Original Article High-resolution genomic profiling of thyroid lesions uncovers preferential copy number gains affecting mitochondrial biogenesis loci in the oncocytic variants

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Abstract: Oncocytic change is the result of aberrant mitochondrial hyperplasia, which may occur in both neoplastic and non-neoplastic cells and is not infrequent in the thyroid. Despite being a well-characterized histologic phenotype, the molecular causes underlying such a distinctive cellular change are poorly understood. To identify potential genetic causes for the oncocytic phenotype in thyroid, we analyzed copy number alterations in a set of oncocytic (n=21) and non-oncocytic (n=20) thyroid lesions by high-resolution microarray-based comparative genomic hybridization (aCGH). Each group comprised lesions of diverse histologic types, including hyperplastic nodules, adenomas and carcinomas. Unsupervised hierarchical clustering of categorical aCGH data resulted in two distinct branches, one of which was significantly enriched for samples with the oncocytic phenotype, regardless of histologic type. Analysis of aCGH events showed that the oncocytic group harbored a significantly higher number of genes involved in copy number gains, when compared to that of conventional thyroid lesions. Functional annotation demonstrated an enrichment for copy number gains that affect genes encoding activators of mitochondrial biogenesis in oncocytic cases but not in their non-oncocytic counterparts. Taken together, our data suggest that genomic alterations may represent additional/alternative mechanisms underlying the development of the oncocytic phenotype in the thyroid.

Keywords: Thyroid oncocytic lesions, aCGH, mitochondrial biogenesis

Introduction

Oncocytic change is defined by an aberrant accumulation of mitochondria within cells, which results in a characteristic granular eosinophilia of the cell cytoplasm that confers a distinctive morphologic phenotype easily appreciated by histologic analysis [1]. This phenotype is commonly found in both neoplastic and nonneoplastic cells of the thyroid gland and other endocrine tissues, such as the pituitary gland, adrenal cortex and parathyroid glands [2]. When compared to their non-oncocytic counterparts, oncocytic lesions have often been shown to harbor distinct clinicopathological features, including different responses to treatment (e.g. poor response to radioactive iodide treatment in thyroid tumors), different prognosis (e.g. renal oncocytomas versus renal cell carcinomas), as well as different molecular profiles [3, 4]. Nevertheless, up to date no nuclear genetic determinant has been specifically associated with oncocytic tumors. It is not clear whether mitochondrial abnormalities, often associated with disruptive mitochondrial DNA (mtDNA) mutations [5-8], are involved in tumor initiation or represent a secondary change [9]. Oncocytic thyroid lesions are currently classified as hyperplasias, adenomas or carcinomas on the basis of the same general criteria applied for the diagnosis of non-oncocytic neoplasms [10].

Importantly, the molecular events leading to tumorigenesis appear to differ between nononcocytic and oncocytic neoplastic lesions. For example, canonical genomic mutations in BRAF or RAS family oncogenes (H-, N-, K-RAS), commonly detected in Papillary or Follicular Thyroid Carcinoma (PTC or FTC), have been observed at a variable frequency in the oncocytic variants [11-14]. Furthermore, potential involvement of chromosomal DNA alterations in oncocvtic thvroid tumor development has been suggested [14, 15]. Previous high-resolution microarraybased comparative genomic hybridization (aCGH) analysis of oncocytic breast carcinomas (OBCs) revealed a distinct pattern of chromosomal DNA changes in OBCs, which were found to have an enrichment for gene copy number alterations (CNA) targeting genes encoding mitochondrial proteins [3]. These observations suggest that the oncocytic phenotype may be related to specific nuclear DNA alterations, in addition or alternatively to those affecting the mtDNA.

In this study, we sought to define the genetic/ genomic mechanisms that lead to the development of oncocytic phenotype in the thyroid gland, and to investigate whether oncocytic cases carry a specific constellation of nuclear genomic DNA changes. Hence, to define the repertoire of gene copy number and mtDNA alterations in oncocytic thyroid lesions and in their respective non-oncocytic counterparts, we subjected 21 oncocytic and 20 non-oncocytic thyroid lesions to high-resolution aCGH and mtDNA sequencing. These analyses revealed that oncocytic cases, regardless being of neoplastic or non-neoplastic nature, display a distinctive genomic profile and carry a significantly higher number of gains when compared to non-oncocytic thyroid lesions. A significant enrichment of genes encoding factors promoting mitochondrial biogenesis was observed in the oncocytic group, regardless of the presence of mtDNA mutations, indicating a novel mechanism which may contribute to the development of the oncocytic phenotype.

