Somatic complex I disruptive mitochondrial DNA mutations are modifiers of tumorigenesis that correlate with low genomic instability in pituitary adenomas

Ivana Kurelac1, Alan MacKay3, Maryou B.K. Lambros3, Erica Di Cesare1, Giovanna Cenacchi2, Claudio Ceccarelli2, Isabella Morra4, Antonio Melcarne5, Luca Morandi6, Francesco Maria Calabrese7, Marcella Attimonelli7, Giovanni Tallini6, Jorge S. Reis-Filho3 and Giuseppe Gasparre1,*


Received September 19, 2012; Revised and Accepted October 3, 2012

Mitochondrial DNA (mtDNA) mutations leading to the disruption of respiratory complex I (CI) have been shown to exhibit anti-tumorigenic effects, at variance with those impairing only the function but not the assembly of the complex, which appear to contribute positively to cancer development. Owing to the challenges in the analysis of the multi-copy mitochondrial genome, it is yet to be determined whether tumour-associated mtDNA lesions occur as somatic modifying factors or as germ-line predisposing elements. Here we investigated the whole mitochondrial genome sequence of 20 pituitary adenomas with oncocytic phenotype and identified pathogenic and/or novel mtDNA mutations in 60% of the cases. Using highly sensitive techniques, namely fluorescent PCR and allele-specific locked nucleic acid quantitative PCR, we identified the most likely somatic nature of these mutations in our sample set, since none of the mutations was detected in the corresponding blood tissue of the patients analysed. Furthermore, we have subjected a series of 48 pituitary adenomas to a high-resolution array comparative genomic hybridization analysis, which revealed that CI disruptive mutations, and the oncocytic phenotype, significantly correlate with low number of chromosomal aberrations in the nuclear genome. We conclude that CI disruptive mutations in pituitary adenomas are somatic modifiers of tumorigenesis most likely contributing not only to the development of oncocytic change, but also to a less aggressive tumour phenotype, as indicated by a stable karyotype.

INTRODUCTION

Mitochondrial DNA (mtDNA) mutations frequently occur in many human neoplasms, where they may contribute to tumorigenic processes by modifying metabolic, apoptotic or hypoxic mechanisms (1,2). In particular, pathogenic mtDNA mutations leading to the disruption of respiratory complex I (CI) assembly have been shown to block tumour growth in
mice (3,4). These mutations are hallmarks of oncotypic tumours, which often present with an indolent, senescent-like phenotype (5,6). Oncotypic change is not uncommon in neoplasms of epithelial origin and is characterized by the abnormal accumulation of dysfunctional mitochondria in the cell cytoplasm, which arises as a compensatory effect due to the severe mitochondrial damage caused by CI disruptive mutations (7). Conversely, the same type of mutations is rarely encountered in non-oncotypic tumours, which essentially harbour missense mtDNA mutations, that may instead positively contribute to cancer development (2,8,9). Therefore, depending both on the type and mutant load of a mtDNA mutation, mitochondrial genes may act both as tumour suppressors and as oncogenes, and were recently attributed with the functional definition of oncojanus, based on this two-faceted role in tumorigenesis (2,4).

Tumours in which CI disruptive mutations occur in the highest frequency (~80%) are renal oncocytomas (10,11), which are generally associated with a less aggressive behaviour and a relative chromosomal stability (12–14). Given that CI disruptive mtDNA mutations have shown to imply anti-tumorigenic effects through the induction of pro-apoptotic mechanisms (3) and/or by preventing hypoxic adaptation (4), it is reasonable to suggest that, as a consequence, the less aggressive behaviour of tumours bearing such mutations might be reflected in their genomic stability as well. In order to investigate the correlation between CI disruptive mutations and genomic stability in tumours, we here studied a set of pituitary adenomas, rare tumours that mainly present as sporadic lesions (15), although they may also occur as a manifestation of inherited syndromes, including multiple endocrine neoplasia 1 (MEN1) (15–17). Pituitary adenomas are classified based on their similarity to normal parenchymal cells, clinical outcome and the type of hormonal secretion (15). Oncotypic change can be found focally in any adenoma type, but diffuse and extensive mitochondrial proliferation is reported to occur in 6–13% of all pituitary adenomas, which are hence classified as pituitary oncocytomas (15,18). Similar to other tumours presenting with an oncotypic phenotype, the only known genetic hallmark of pituitary oncocytomas are CI disruptive mtDNA mutations, found in 60% of the cases (19).

