oncogenic alteration the whole carcinoma will be composed by Hürthle cells, whereas if the mitochondrial alterations occur after the oncogenic clonal expansion has been established, the end result

will be a carcinoma only partially composed by Hürthle cell (Fig. 1; Sobrinho-Simões *et al.* 2005).

At variance with GRIM-19, no association was found between the expression of ubiquinol–cytochrome *c* reductase core protein 1 (*UQCRC1*), a gene of MRC complex III, and Hürthle cell tumors (Máximo *et al.* 2004).

Approximately 99% of the mitochondrial proteins are encoded by the nuclear genome and therefore need to be imported from the cytosol; this transport relies on translocases (Rehling *et al.* 2004). Translocase of inner mitochondrial membrane 44 homolog (*TIMM44*) is part of this mitochondrial protein import system, being important for a normal mitochondrial function (Rehling *et al.* 2004). Two novel variants in *TIMM44* were identified in two out of eight families with Hürthle cell tumors; these variants respectively in exon 9 and exon 13 of *TIMM44*, cosegregate with the Hürthle cell phenotype, being absent in a large group of controls (Bonora *et al.* 2006a).

The available evidence supports the existence of defects in the energy production machinery in Hürthle cell tumors and suggests that the increased mitochondrial content of such tumors may reflect a compensatory mechanism. Interestingly, lack of complex I has been proposed to prevent tumor cells from undergoing apoptosis (Zimmermann *et al.* 2009). The data on mtDNA and nDNA mutations in Hürthle cell tumors are summarized in Table 2.

Alterations in gene expression in Hürthle cell tumors

Using a two-step differential expression analysis of six Hürthle cell FTA and their paired normal tissues, Jacques *et al.* (2005) found 30 overexpressed sequences in the tumoral tissues. Out of these 30 sequences, 12 were mtDNA encoded (ten genes from *OXPHOS*, the 16S rRNA gene and one noncoding sequence from D-Loop) and 18 nuclear encoded.

Figure 1 Schematic representation of (A) a case with partial Hürthle cell transformation and (B) a case with total Hürthle cell transformation. If the oncogenic step precedes mitochondrial abnormality, the tumor may have partial Hürthle cell transformation (A); if the mitochondrial abnormality precedes the oncogenic step, the tumor will probably display total Hürthle cell transformation (B).

Table 2 Summary of specific genetic alterations in Hürthle cell tumors

Gene	mtDNA		nDNA	
	mtDNA CD (several mtDNA genes)	Complex I genes	<i>GRIM-19</i>	<i>TIMM44</i>
Protein location	Mitochondria	Mitochondria	Mitochondria/cytoplasm?	Mitochondria
Protein function	OXPHOS	OXPHOS	OXPHOS/cell death	Translocation of mitochondrial proteins
Mutation type	Somatic	Somatic	Somatic/germinative	Germinative
References	Máximo <i>et al.</i> (2002) and Máximo & Sobrinho-Simões (2000a,b)	Bonora <i>et al.</i> (2006b), Gasparre <i>et al.</i> (2007), Zimmermann <i>et al.</i> (2011) and Pereira <i>et al.</i> (2012)	Máximo <i>et al.</i> (2005a,b)	Bonora <i>et al.</i> (2006a)

mtDNA, mitochondrial DNA; nDNA, nuclear DNA; CD, common deletion; *GRIM-19*, gene associated with retinoid–IFN-induced mortality-19; OXPHOS, oxidative phosphorylation; *TIMM44*, translocase of inner mitochondrial membrane 44 homolog.