Materials and methods

Case selection: oncocytic and non-oncocytic thyroid lesions

The study included 41 samples comprising 21 oncocytic (OT) and 20 non-oncocytic (T) primary thyroid lesions. To distinguish whether the difference between oncocytic and non-oncocytic lesions would be independent from histologic type, cases with different diagnoses were selected for each group. Oncocytic thyroid lesions included 9 hyperplastic nodules (HYP-0), 5 follicular adenomas (FA-0), 5 follicular carcinomas (FTC-O) and 2 papillary carcinomas (PTC-O). Non-oncocytic lesions included 5 hyperplastic nodules (HYP), 6 follicular adenomas (FA), 4 follicular thyroid carcinoma (FTC) and 5 papillary thyroid carcinomas (PTC), out of which 2 were classic PTC and 3 papillary carcinoma-follicular variant (PTC-FV). This project was approved by the local research ethics committee. Samples were anonymized and handled in compliance with the Helsinki Declaration. Follow-up information was not used.

Microdissection and DNA extraction

Extraction of DNA from formalin fixed paraffin embedded (FFPE) tissue was performed using DNeasy Blood&Tissue Kit (Qiagen, Milan, Italy), following the manufacturer's instructions. All tumor samples were microdissected to ensure >70% purity of neoplastic cells. Microdissection was performed with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan) from 6-10 consecutive 8µm thick sections stained with nuclear fast red [16]. DNA concentration was measured with the Quantit[™] DNA HS kit (Invitrogen, Carlsbad, California, USA). The DNA quality was assessed by multiplex PCR following the "van Beers" protocol [17].

Array comparative genomic hybridization analysis

The aCGH platform comprises ~32,000 bacterial artificial chromosome (BAC) clones tiled across the genome and it is appropriate for analysis of DNA samples extracted from FFPE tissue. The resolution is comparable to that of high density oligonucleotide arrays [18]. Labeling, hybridization, washes, image acquisition and data analysis were carried out as previously described [16]. To convert smoothed



Figure 1. Dendogram of unsupervised hierarchical clustering of aCGH categorical data deriving from the analysis of 21 oncocytic and 20 non-oncocytic thyroid lesions.

Log, ratios into categorical data, previously validated thresholds were applied, where low level gain was defined as a cbs-smoothed Log, ratio of between 0.12 and 0.45, corresponding to approximately 3-5 copies of the locus, whereas gene amplification was defined as having a Log₂ ratio >0.45, corresponding to more than five copies. Losses were defined as cbssmoothed Log, ratio <-0.12. Cbs-smoothed Log, ratio values and categorical data were used for hierarchical clustering, considering correlation distance and using a Wards clustering algorithm. The stability of the clusters was defined using pvClust [19]. To identify statistically significant differences between the genomic profiles of oncocytic versus non-oncocytic thyroid tumor samples, categorical aCGH data were subjected to multi-Fisher's exact test with adjustment for multiple testing using the step-down permutation procedure maxT, providing strong control of the family-wise type I error rate (FWER). aCGH data on CNAs affecting sex chromosomes were excluded from the final analysis given that female DNA was used as a reference for both male and female cases. The complete aCGH data set has been deposited and is available at http://webservice.cloud. ba.infn.it/hmtdb/GPR_files.rar. Sample codes are available in Supplementary Table 1: http:// webservice.cloud.ba.infn.it/hmtdb/Supp_Table 1_Samples_gpr_files.xls.

Functional categories and pathways analysis

Genes encompassed in gained and lost chromosomal regions were annotated for each

sample and association between genes and specific functional categories was carried out in each oncocytic and non-oncocytic sample. Copy number aberrations on sex chromosomes were excluded from this analysis. In particular, all the gained and lost genes found in each oncocytic and non-oncocytic sample were searched within 5 functional categories/pathways: the MitoCarta human set of 1023 genes, the mTOR (hsa04150)

and PI3K/AKT (hsa04151) human KEGG (http://www.genome.jp/kegg) pathways containing 55 and 350 genes respectively, the mitochondrial respiratory chain complex gene family available upon the HUGO website (http:// www.genenames.org/) containing 129 genes and a manually-curated group of 14 genes encoding for mitochondrial biogenesis promoters. The latter was created by using "mitochondrial biogenesis" and "homo" as keywords in NCBI Gene section. Out of 160 results, for 14 genes literature was available demonstrating their link with increase of mitochondrial biogenesis (CREB1, ESRRA, NRF1, PPARA, PPARG, PPARGC1A, PPARGC1B, PPRC1, TFAM, DCTN, NR1D1, POLG, THG1L, TK2) [20-31]. Two-tailed Mann-Whitney test was applied on fractions of gained and lost genes belonging to the specific category or pathway (N of sample-specific genes gained in a category/total N of samplespecific genes gained), to find statistically significant differences between the whole number of oncocytic samples and the non-oncocytic group.

Sequencing analysis

BRAF analysis was performed using the allele specific locked nucleic acid technique (ASLNAqPCR) for detection of BRAF^{V600E} mutation (1). Primers and corresponding annealing temperatures used for analysis are available on request. *KRAS*, *HRAS* and *NRAS* sequencing was performed using a CEQ2000 Genetic Analysis Systems (Beckman Coulter, Fullerton, CA, USA) as previously described (2). Sequences were analyzed using CEQanalyzer software (Beckman Coulter, Fullerton, CA, USA).