An overlooked issue concerning mtDNA mutations is whether they occur as germ-line predisposing hits or whether they are truly somatic, randomly arising events, which may modify tumour progression and result in the development of a low-proliferative oncotypic versus a more aggressive tumour. Since multiple copies of mtDNA are present in each cell, mutant and wild-type molecules may co-exist in a condition known as heteroplasmacy and mutant loads may therefore vary. This aspect of mitochondrial genetics has rarely been taken into account when analysing mtDNA mutations in tumours. For instance, assumptions about the somatic nature of mtDNA mutations have been made on the basis of analyses performed with techniques like Sanger sequencing, which are not sufficiently sensitive to detect low-level heteroplasmacy (20–22). However, recent reports based on highly sensitive next-generation sequencing techniques have revealed that low-level mutant loads may be present in the germ line of cancer patients (23), and the occurrence of low loads of germ-line CI disruptive oncojanus-associated mtDNA mutations has been reported in single cases (24,25).

Aims of this study were (i) to investigate the correlation between CI disruptive mtDNA mutations and genomic aberrations in pituitary adenomas and (ii) to determine whether these mutations act as germ-line or somatic players. A thorough genetic characterization of pituitary adenomas (oncojanus and non) was therefore performed, both in terms of mtDNA and of chromosomal DNA alterations. Our results corroborated the association between CI disruptive mutations and oncojanus phenotype, provided evidence that such mutations are unlikely to exist as germ-line predisposing elements in pituitary adenoma patients and showed that somatic CI disruptive mtDNA mutations significantly correlate with a stable complement of chromosomal DNA, which suggested their modifying role in pituitary tumour progression, likely contributing through the double-faceted oncojanus effect (2,4).

RESULTS

Pathogenic mtDNA mutations are somatic events frequently occurring in pituitary oncocytomas

With the aim to correlate mtDNA data with the degree of chromosomal number aberrations, we expanded a previously characterized sample set of 44 pituitary adenomas (19) with additional 20 pituitary oncocytoma cases, thus increasing statistical power. The cases were carefully selected through histological and ultrastructural examination (Fig. 1). Corresponding blood samples for the 20 novel cases were also available, allowing us to investigate whether mtDNA mutations may exist in the germ line as predisposing elements for oncotypic transformation of pituitary adenomas.

The mtDNA characterization of the novel cases revealed that 12 out of 20 (60%) presented with tumour-specific mtDNA mutations, 6 of which were novel, whereas 6 have previously been associated with a known disease (Table 1), such as m.12421delA in MT-ND5 described in cases with isolated CI deficiency (26). The m.11873insC MT-ND4 mutation, which results in a shift of the open reading frame and in the generation of a premature stop codon, has already been reported and shown to cause CI derangement (19,27). Seven out of the 12 cases (58%) carried mutations in coding regions, 2 in MT-RNR2 (16SrRNA) and 3 in tRNA genes. On the other hand, nine mtDNA changes not defining the haplogroup and hence potentially somatic were nonetheless found both in tumour and blood samples of seven cases (Supplementary Material, Table S1). All of these non-tumour-specific mutations were found in homoplasmacy in both tissues, indicating their probable polymorphic nature.