Out of the 18 were nuclear-encoded sequences, two encode transcription factors (zinc finger MYND domain-containing protein 12 (*ZMYND12*) and transcription factor 8 (*TCF8*)), and seven genes are involved at multiple levels of protein metabolism: mRNA transport (protein virilizer homolog isoform 2), posttranslational modifications (beta-3-glycosyltransferase-like (*B3GTL*; *B3GALT1*)), protein trafficking (sorting nexin 19 (*SNX19*)), ubiquitination/proteasome (retinitis pigmentosa 42 (*RP42* protein)), and proteolysis (cathepsin B (*CTSB*), SUMO/sentrin specific peptidase family member 1 (*SENPI1*), and ubiquitin-conjugating enzyme E2D 3 (*UBE2D3*)). Other over-expressed genes were ferritin heavy chain 1 (*FTH1*), integrin α -V (*ITGAV*), erythrocyte membrane protein band 4.1 like 4A (*EPB41L4A*), ALL1-fused gene from chromosome 1q (*AF1Q* (*MLLT11*)), and five genes with unknown function. Hürthle cell tumors display a striking upregulation of mtDNA genes and of genes implicated in protein metabolism. The authors concluded that mitochondrial proliferation appears to be associated with intense protein turnover, suggesting that such turnover is necessary for the mitochondrial proliferation and/or that the mitochondrial saturation of the cytoplasm disturbs cell trafficking and processing of proteins (Jacques *et al.* 2005). It would be interesting to compare these results with those obtained in non-Hürthle cell tumors to evaluate the specificity of the data.

Death-associated protein 3 (*DAP3*), a GTP-binding constituent of the small subunit of the mitochondrial ribosome with a proapoptotic function, was found overexpressed in Hürthle cell FTC (Jacques *et al.* 2009). The authors studied 61 normal thyroids and 100 cases of different thyroid tumor histotypes, by tissue array, and found that *DAP3* was overexpressed in 7/9 Hürthle cell FTC, 26/37 Hürthle cell FTA and in

16/34 PTCs, being absent in 2/4 PDTs, 5/5 FTCs, 3/3 tumors of uncertain malignancy potential, and 8/8 FTA. It was concluded that *DAP3* overexpression appears to be dependent on the cell mitochondrial content, suggesting that *DAP3* may be necessary for an increased mitochondrial biogenesis, regulating the mitochondrial protein synthesis (Jacques *et al.* 2009).

The comparison of gene expression profile of six Hürthle cell FTC and six mitochondrion-rich PTC disclosed 83 genes differentially expressed between the two groups (Baris *et al.* 2005). Interestingly, most of the genes (83.3%) overexpressed in Hürthle cell FTC and mitochondrion-rich PTC are involved in metabolic functions, namely overexpression of complex III and IV genes, and clearly distinguish Hürthle cell FTC from mitochondrion-rich PTC (Baris *et al.* 2005).

The expression of three genes (PGC1-related coactivator (*PRC* (*PPRC1*)), mitochondrial transcription factor A (*TFAM*), and nuclear respiratory factor 1 (*NRF1*)) involved in mitochondrial biogenesis was studied in a series of 30 Hürthle cell tumors and corresponding normal tissues were found to be over-expressed in tumoral tissues (Savagner *et al.* 2003). These data reinforce the assumption that there is increased proliferation of mitochondria in Hürthle cell tumors associated with the overexpression of factors involved in mitochondrial biogenesis (Savagner *et al.* 2003; Table 3).

Methylation alterations in Hürthle cell tumors

Among many studies dealing with hypermethylation and, hence, inappropriate gene silencing, in thyroid cancer (Xing 2007, Greco *et al.* 2009, Eze *et al.* 2011)

Table 3 Summary of epigenetic alterations in Hürthle cell tumors

Type of alteration	Expression profile	Methylation profile	microRNAs profile
Function/genes	OXPHOS/OXPHOS genes and <i>16S rRNA</i> Apoptosis/ <i>DAP3</i> Transcription factors/ <i>TFAM</i> ; <i>NRF-1</i> ; <i>ZMYND12</i> , and <i>TCF8</i> Protein metabolism/ <i>KIAA1429</i> ; <i>B3GTL</i> ; <i>SNX19</i> ; <i>RP42</i> ; <i>CTSB</i> ; <i>SENP1</i> ; <i>UBE2D3</i> Mitochondrial biogenesis/ <i>PRC</i>	Cell–cell adhesion/ <i>CDH1</i> Iodine uptake/ <i>NIS</i> Zinc-finger protein/ <i>RIZ1</i>	NA/miRNA-221; miRNA-339; miRNA-183; mir-197 and miRNA-31
Changes	Overexpression	Hypermethylation	Upregulation
References	Savagner <i>et al.</i> (2003), Baris <i>et al.</i> (2005) and Jacques <i>et al.</i> (2005, 2009)	Graff <i>et al.</i> (1998), Huang (1999), Venkataraman <i>et al.</i> (1999), Rocha <i>et al.</i> (2001) and Galusca <i>et al.</i> (2005)	Nikiforova <i>et al.</i> (2008)