Sequencing of the entire mtDNA was performed according to standard, previously published protocols in 2 cases (OT9 e OT10). In the remaining cases the status of mtDNA had been previously characterized and reported [5]. Among the samples analyzed, 2/14 oncocytic and 5/12 non-oncocytic samples are novel and are described here for the first time. Mitochondrial genomes of both already published and novel cases have been deposited in HmtDB (http://www.hmtdb.uniba.it/hmdb/). Sequencing was performed as previously described [32]. The status of pathogenic mtDNA mutations was assigned as previously described [32], but using both rCRS (GenBank: NC_012920) and RSRS [33] as reference sequences. Moreover, three different methods of pathogenicity prediction were applied, in particular SNPS & Go [34], MutPred [35] and Polyphen-2 [36]. Nucleotide variability, calculated on 14,144 healthy individuals available upon HmtDB (update of November 2013) with the SiteVar software [37], was evaluated for each mitochondrial mutant position. MtDNA mutations, with respect to both rCRS and RSRS, predicted as pathogenic by more than one method of prediction and associated to low variability values were considered possible candidatescontributingtoamitochondrialdysfunction.

Immunohistochemistry (IHC)

Immunohistochemical analysis of NDUFB6 and NDUFB8 complex I subunits levels was performed using the mouse monoclonal antibody clone 21C11 (MIM: 603322, dilution 1:600, Invitrogen, USA) and mouse monoclonal antibody clone 20E9 (MIM: 602140, dilution 1:400, Invitrogen), respectively [38]. Sections were dewaxed, rehydrated, and retrieved using a Tris-EDTA pH 9.0 solution (20 min at 98°C). Endogenous peroxidase activity was quenched with a methanol/H₂O₂ 1.5% solution. Primary antibodies were incubated overnight and processed with a non-biotin-amplified system (NovoLink, Novocastra, UK) according to the manufacturer's instructions. The reaction was developed with 3-3'-diaminobenzidine tetrahydrochloride/H₂O₂ solution. Sections were counterstained with hematoxylin, dehydrated, and mounted in Bio-Mount (Bio-Optica, Milan, Italy). The loss of expression was defined as absence of immunostaining (<1% of positive neoplastic cells). Loss of at least one of the two subunits was considered as indicator of complex dysfunction. Complex V ATP5B subunit (MIM: 102910) immunostained population was used as a positive control (dilution 1:1000, mouse monoclonal 7F9, Invitrogen).

Statistics

If not stated otherwise, all statistical analyses were performed using Mann-Whitney two-tailed test.

Results

Oncocytic lesions harbor genomic profiles distinct from those of their non-oncocytic counterparts

In some contexts, oncocytic variants have been shown to feature distinct molecular profiles when compared to their non-oncocytic counterparts, including profiles of microRNA expression [4] and patterns of gene CNAs [3]. Thyroid oncocytic lesions were shown to be characterized by frequent chromosomal CNAs but it is not known whether these genetic lesions have a functional role in the development of oncocytic phenotype [14]. To assess whether oncocytic lesions in our sample set may be a discrete molecular entity with respect to the pattern of their chromosomal changes, 21 thyroid lesions with oncocytic phenotype and 20 non-oncocytic thyroid lesions as controls were subjected to high-resolution aCGH. The analysis resulted in a dataset comprising 31,367 clones with unambiguous mapping information according to the August 2009 build (hg19) of the human genome (www.ensembl.org).

Unsupervised hierarchical cluster analysis (correlation) was performed based on categorical aCGH data, excluding the sex chromosomes, and resulted in distinct branches in which samples with oncocytic phenotype clustered together (**Figure 1**). In the 'oncocytic branch', 19/25 samples were oncocytic variants, while in the 'non-oncocytic branch' 14/16 lesions were those lacking oncocytic features (Two-tailed Fisher's exact test *P*-value<0.0001). Such result was surprising since we did not expect hyperplasia to cluster together with transformed, malignant lesions. Therefore, in order to ensure the robustness of such result,