All novel tumour-specific mutations affecting coding regions were predicted as probably damaging by PolyPhen1, having high position-specific independent count (PSIC) scores >2 (see Materials and Methods). Alignment with ClustalW2 (Fig. 2A) showed that mutations m.3413G>A, m.3457G>A and m.15092G>A affect amino acids highly conserved between different metazoan phyla (from Porifera to Great Apes and Homo sapiens). Among the three somatic mutations affecting tRNAs, m.1644G>A was previously
described as associated with adult Leigh syndrome, hypertrophic cardiomyopathy and mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes (MELAS) syndrome (28). The remaining two tRNA mutations were shown to induce a significant change in the minimal free energy (ΔG) of the respective secondary structures by RNA fold (Fig. 2B). Moreover, the application of tRNAscan-SE confirmed the effect of the two mutations on the functional conservation of two tRNAs: m.12276G>A found in the H9 sample was predicted to have a less stable D-stem in

Table 1. Somatic mtDNA mutations in pituitary oncocytomas (H)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Base change</th>
<th>AA change</th>
<th>Gene</th>
<th>Mutant load (%)</th>
<th>PSIC/ΔG</th>
<th>Annotation status</th>
</tr>
</thead>
<tbody>
<tr>
<td>H1</td>
<td>m.3094G&gt;A</td>
<td>–</td>
<td>MT-RNR2</td>
<td>50</td>
<td>–</td>
<td>Colonic crypts biochemical defects (76)</td>
</tr>
<tr>
<td>H2</td>
<td>m.3413G&gt;A</td>
<td>G36D</td>
<td>MT-ND1</td>
<td>100</td>
<td>2.396</td>
<td>Novel</td>
</tr>
<tr>
<td>H3</td>
<td>m.3457G&gt;A</td>
<td>D51N</td>
<td>MT-ND1</td>
<td>100</td>
<td>2.075</td>
<td>Novel</td>
</tr>
<tr>
<td>H8</td>
<td>m.2993T&gt;C</td>
<td>–</td>
<td>MT-RNR2</td>
<td>50</td>
<td>–</td>
<td>Novel</td>
</tr>
<tr>
<td>H9</td>
<td>m.12276G&gt;A</td>
<td>–</td>
<td>MT-TL2</td>
<td>50</td>
<td>From −18.50 to −11.90 kcal/mol</td>
<td>Chronic external ophthalmoplegia (77)</td>
</tr>
<tr>
<td>H11</td>
<td>m.15092G&gt;A</td>
<td>G116S</td>
<td>MT-CYB</td>
<td>50</td>
<td>2.112</td>
<td>Novel</td>
</tr>
<tr>
<td>H12</td>
<td>m.11873insC</td>
<td>–</td>
<td>MT-ND4</td>
<td>50</td>
<td>–</td>
<td>Pituitary adenoma (19)</td>
</tr>
<tr>
<td>H13</td>
<td>m.12421delA</td>
<td>–</td>
<td>MT-ND5</td>
<td>30</td>
<td>–</td>
<td>Isolated CI deficiency (26)</td>
</tr>
<tr>
<td>H14</td>
<td>m.1644G&gt;A</td>
<td>–</td>
<td>MT-TV</td>
<td>100</td>
<td>No change in ΔG</td>
<td>Adult Leigh syndrome (28), hypertrophic cardiomyopathy and MELAS (78)</td>
</tr>
<tr>
<td>H17</td>
<td>m.3457G&gt;A</td>
<td>D51N</td>
<td>MT-ND1</td>
<td>100</td>
<td>2.075</td>
<td>Novel</td>
</tr>
<tr>
<td>H18</td>
<td>m.12421delA</td>
<td>–</td>
<td>MT-ND5</td>
<td>45</td>
<td>–</td>
<td>Novel</td>
</tr>
<tr>
<td>H19</td>
<td>m.4427G&gt;A</td>
<td>–</td>
<td>MT-TM</td>
<td>100</td>
<td>From −15.82 to −12.15 kcal/mol</td>
<td>Novel</td>
</tr>
</tbody>
</table>

AA, amino acid; PSIC, position-specific independent counts; CI, complex I; MELAS, mitochondrial myopathy, encephalopathy, lactic acidosis and stroke-like episodes.