NA, not applicable; *B3GTL*, beta-3-glycosyltransferase-like; *CDH1*, cadherin 1; *CTSB*, cathepsin B; *DAP3*, death-associated protein 3; *KIAA1429*, protein virilizer homolog isoform 2; *NIS*, sodium–iodide symporter; *NRF-1*, nuclear respiratory factor-1; OXPHOS, oxidative phosphorylation; *PRC*, PGC1-related coactivator; *RIZ1*, retinoblastoma protein-binding zinc-finger protein RIZ; *RP42*, retinitis pigmentosa 42; *SENP1*, SUMO/setrin specific peptidase family member 1; *SNX19*, sorting nexin 19; *TCF8*, transcription factor 8; *TFAM*, transcription factor A, mitochondrial; *UBE2D3*, ubiquitin-conjugating enzyme E2D3; *ZMYND12*, zinc finger MYND domain-containing protein 12.

only a few have specifically addressed Hürthle cell tumors.

Global DNA methylation, using a monoclonal antibody specific for 5-methylcytidine (5-mc), was evaluated in a large series of thyroid tumors (including ten Hürthle cell FTA) and tumor-like lesions (Galusca *et al.* 2005). The similar levels of 5-mc in FTA and in Hürthle cell FTA indicate that the global DNA methylation levels cannot be used to separate Hürthle from non-Hürthle cell tumors, but it would also be interesting to evaluate such levels in Hürthle cell FTC.

The loss of expression of *CDH1* has been associated with hypermethylation of *CDH1* 5' CpG island in a panel of human thyroid cancer cell lines, 83% of PTC and 40% of Hürthle cell FTC, but the series is too small (two out of five cases) to allow any definitive conclusion that the role of loss of *CDH1* may play in Hürthle cell tumors (Graff *et al.* 1998). The same holds true regarding the transcriptional failure of the sodium–iodide symporter and the loss of repression of retinoblastoma protein-binding zinc-finger protein RIZ (*RIZ1*) due to promoter methylation (Venkataraman *et al.* 1999, Lal *et al.* 2006; Table 3).

microRNA alterations in Hürthle cell tumors

Several independent studies have analyzed miRNA expression in different types of thyroid tumors, showing particular miRNA profiles in some of them (reviewed in Nikiforova *et al.* (2008), Pallante *et al.* (2010), and Wilson (2010)). Two studies have

specifically targeted Hürthle cell tumors (Nikiforova *et al.* 2008, Kitano *et al.* 2011), and in one of them Hürthle cell tumors were considered as a separate group (Nikiforova *et al.* 2008).

A unsupervised hierarchical analysis of miRNA expression in a series of 60 thyroid neoplastic and nonneoplastic samples, including 23 PTC (classical and follicular variant), nine FTC (conventional and Hürthle cell FTC), eight FTA (conventional and Hürthle cell FTA), four anaplastic carcinomas, four PDTC, two MTC, five hyperplastic nodules, and five samples of normal thyroid tissue revealed four major clusters: Hürthle follicular tumors (adenomas and carcinomas), conventional follicular tumors (adenomas and carcinomas), PTC, and MTC (Nikiforova *et al.* 2008). The first three clusters were closer to each other, whereas the MTC cluster was at a larger distance, consistent with their origin from a different cell type. The Hürthle cell tumor cluster was the most clearly segregated of the three follicular cell-derived clusters. Among the several miRNAs upregulated in the different types of tumors, miR-221, miRNA-339, miR-183, and miR-197 were highly expressed in Hürthle cell FTC, and miR-31, miRNA-339, and miR-183 were highly expressed in Hürthle cell FTA; miR-197 was found to be specifically upregulated in Hürthle cell FTC, and miR-31 in the top of the list (increased 35.2-fold) in Hürthle cell FTA, also being upregulated in PTC (increased 7.5-fold). The authors claim that their results show that Hürthle cell tumors have a distinct set of upregulated miRNA and cluster separately from conventional follicular tumors; the

demonstration that the clusters of follicular tumors and Hürthle cell tumors included both adenomas and carcinomas supports the assumption of a stepwise progression from one tumor type to the other in each category (Nikiforova *et al.* 2008). The cluster of similar miRNAs in benign and malignant follicular tumors displaying Hürthle cell features supports also the assumption that such features represent a phenotype that is superimposed on different oncogenic genotypes. This assumption is further reinforced by the upregulation of miR-221 in renal oncocytomas (Fridman *et al.* 2010; Table 3).