Case	Lesion type	RAS and BRAF status	CI mtDNA Mutations	Mitochondrial biogenesis genes gained	
T1	HYP	WT	WT		
Т2	HYP	WT	WT		
ТЗ	HYP	WT	WT		
T4	FA	WT	WT		
Т5	FA	WT	WT		
Т6	FA	WT	WT		
Т7	FA	WT	WT		
Т8	PTC	BRAF V600E	WT	ESRRA	
Т9	PTC	BRAF V600E	WT		
T10	PTC-FV	WT	WT	ESRRA, PPRC1, NR1D1	
T12	FTC	KRAS Q61R	WT		
OT1	HYP-0	WT	m.13414G>A	ESRRA, PPRC1	
OT2	HYP-0	WT	m.5185G>A	ESRRA, PPARA, PPARGC1B, PPRC1, NR1D1, TK2	
OT3	HYP-O	WT	m.11613T>C	TK2	
OT4	HYP-O (HT)	WT	WT	ESRRA, NR1D1	
OT5	HYP-O (HT)	WT	WT		
OT6	FA-O	WT	WT	ESRRA, NRF1, PPARGC1B, NR1D1, THG1L, TK2	
OT7	FA-O	WT	WT	NRF1, PPARGC1B, PPRC1, NR1D1, THG1L, TK2	
0T8	FA-O	WT	m.11403G>A	ESRRA	
OT9	FA-O	WT	WT	ESRRA, PPRC1, NR1D1	
OT10	FA-O	WT	m.14081G>A	ESRRA, NRF1, NR1D1, TK2	
OT11	PTC-0	WT	m.3331del242bp	TK2	
0T12	FTC-O	WT	m.13235insT		
0T13	FTC-0	WT	m.4720G>A	NRF1, NR1D1, TK2	
OT14	FTC-0	WT	m.11038delA	ESRRA, PPARG, PPARGC1B, PPRC1, NR1D1, TK2	

 Table 1. Histologic type and molecular characteristics of the cases

PTC-FV, Papillary Thyroid Carcinoma Follicular Variant; HYP-O, Hyperplastic nodule with Oncocytic features; HYP-O (HT), Hyperplastic nodule with Oncocytic Features in Hashimoto's thyroiditis; FA-O, Follicular Adenoma-Oncocytic variant; PTC-O, Papillary Thyroid Carcinoma-Oncocytic variant; FTC-O, Follicular Thyroid Carcinoma-Oncocytic variant; WT, wild-type; del, deletion; ins, insertion.

we subsequently performed a stringent quality control, excluding samples of doubtful genome quality and ensuring that only data without background noise be included in the cluster analysis. The filtered sample set included 25 cases comprising 14 oncocytic (OT) and 11 non-oncocytic (T) primary thyroid lesions (Table 1). Oncocytic thyroid lesions included 5 hyperplastic nodules (HYP-O), 5 follicular adenomas (FA-O), 3 follicular carcinomas (FTC-O) and 1 papillary carcinoma (PTC-O). Non-oncocytic lesions included 3 hyperplastic nodules (HYP), 4 follicular adenomas (FA), 1 follicular thyroid carcinoma (FTC) and 3 papillary thyroid carcinomas, out of which 2 classic PTCs and 1 papillary carcinoma-follicular variant (PTC-FV). The second cluster analysis again resulted in two distinct and robust branches in which the samples with oncocytic phenotype clustered together (Figure 2). Both clusters showed high measures of stability, as shown by the pvClust analysis (**Figure 3**). In the 'oncocytic branch', 12/15 samples were oncocytic variants, while in the 'non-oncocytic branch' 8/10 lesions were those lacking oncocytic features (Two-tailed Fisher's exact test *P*-value = 0.005). Co-clustering of hyperplasias with malignant lesions, after ensuring the cluster robustness, did not prevent us from carrying on with the differential analysis, since our aim was to detect genomic changes specifically involved in the development oncocytic phenotype-regardless of the tumor histology.

Copy number alterations are frequent genetic alterations in oncocytic thyroid lesions

We next proceeded to define the repertoire of CNAs in oncocytic thyroid lesions and their respective non-oncocytic counterparts. Upon

aCGH profiling of oncocytic thyroid lesions



Figure 2. Dendrogram and heatmap of unsupervised hierarchical clustering of aCGH categorical data deriving from 14 oncocytic and 11 non-oncocytic thyroid lesions. Rows present cbs-log₂ ratios categorized as amplifications (dark red), gains (red), losses (blue) and no change (white) for each BAC clone in genomic order. The main branching distinguished "flat" non-oncocytic branch versus more complex oncocytic branch. OT-oncocytic, T-non oncocytic.

exclusion of the data from sex chromosomes, 915 CNAs (599 gains, 284 losses and 32

amplifications) were detected in 25 samples (Supplementary Table 2A: http://webservice.

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Figure 3. Cluster stability analysis of the unsupervised hierarchical clustering performed using aCGH categorical data deriving from 14 oncocytic and 11 non-oncocytic thyroid lesions.

cloud.ba.infn.it/hmtdb/Supp_Table_2A_aCG-Hevents_phenotype.xls). In oncocytic cases there was a statistically significant higher number of aCGH events compared to the non-oncocytic group (P-value = 0.038). In particular, 623 CNAs were detected in oncocytic samples (410 gains, 192 losses and 21 amplifications, average of 44.5 ± SD 25.7 CNAs per sample), whereas non-oncocytic cases harbored 292 CNAs (189 gains, 92 losses and 11 amplifications, average of 26.5 ± SD 17.5 CNAs per sample). The percentage of the genome and the number of genes involved in CNAs in oncocytic cases was significantly higher than that of nononcocytic samples (P-value = 0.025 and P-value = 0.005, respectively; Figure 4). In particular, the number of genes involved in gains was sig-

nificantly higher in oncocytic cases than in nononcocytic lesions (*P*-value = 0.002; <u>Supplementary Table 2A</u>: http://webservice.cloud.ba. infn.it/hmtdb/Supp_Table_2A_aCGHevents_ phenotype.xls). It is important to note that oncocytic cases constitute a heterogeneous set of lesions in regards to the number and repertoire of CNAs, since the copy number alterations were not normally distributed in our oncocytic group. This means that any common copy number aberration may be considered encouraging in the search for oncocytic-specific determinants.