Figure 1. Morphology of oncocytomas and non-oncocytic pituitary adenomas. Haematoxylin/eosin staining (magnification ×200) showing monomorphic adenomatous cells, with eosinophilic cytoplasms and small nuclei without atypia in pituitary oncocytomas (A). Mitotic figures were rare (less than 1 mitotic figure/10 high-power fields), and tumour vasculature was delicate. On the other hand, in non-oncocytic pituitary adenoma, nuclei occupied most of the cytoplasm as presented by predominant haematoxylin staining (B). Electron micrographs of oncocytomas (C) showed cell cytoplasm occupied by innumerable densely packed mitochondria characterized by partial loss of matrix and irregularly organized cristae lacking intra-mitochondrial dense granules. A few rough endoplasmic reticulum profiles were observed, relatively small Golgi apparatus, sparsely distributed secretory granules and a variable accumulation of mitochondria, which appeared to occupy up to 50% of the cytoplasmic compartment. These mitochondria were generally swollen, with partial or complete cavitation of the matrix and a variable loss of cristae. Some mitochondria featured an irregular pattern of residual cristae which appeared short, in a crescent or concentric array. Non-oncocytic pituitary adenomas selected for our analysis did not show gross mitochondrial alterations and often abundance of secretory granules was evident (D).
comparison with the wild-type MT-TL2 (data not shown), whereas the m.4427G>A mutation found in the H19 sample was shown to completely prevent the formation of MT-TM.

The nine germ-line mtDNA mutations presented low PSIC values, high amino acid variability reported in HmtDB, no influence on minimal free energy in the case of tRNA mutations and/or were as already known polymorphisms (Supplementary Material, Table S1).

To determine whether mtDNA mutations may be predisposing or modifying events, DNA from the blood of the 20 pituitary oncocytoma patients was analysed by fluorescent PCR (F-PCR) for indels and by allele-specific locked nucleic acid quantitative...
real-time PCR (ASLNAqRT-PCR) for point mutations, which allow detection of 2% (29) and 0.1% (30) mutant load, respectively. The assays were set up for all mutations detected by Sanger sequencing in the tumour but not in corresponding blood samples. No low-level heteroplasmy was found in the blood samples upon F-PCR and ASLNAqRT-PCR analyses (Supplementary Material, Figs S1 and S2). These findings suggest that mtDNA mutations are not an element predisposing to oncogenic transformation in pituitary adenomas, but most likely a secondary event. The entire set of the complete mitochondrial genome sequences from tumour samples described in the present study was deposited in the public resource HmtDB (31), and the list of the HmtDB identifiers is presented in Supplementary Material, Table S2.

Finally, haplogroup comparison with a set of mitochondrial genomes of healthy Italian individuals available in HmtDB did not highlight any correlation between a particular haplogroup and the development of oncogenic phenotype. This finding was further supported by implementing our data with previously published pituitary oncocytomas and non-oncocytic pituitary adenomas (Supplementary Material, Fig. S3).

mtDNA mutations found in pituitary oncocytomas prevent proper CI assembly

In order to stratify our samples for array comparative genomic hybridization (aCGH) analysis, we next determined the functional effect of the mtDNA mutations on CI assembly.

The status of CI disruptive mutation was assigned to (i) mtDNA changes which introduced a premature stop codon in coding regions of CI subunit genes, (ii) previously described mutations causing CI deficiency, (iii) novel variants found in a highly conserved site, at which amino acid variability in the general population as determined by HmtDB was under 1% for coding mutations, or which substantially influenced ΔG for tRNA mutations, and (iv) novel variants found in conserved D-loop functional regions (promoters PH1 and PL).

Furthermore, in order to validate our criteria for mutation pathogenicity, immunohistochemistry (IHC) for nuclear encoded NDUFb6 and NDUFb8 subunits of CI was performed on samples for which material was available, since negative staining for at least one of the two subunits is indicative of CI disassembly (24). Complex V ATP5B subunit was used as an indicator of mitochondrial abundance. All of the tested samples showed to be uniformly positive for ATP5B, indicating homogeneous oncogenic areas (Fig. 2C). On the other hand, although samples without mtDNA mutations showed positive NDUFb6 and NDUFb8 staining, the staining of at least one of these subunits was negative or very weak in samples carrying mutations in mtDNA genes (Fig. 2C).

Taking together, considering the bioinformatics predictions and IHC data, 17 cases were defined as bearing CI disruptive mutations (Supplementary Material, Table S3).