Hürthle cell tumors and apoptosis

To date, no large study on Hürthle cell carcinomas and apoptosis has been reported. The overexpression of p53 in a high percentage of both Hürthle cell FTA and Hürthle cell FTC was shown to be inversely related to Bcl2 immunostaining (Muller-Hocker 1999). *In situ* end-labeling applied to a series of 52 thyroid tumors, including both Hürthle and non-Hürthle cell tumors, showed strong nuclear reactivity in almost all Hürthle cell tumors in contrast to non-Hürthle cell tumors, in the absence of morphological apoptotic changes and caspase activation (Volante *et al.* 2001). A cytoplasmic staining, probably revealing mtDNA fragmentation, was also observed in Hürthle cell tumors (Volante *et al.* 2001). The authors concluded that this DNA fragmentation is a key feature of Hürthle cell tumors underlying a rigid response to ischemic stimuli, leading to a necrotic rather than an apoptotic response to stress conditions, probably related to a blockage of the apoptotic processes due to the presence of mitochondrial abnormalities, as we had previously suggested (Máximo & Sobrinho-Simões 2000a, Volante *et al.* 2001).

Following an oxidative proapoptotic stress, XTC.UC1 cells (a Hürthle cell tumor-derived cell line) displayed extensive DNA fragmentation (up to 70% of cells), dramatically exceeding that observed in WRO cells (non-Hürthle cell tumor-derived cell line; Allia *et al.* 2003). At variance with this, the oxidative stimulus induced a remarkable apoptosis gene activation in non-Hürthle WRO cells only. These results suggest that Hürthle cells may have a (unique?) silent activation of proapoptotic mechanism(s), which may be responsible for the DNA instability leading to the frequently observed cell death *in vivo* and increased sensitivity to ischemic stresses (Allia *et al.* 2003). The mitochondrial abnormalities that are the hallmark of Hürthle cell tumors may contribute to this predisposition for necrosis instead of apoptosis, in a similar way

to that observed in ischemic hearts after reperfusion (Borutaite & Brown 2003).

Hürthle cell tumors and hypoxia-inducible factor 1 stabilization and angiogenesis

There is evidence suggesting that mitochondrial dysfunction may lead to the stabilization of hypoxia-inducible factor 1 α (HIF1 α (HIF1A)), therefore inducing a pseudohypoxic condition (activation of cellular hypoxia response pathways in the presence of normal oxygen tension) that would trigger a tumorigenic hypoxia pathway. The activation of this pathway results in the transcription of a number of genes known to be associated with human tumorigenesis, such as those involved in glucose metabolism, angiogenesis, extracellular matrix modification, motility, and survival (Pouyssegur *et al.* 2006, Higgins *et al.* 2007, Bristow & Hill 2008, Yang *et al.* 2008). At variance with this hypothesis, it was shown that tumors harboring complex I homoplasmic mutations are not pseudohypoxic since they do not accumulate succinate and stabilize HIF1 α (Porcelli *et al.* 2010). The immunohistochemical study of VEGF (an HIF1 α target gene) in a series of 117 thyroid tumors, including six Hürthle cell FTA and two Hürthle cell FTC, showed that all PTC and Hürthle cell tumors displayed a strong, diffuse staining reaction, whereas anaplastic carcinoma usually exhibited weak and infrequent immunoreactivity (Huang *et al.* 2001). These data support the assumption that HIF1 α pathway is active in both benign and malignant Hürthle cell tumors (Huang *et al.* 2001).

Etiopathogenesis of Hürthle cell tumors: experimental models: cybrid cell lines

The hypothesis that mitochondrial OXPHOS dysfunction leads to Hürthle cell phenotype is yet unproved, one of the reasons being that the usual *in vitro* assays (gene transfections, siRNA, shRNA) to assess gene function are not appropriate to study mtDNA; to overcome this limitation, the cybrid cell lines (cybrids) methodology has been developed, allowing the distinction of nDNA-induced from mtDNA-induced cell phenotypes. The major advantage of cybrids is that the introduction of mutant or wild-type (wt) mtDNA in a common nuclear background allows the association of the observed cellular effect(s) with the mtDNA mutation under study.