When samples were grouped based on their histology, regardless of the oncocytic phenotype, no significant differences in the number

Reference	Type of Analyzed oncocytic samples (n= case number)	BRAF V600E mutation frequency (type of mutated thyroid tumor)	H-, N-, K-RAS mutations frequency (type of mutated thyroid tumor)
[51]	FTC-0 (n=11)	NP	55% (FTC-O)
[14]	FA-O, FTC-O (n=11)	NP	9.1% (FTC-O)
[52]	FTC-0 (n=3)	0%	NP
[53]	FTC-0, FA-0 (n=22)	0%	NP
[13]	FTC-0, FA-0 (n=32)	NP	9.4% (FTC-O, FA-O)
[54]	FA-0, FTC-0, PTC-0 (n=39)^	27.3% (PTC-O)	NP
[55]	FTC-0 (n=9)	0%	0%§
[56]	PTC-0 -FV (n=3)	0%	0%
[57]	FA-O, FTC-O, PTC-O (n= 44)	0%	0%
[58]	FA-O, FTC-O (n=70)	NP	11.4% (FTC-O)
[59]	PTC-0 (n=9)	0%	NP
[11]	FTC-0, PTC-0 (n=13)	23% (PTC-O)	O%
[60]	PTC-0 (n=8)	87.5% (PTC-O)	NP
[61]	FTC-0, FA-0 (n=20)	0%	5.8% (FTC-O)
[12]	FA-O, FTC-O, (n=27)	0%	11% (FTC-O)
Present study	Hyp-O, FA-O, PTC-O, FTC-O (n=14)	0%	0%

 Table 2. Summary of canonical BRAF and RAS hot-spot mutations frequency in oncocytic thyroid lesions

^Warthin-like PTCs were not considered; §Only N-RAS gene was analyzed.

of aCGH events were identified between hyperplasias, adenomas and carcinomas, apart from the significantly higher number of amplifications detected in PTCs, compared to the hyperplasias (P-value = 0.016) and significantly higher number of genes involved in amplifications in PTCs, compared to hyperplasias (P-value = 0.016) (Supplementary Table 2B: http://webservice.cloud.ba.infn.it/hmtdb/Supp_Table_ 2B_aCGHevents_histology.xls). This result was expected since cancer cells (PTC) were compared to non-transformed cells (hyperplasia), and it was therefore not striking that the differences were statistically significant despite the low number of cases. On the other hand, lack of significance in the number of aCGH events for other case groups in spite of their histological heterogeneity was an encouraging result in the search for oncocytic-specific determinants.

Furthermore, to investigate whether oncogene mutations most commonly involved in thyroid tumorigenesis are equally distributed in oncocytic and non-oncocytic cases, we screened all cases for canonical mutational hotspots (**Table 1**). The BRAF^{V600E} mutation was detected in two out of 11 non-oncocytic cases (16.6%), and both were papillary thyroid carcinomas. No *BRAF* mutations were observed either in the

remaining non-oncocytic samples or in any of the oncocytic cases. The *KRAS* c.182A>G (Q61R) mutation was detected in only one (8.3%) non-oncocytic case (a follicular thyroid carcinoma). No mutations in *KRAS*, *HRAS* and *NRAS* genes were found in the oncocytic group. Regardless of the small sample set, our data is consistent with the literature in that oncocytic variants display a low frequency of oncogene mutations (**Table 2**) [11-14].

Oncocytic lesions present with no significant losses of nuclear genes involved in electron transport chain

Next, we sought to define differences in the repertoire of genes involved in copy number gains and losses in oncocytic and non-oncocytic cases. Supervised analysis based on a multi-Fisher's exact test adjusted for false discovery was performed employing the categorical aCGH data to detect the regions specifically associated with the oncocytic phenotype. With this analysis we identified the regions that were differentially gained, lost or amplified between the 14 oncocytic and 11 non-oncocytic thyroid samples. A more complex genomic constellation is appreciated in the oncocytic group, strengthening the hypothesis that CNAs might





Figure 4. Number of genes found in all (A), gained (B), lost (C) and amplified (D) copy number aberrations of 14 oncocytic (white) and 11 non-oncocytic (black) thyroid tumor samples.