Occurrence of CI disruptive mtDNA mutations correlates with lower genomic instability in pituitary adenomas

To determine the repertoire of nuclear numerical genomic aberrations found in pituitary adenomas, 44 previously characterized pituitary adenomas (19) together with the 20 additional new cases described in this study were subjected to PCR-based DNA quality control. Eventually, 48 out of 64 pituitary adenomas were shown to have DNA of sufficient quality to be subjected to high-resolution aCGH (Supplementary Material, Table S3). The aCGH analysis produced a data set which comprised 31 367 bacterial artificial chromosome (BAC) clones with unambiguous mapping information according to the August 2009 build (hg19) of the human genome (www.ensembl.org). The complete aCGH data set has been deposited and is available at http://rock.iarc.fr/collaborations/Mackay/pituitary_adenoma_aCGH/. Upon exclusion of the data from sex chromosomes, 2984 copy number aberrations (i.e. 1774 gains, 1149 losses and 61 amplifications) were detected in the 48 pituitary adenomas. Pituitary adenomas revealed an average of 9.56% (± SD 15.5) of the genome involved in copy number aberrations.

Since mtDNA mutations leading to CI disassembly have been shown to exhibit a suppressive effect on tumour progression (3,4,19), we hypothesized that they might be associated with the relatively low genomic instability observed in pituitary adenomas. In order to investigate the potential modifying function of CI disruptive mutations, we correlated mtDNA genotype with the nuclear genetic features in our data set of pituitary tumours for which both mtDNA sequence and aCGH data were available (Supplementary Material, Table S3).

Tumours lacking CI disruptive mtDNA mutations presented with an average of 12.73% (± SD 18.37%) of the genome involved in copy number aberrations. On the other hand, copy number aberrations involved an average of 3.78% (± SD 4.32%) of the genome in pituitary adenomas harbouring CI disruptive mutations. In general, a greater number and complexity of chromosomal copy number aberrations (>5% of the genome involved) were found in tumours without CI disruptive mutations (16 out of 31), whereas those harbouring these mutations mostly displayed 'flat' genomes (15 out of 17) (two-tailed Fisher’s exact; P = 0.011).

Since the ‘flat’ pattern of genomic profiles as defined by aCGH is often associated with microsatellite instability (MSI), we sought to determine whether pituitary adenomas in our set displayed MSI. Analysis based on BAT26 and BAT40 markers was performed, revealing that all pituitary adenomas analysed were microsatellite-stable (Supplementary Material, Fig. S4). Another potential explanation for apparently ‘flat’ aCGH profiles is the multiclonality of the cases analysed, i.e. tumours composed of mosaics of cancers cells that, in addition to the founder genetic aberrations, harbour private mutations. Although this question was not directly addressed in the samples included in this study, it is well known that pituitary adenomas are clonal (32,33).

Moreover, to exclude that the differences in genomic profiles could be due to mutations in the TP53 tumour suppressor, a gene whose inactivation is associated with higher levels of chromosomal aberrations (34), we sequenced the whole TP53 coding sequence in the 48 pituitary adenomas included in aCGH analysis. Neither TP53 pathogenic mutations nor loss of heterozygosity affecting the TP53 locus was found.

Last, since mutations in the oncogene GNAS have been reported to occur in 40% of pituitary somatotropinomas (35) but not in non-functioning pituitary tumours (36), we investigated whether its mutations might be associated with the
differences in genomic profiles of pituitary adenomas. \textit{GNAS} mutational hotspot analysis revealed the presence of the previously reported mutation c.2530C>T (p.844R>C) in two cases (H40 and CNTRH118), none of which harboured disruptive mtDNA mutations (Supplementary Material, Fig. S5) (35).

Taken together, our findings demonstrate that, independent of microsatellites stability, \textit{TP53} and \textit{GNAS} genotype, stable nuclear genomic architecture is a feature of pituitary adenomas bearing CI disruptive mutations.