It was observed that primary cultures derived from Hürthle cell thyroid tumors harboring complex I mtDNA disruptive mutations failed to maintain both

the mutations and the Hürthle cell phenotype, thus supporting the assumption that the Hürthle cell phenotype is induced by mtDNA mutations (Gasparre et al. 2007). In the same study, it was shown that, after a number of passages, the cells lose their Hürthle cell phenotype, and also the homoplasmy status of the mtDNA mutations that evolve progressively to lower and lower levels of heteroplasmy (Gasparre et al. 2007). These observations highlight the role that microenvironment and stroma play in the acquisition and maintenance of the Hürthle cell phenotype, a finding that needs to be further explored in the future.

Other studies support the causal link between mtDNA mutations and the Hürthle cell phenotype. The XTC.UC1, a Hürthle cell thyroid cell line, displays a significant reduction in the activity of complexes I and III as a result of two mtDNA mutations, one in the *ND1* (*MT-ND1*) gene (complex I) and another in *CYTB* (*MT-CYB*) gene (complex III) (Bonora et al. 2006b); further experiments with cybrids derived from the XTC.UC1 proved that the aforementioned observations were independent of the nuclear background (Bonora et al. 2006b).

mtDNA mutations were shown to be capable of modulating the tumorigenic potential of a cancer cell line: cells with mutant mtDNA lead to significantly larger tumors in nude mice than cells with wt mtDNA (Petros et al. 2005). Similarly, it was observed that mutant ATP6 cybrids increased faster than wt in culture, and conferred an advantage in the early stage of tumor growth in nude mice (Shidara et al. 2005). Interestingly, upon transfection of a wt nuclear version of the *ATP6* (*MT-ATP6*) gene in the mutant cybrids, these reverted the phenotype, thus reinforcing the functional effects of the mtDNA mutations (Shidara et al. 2005).

A role for mtDNA mutations in metastization was proposed after the observation that the metastatic potential of two mouse tumor cell lines was dependent on their mtDNA (Ishikawa et al. 2008). Furthermore, the mtDNA of the highly metastatic cell line contained two *ND6* mutations – G13997A and 13885insC – associated with complex I deficiency and overproduction of ROS. The role played by ROS in the metastatic potential was confirmed after observing that pretreatment with ROS scavengers abolished metastasis formation (Ishikawa et al. 2008).

The evaluation of the protumorigenic role of mtDNA mutations was addressed using cybrids harboring the *ND1* mutation (Gasparre et al. 2011). After obtaining cybrids with different levels of mutation load, a threshold level for the tumorigenic effect of the *ND1* mutation was defined, above which

tumor growth and invasiveness were reduced significantly. Notably, the decreased tumorigenic effect of high *ND1* mutation load was associated with HIF1 α destabilization and downregulation of HIF1 α -dependent genes (see above). These results suggest that the effect of OXPHOS dysfunction is dose-dependent: complete loss of OXPHOS hampers tumor development, while a protumorigenic effect is induced upon limited OXPHOS dysfunction (Gasparre et al. 2011).

Familial clustering of Hürthle cell tumors

The case for the existence of a distinct familial/hereditary form of Hürthle cell thyroid tumors is building up as significant clinicopathologic presentations are put together with an increasing body of genetic data.

From the clinicopathologic standpoint, the observations suggestive of genetically determined form(s) of Hürthle cell thyroid tumors comprise: a) presentation in monozygotic twins, b) nonrandom familial aggregation according to a Mendelian transmission pattern, and c) concordant, unusual/peculiar morphology in multifocal tumors.

Very early reports have drawn attention to the presentation of Hürthle cell tumors in monozygotic twins (Firminger & Skelton 1953, Nagamachi et al. 1973, Katoh et al. 1998), a finding that points to a hereditary etiology.

Aside from the occurrence in twins, the patterns of clustering of Hürthle cell thyroid tumors in families (excluded radiation exposure) are compatible with an autosomic dominant Mendelian mode of inheritance (Canzian et al. 1998, Katoh et al. 1998, McKay et al. 2004). In such families, early age of onset and increased frequency of multifocal disease are quite impressive (Katoh et al. 1998). When detailed histologic analysis was performed, both benign and malignant thyroid tumors in the same family frequently exhibited a large extent of Hürthle cell transformation (Canzian et al. 1998, Katoh et al. 1998).