contribute to development of oncocytic phenotype (**Figure 5**). Regions found to be significantly more often gained/lost in oncocytic samples are reported in <u>Supplementary Table 3</u>: http:// webservice.cloud.ba.infn.it/hmtdb/Supp_Table_4_Multi_Fisher_Results.xls. Since PI3K-

Sample	HmtDB identifier	mtDNA mut	Mutant load	Gene	AA change	Polyphen/ ΔG prediction	HMTDB nt variability	Complex I IHC
OT1	PA_EU_IT_0057	m.13414G>A	homoplasmic	MT-ND5	Premature stop codon		0	NDUFB6-, NDUFB8↓
		m.13271T>C	homoplasmic	MT-ND5	L312P	Probably damaging	0	
0T2	PA_EU_IT_0060	m.5185G>A	homoplasmic	MT-ND2	Premature Stop Codon		0	NDUFB6-, NDUFB8+
0T3	PA_EU_IT_0065	m.11613T>C	homoplasmic	MT-ND4	L285P	Probably damaging	0	NDUFB6↓, NDUFB8+
0T4	PA_EU_IT_0087	-						NDUFB6+, NDUFB8+
0T5	PA_EU_IT_0088	-						NDUFB6+, NDUFB8-
0T6	PA_EU_IT_0077	-						nd
OT7	PA_EU_IT_0085	-			-			NDUFB6-, NDUFB8-
0T8	PA_EU_IT_0091	m.11403G>A	homoplasmic	MT-ND4	Premature Stop codon		0	nd
OT9	PA_XX_XX_0488	-						NDUFB6↓, NDUFB8+
OT10	PA_XX_XX_0489	m.14081G>A	homoplasmic	MT-ND5	G582D	Probably damaging	0	NDUFB6↓, NDUFB8+
0T11	PA_EU_IT_0070	m.3331del242bp	heteroplasmic (70%)	MT-ND1			0	NDUFB6+, NDUFB8+
0T12	PA_EU_IT_0082	m.13235insT	homoplasmic	MT-ND5	frameshift		0	NDUFB6-, NDUFB8+
0T13	PA_EU_IT_0086	m.4720G>A	homoplasmic	MT-ND2	W84X (frameshift)		0	nd
		m.4996G>A	homoplasmic	MT-ND2	R176H	Benign	0	
0T14	PA_EU_IT_0081	m.11038delA	homoplasmic	MT-ND4	99X (frameshift)		0	NDUFB6+, NDUFB8+
		m.4831G>A	homoplasmic	MT-ND2	G121D	Probably damaging	0	

 Table 3. mtDNA mutations, HmtDB identifiers and complex I IHC of oncocytic lesions. Cases in bold show maintenance of NDUFB6 and NDUFB8 staining in oncocytic cells

+positive staining; -negative staining; 1downmodulated. AA, amino acid; nt, nucleotide; del, deletion; ins, insertion; bp, base pairs; nd, not determined.



Figure 5. Comparative analysis of the prevalence of chromosomal gains, losses and amplifications found in the thyroid oncocytic samples and their non-oncocytic counterparts. The proportion of samples in which each clone is gained (green bars) or lost (red bars) is plotted (Y-axis) for each BAC clone according to genomic location (X-axis), sex chromosomes excluded. Multi-Fisher's exact tests were performed with cbs Log₂ ratios for each clone, and those with an adjusted *p*-value of less than 0.05 are plotted (inverse Log₁₀, Y-axis) according to genomic location (X-axis).

AKT-mTOR pathway has recently been associated with oncocytic thyroid carcinomas [12], we performed a functional annotation of the genes pertaining to the human mTOR and PI3K/AKT KEGG pathways that mapped to regions significantly differentially gained or lost in oncocytic samples. No significant fractions of gained or lost genes involved in the mTOR pathway were observed between oncocytic and non-oncocytic lesions (Supplementary Table 4: http://webservice.cloud.ba.infn.it/hmtdb/Supp_Table_5_ Pathways_enrichment.xls). Nevertheless, as seen from the multi-Fisher analysis, the gain of 14g32.32-g32.33 (103.75 Mb - 106.03 Mb), encompassing AKT1, was observed in seven oncocytic and in none of the non-oncocytic lesions (Supplementary Table 3: http://webservice.cloud.ba.infn.it/hmtdb/Supp_Table_4_ Multi_Fisher_Results.xls).

One of the hypotheses explaining development of oncocytic phenotype is that this process constitutes a compensatory mechanism triggered

by the respiratory chain defects often observed in lesions with abnormal accumulation of mitochondria [39-41]. The most well known causes of such a defect are disruptive mtDNA mutations, detected with high frequency in oncocytic tumors [2]. Interestingly, mutations in nuclear genes encoding mitochondrial respiratory chain complexes subunits have been rarely detected in oncocytic lesions [42, 43]. We therefore hypothesized that genomic losses encompassing genes involved in respiratory chain might constitute a genetic hit leading to the oncocytic phenotype. To test the hypothesis of a nuclear ETC damage which would trigger oncocytic development, the frequency of CNAs involving nuclear genes with mitochondrial functions was compared between oncocytic and non-oncocytic thyroid samples. However, neither Mitocarta nor respiratory complexes gene sets were significantly enriched in the losses observed in the oncocytic group, suggesting that loss of nuclear mitochondrial genes is not related to the development of



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Figure 6. A. Immunohistochemical staining of mitochondrial complexes I (NDUFB6 and NDUFB8) and V (ATP5B) in thyroid oncocytic lesions (OT). B. Model of oncocytic phenotype development in transformed thyroid lesions. Oncogenic hit may be independent from mitochondrial biology, as in the case of BRAF/RAS activation where oncocytic phenotype is probably due to modifying secondary events such as gains of genes encoding promoters of mitochondrial biogenesis (MBGs) or occurrence of disruptive mtDNA mutations. On the other hand, in the case of chromosomal gains of Akt, PI3K-AKT-mTOR pathway may promote both cell proliferation and mitochondrial biogenesis simultaneously. The latter scenario may subsequently involve occurrence of disruptive mtDNA mutations (dotted line 1). It may not be excluded that the damage caused by mtDNA mutations could represent a selective pressure which preferentially selects cells carrying chromosomal gains with genes encoding mitochondrial biogenesis activators (dotted line 2). Importantly, it must be noted that oncocytic change may also arise independently of transformation, as in the case of hyperplasias (not depicted).

oncocytic phenotype, at least in our sample set (<u>Supplementary Table 4</u>: http://webservice. cloud.ba.infn.it/hmtdb/Supp_Table_5_Pathways_enrichment.xls).

Gains of nuclear genes encoding activators of mitochondrial biogenesis are a distinctive hallmark of lesions with oncocytic phenotype

We next hypothesized that an alternative mechanism contributing to mitochondrial accumulation may stem from CNAs involving genes that encode mitochondrial biogenesis activators. The relevant literature was curated to create a gene category of positive regulators of mitochondrial biogenesis (see Methods) and the fraction of those genes gained and lost in each sample was calculated in the aCGH data set (Supplementary Table 4: http://webservice. cloud.ba.infn.it/hmtdb/Supp_Table_5_Pathways_enrichment.xls). Copy number gains affecting genes from the mitochondrial biogenesis category were found significantly more frequently in oncocytic rather than in non-oncocytic samples (Table 1 and Supplementary Table http://webservice.cloud.ba.infn.it/hmtdb/ 4: Supp_Table_5_Pathways_enrichment.xls). In particular, 11/14 (78.6%) oncocytic samples harbored gains carrying at least one of the genes encoding for mitochondrial biogenesis activators, whereas only 2/11 (18.2%) nononcocytic samples displayed copy number gains of these genes. Moreover, the difference in the fraction of copy number gain events carrying mitochondrial biogenesis genes was significantly higher in oncocytic than in non-oncocytic lesions (P-value = 0.003). Furthermore, the analysis of recently published genomic data revealed that regions of recurrent chromosomal gains detected in oncocytic carcinomas carried several genes encoding activators of mitochondrial biogenesis (e.g. PPARGC1B, THGIL, NRF1 and NR1D1) [12]. Taken together, it seems that gains in copy number of genes encoding activators of mitochondrial biogenesis may underpin, at least in part, the development of the oncocytic phenotype in thyroid context.

It has been suggested that oncocytic change is followed by mitochondrial damage, particularly in the form of mitochondrial complex I dysfunction caused by mutations in mtDNA [40, 41]. However, conversely from mtDNA mutations, gains of mitochondrial biogenesis promoters, which we identify here as frequent events in oncocytic samples, should not induce damage in respiratory chain complexes. To understand whether mitochondrial function is maintained in some oncocytic variants, especially those harboring gains of mitochondrial biogenesis promoters, we next defined the status of mitochondrial complex I in our oncocytic samples. In particular, mtDNA screening and mitochondrial complex I IHC staining (indicative of the complex integrity) were performed in the samples for which material was available (Table 3).

Positive IHC staining was observed for both NDUFB6 and NDUFB8 mitochondrial complex I subunits in 27.3% (3/11) of the oncocytic cases analyzed (Figure 6A; Table 3), suggesting that certain oncocytic lesions maintain complex I function. However, no specific common denominators were identified for these complex I "positive" samples and thus further analysis with greater sample numbers is necessary in order to understand the consequence of mitochondrial function maintenance in thyroid oncocytic lesions. It is interesting to note that there was no correlation between occurrence of mtDNA mutations and gains of mitochondrial biogenesis promoters, which implies that the latter are not a mere result of a selective pressure created by mitochondrial damage (compensatory effect) but may rather be a cause of an abnormal mitochondrial accumulation independent of the mitochondrial function status.