The occurrence of multifocal tumors with Hürthle cell features adds further consistency to the hypothesis of (a) germline mutation(s) conferring liability to Hürthle cell tumorigenesis (Prazeres et al. 2008, 2010).

A number of studies have been undertaken in order to map the location of putative genes underlying familial Hürthle cell tumors. Microsatellite-based linkage analysis, performed in a single large French family with an unusual predominance of Hürthle cell tumors, resulted in the identification of a specific locus, at chromosomal region 19p13.2, that cosegregated with both benign and malignant disease phenotypes in affected individuals (Canzian et al. 1998).

Figure 2 Schematic representation of the putative role of mitochondrial abnormalities in oncocytic transformation and tumorigenesis. Mutations in oncogenes and/or tumor suppressor genes are probably necessary for the malignant transformation of a Hürthle cell tumor.

This association was further validated in a series comprising ten independent families, nine of which presented with Hürthle cell tumors (McKay *et al.* 2004). Moreover, loss of heterozygosity was demonstrated at this region in both familial (Prazeres *et al.* 2008) and sporadic Hürthle cell thyroid tumors (Stankov *et al.* 2004), in keeping with the assumption that a tumor suppressor gene is involved. Out of the 14 candidate genes mapping within the 19p13.2 region of linkage, two novel variants in TIMM44 (see above), were found (Bonora *et al.* 2006a). The involvement of a gene with mitochondrial functions would make sense in this setting; despite this, no major loss of function was detected. Moreover, analysis of independent series of families failed to detect these or other TIMM44 variants (V Máximo, J Lima, P Soares and M Sobrinho-Simões M 2009, and H Prazeres, P Soares and M Sobrinho-Simões 2009, unpublished observations), thus turning the role of TIMM44 in familial Hürthle cell tumors uncertain at this time.

Familial Hürthle cell tumors can also be included the umbrella entity known as familial non-MTC (FNMTTC) that refers to the familial occurrence of follicular cell-derived thyroid carcinomas, PTC or FTC, with or without Hürthle cell features (reviewed by Prazeres *et al.* (2010)). It is uncertain whether, like in the sporadic setting, familial Hürthle cell tumors should be regarded just as a sort of phenotype of FNMTTC. To date, besides the abovementioned 19p13.2 region, FNMTTC loci have been proposed at 14q (Bignell *et al.* 1997), 1q21 (Malchoff *et al.* 2000),

2q21 (McKay *et al.* 2001), 8p23 (Cavaco *et al.* 2008), 8q24 (He *et al.* 2009), 1q21, and 6q22 (Suh *et al.* 2009); yet, a specific genetic alteration remains to be detected (Prazeres *et al.* 2010).

Therapy selection in Hürthle cell carcinomas

Besides the aforementioned issues on Hürthle cell tumors (etiopathogenesis, diagnosis, and prognosis), the treatment of patients emerges as a crucial item. Regardless of accepting or not the concept of Hürthle cell as a phenotype that can be found in almost every histotype of thyroid carcinoma, it is tempting to use the mitochondrial and metabolic abnormalities that are shared by carcinomas composed by Hürthle cells to look for specific therapy targets. These targets may be complementary to those identified by the oncogenic mutations such as RAS, BRAF, and *RET* mutations and *RET* mutations/rearrangements (Couto *et al.* 2009, Máximo *et al.* 2009).

Concluding remarks

The clinicopathologic and molecular evidence on record indicates that Hürthle cell appearance represents a phenotype driven mainly by mitochondrial alterations secondary to mtDNA and nDNA mutations coding for OXPHOS proteins. It remains to be fully clarified that the precise mechanism(s) through which

such mitochondrial abnormalities may be involved in tumorigenesis (Fig. 2).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the review reported.

Funding

This work was partially supported by the Portuguese Science and Technology Foundation (FCT) through the grant SFRH/BPD/72004/2010 (H Prazeres), through the Programs Ciência 2007 (V Máximo), and Ciência 2008 (J Lima) and through the project (PIC/IC/83037/2007). IPATIMUP is an Associate Laboratory of the Portuguese Ministry of Science, Technology and Higher Education and is partially supported by the FCT.

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Received in final form 23 March 2012

Accepted 18 April 2012

Made available online as an Accepted Preprint
18 April 2012