Discussion

The same diagnostic criteria employed for the histologic typing of conventional (non-oncocytic) thyroid lesions are applied to the diagnosis of oncocytic variants [10], meaning that mitochondrial accumulation, although distinctive morphologically, is not considered relevant from the clinical and diagnostic pathology standpoints. Nevertheless, there is burgeoning data to demonstrate the importance of mitochondria and mitochondrial function in tumor biology. Certain aspects of mitochondrial function, such as those related to mtDNA mutations which often occur in thyroid oncocytic lesions, may impact on tumor progression and behavior [44, 45]. In addition, molecular pathways leading to the development of oncocytic versus conventional thyroid lesions may be distinct, as in the case of the recently defined microRNA profiles in oncocytic follicular carcinomas [4]. Therefore, understanding the molecular underpinning of the oncocytic phenotype in the thyroid lesions (neoplastic and non-neoplastic) may provide important information in regards to the etiology and management of thyroid cancer patients.

It should be noted that despite the small sample size our results provide evidence to suggest that oncocytic lesions do display a distinct repertoire of CNAs that may play a role in the biology of these lesions. Hierarchical clustering of categorical aCGH data obtained from our sample set revealed that thyroid samples formed stable clusters, preferentially clustering on the basis of the oncocytic phenotype rather than histologic type, as opposite to the expected clustering primarily according to histologic diagnoses (hyperplastic nodules, follicular adenoma and follicular/papillary carcinoma). It should be noted that in our study there was no perfect matching of samples displaying oncocytic phenotype and controls, given the rarity of some of the cases. Therefore, given the limited sample size and the imperfect matching of lesions, our results should be perceived as exploratory and hypothesis generating. Nevertheless, our findings clearly demonstrate that oncocytic thyroid lesions, regardless of whether they are biologically or clinically malignant, harbor distinct molecular features when compared to their non-oncocytic counterparts, meaning that there seems to be a common, copy number aberration-based background that leads to the development of oncocytic phenotype.

Furthermore, our aCGH analyses have revealed that, at variance with breast carcinomas which harbor high number of genomic alterations [3], thyroid lesions have relatively simple gene copy number profiles, similarly to those of pituitary tumors [32].

The genomic regions more significantly affected in the oncocytic group (both hyperplastic nodules and tumors) included, among other, gains of chromosomes 7 and 12, and loss of chromosome 2, all genomic events previously associated with oncocytic change in thyroid lesions [11, 14, 15]. Our observation that gains of AKT1 are significantly more frequent in oncocytic than in non-oncocytic lesions is consistent with recent findings suggesting that the PI3K-AKT-mTOR pathway is potentially involved in the development of oncocytic thyroid carcinomas [12] and with the notion that this pathway supports mitochondrial biogenesis [46]. It is plausible that, together with its prosurvival functions, activation of mTOR may also contribute to trigger mitochondrial hyperplasia in thyroid lesions, however we did not observe a significant enrichment for CNAs affecting PI3K-AKT-mTOR-related genes in oncocytic lesions. Given the limited DNA availability for the samples analyzed, we were unable to investigate the presence of somatic mutations affecting the genes pertaining to the PI3K pathway. Further studies to define the functional impact of AKT1 copy number gains in thyroid lesions are therefore warranted.

Currently, the most widely accepted theory to explain the development of the oncocytic phenotype stems from the finding of disruptive mtDNA mutations causing a bioenergetic defect, and posits that these defects may trigger a compensatory upregulation of mitochondrial biogenesis (for a review see [47]). Here we identified genomic instability, in particular gains of mitochondrial biogenesis promoter genes, as a potential novel mechanism for the development of the oncocytic phenotype. There was no correlation between gains of mitochondrial biogenesis genes and complex I function, suggesting that aCGH events involving such gains are not merely a consequence of a preferential selection due to the mitochondrial damage and a response to a compensatory stimulus, but may independently contribute to the development of oncocytic phenotype. In this context, it is important to note the energy metabolism of oncocytic cells bearing mitochondrial damage is likely to be substantially different from that of cells in which mitochondrial accumulation is a consequence of gains of mitochondrial biogenesis genes, or other events which do not result in mitochondrial dysfunction (**Figure 6B**). Therefore, distinguishing the origin of mitochondrial abundance and defining whether functional or non-functional mitochondria accumulate in an oncocytic thyroid lesion are of importance, given that distinct causes of mitochondria accumulation may impart diverse functional consequences on tumor bioenergetics and, ultimately, on clinical behavior and prognosis.

Up to date, it has not been widely investigated whether accumulated mitochondria in oncocytic cells are functionally excessive or dormant. Considering that cancer metabolism has been shown to represent a valid therapeutic target [48], the stratification of oncocytic thyroid variants into those with functional versus non-functional mitochondria may contribute to therapeutic choices, particularly in therapies whose efficacy depends on the competence of oxidative phosphorylation in a cancer cell, such as in the case of metformin [49, 50].

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Disclosure of interest of conflict

None.

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