Low level of copy number aberrations is also a feature of oncocytic phenotype in pituitary adenomas

It is generally accepted that CI disruptive mutations are hallmarks of oncocytic phenotype. Indeed, this was confirmed in our ‘expanded’ sample set, where the occurrence of CI disruptive mtDNA mutations significantly correlated with oncocytic phenotype (two-tailed Fisher’s test; \( P = 0.0002 \)). In order to investigate whether the high genomic stability observed in pituitary adenoma cases carrying CI disruptive mutations correlates with the occurrence of oncocytic phenotype, we analysed the aCGH data by taking into consideration the oncocytic versus non-oncocytic phenotype. To determine the repertoire of nuclear numerical genomic aberrations found in pituitary oncocytomas and the differences in the patterns of genomic aberrations, we compared aCGH data relative to 31 pituitary oncocytomas versus 17 non-oncocytic pituitary adenomas from our sample set (Supplementary Material, Table S3).

Since CI disruptive mutations are known hallmarks of oncocytic phenotype, we hypothesized that the high genomic stability observed in pituitary adenoma cases carrying CI disruptive mutations could also be translated to the occurrence of oncocytic phenotype. In fact, the vast majority (77%) of pituitary oncocytomas displayed minimal levels of numerical chromosomal aberrations (i.e. ‘flat’ genomes). A statistically significant lower number of BAC clones harbouring gains, losses and amplifications were found in pituitary oncocytomas compared with non-oncocytic adenomas (two-tailed t-test; \( P = 0.03 \)). In total, 1564 aberrations were detected in oncocytoma cases (50 per sample, \( + \text{SEM} 4.3 \)), and on average corresponding to 5.02% of the genome involved in copy number aberrations (range 0.74–33.92%). On the other hand, non-oncocytic pituitary adenomas carried a total of 1420 copy number aberrations (83 per sample, \( + \text{SEM} 15.5 \)), which on average corresponded to 17.84% of the genome involved in chromosomal aberrations (range 0.72–81.77%).

In addition, copy number aberrations in non-oncocytic pituitary adenomas often involved large parts of the genome (Fig. 3A); 11 out of 17 samples (65%) displayed >5% of their genomes affected by a numerical chromosomal aberration, whereas only 7 out of 31 (23%) oncocytomas presented with this level of copy number aberrations (Supplementary Material, Table S3).

Furthermore, unsupervised hierarchical cluster analysis was performed based on categorical aCGH data derived from 31 367 BACs, which revealed no consistent clustering based on the type of the lesion (Supplementary Material, Fig. S6), which indicates that there are no particular copy number aberrations associated with oncocytic phenotype. However, the two main branches may be distinguished as containing ‘flat’ (Branch 1) versus more complex (Branch 2) genomes. Both oncocytic phenotype and the occurrence of CI disruptive mtDNA mutations significantly correlated with the ‘flat’ genomes of Branch 1 (two-tailed Fisher’s test; \( P = 0.039 \) and \( P = 0.036 \), respectively), which additionally corroborated our previous finding on the association of CI disruptive mutations and oncocytic phenotype with the less complex genomic architecture of pituitary adenomas.

Distinct copy number aberrations in pituitary oncocytomas do not involve nuclear genes with a known mitochondrial function

Although copy number aberrations have been extensively studied in other pituitary tumours (37–40), as well as in oncocytic tumours of other tissue types (5,41,42), limited information is available on chromosomal alterations in pituitary oncocytomas, partly also due to the generally limited understanding of the molecular pathways leading to tumorigenesis in the pituitary gland (32,33,43,44). In order to define the qualitative differences in the repertoire of gene copy number changes found in pituitary oncocytic and non-oncocytic adenomas, a multi-Fisher’s exact test adjusted for false discovery was applied to compare genomic profiles of the 31 oncocytomas versus 17 non-oncocytic pituitary adenomas. Among the regions significantly gained, lost or amplified between the two groups (Fig. 3B), there was only one apparently oncocytoma-associated copy number aberration: a loss of 4p16.3. It must be underlined that this region maps to a locus reported to be uncommonly affected by germ-line copy number polymorphisms, is peri-telomeric and prone to aCGH artefacts; hence, this finding ought to be perceived as hypothesis-generating. The loss within chromosomal band 4p16.3 was found in 10 out of 31 (32%) oncocytomas, whereas no copy number losses affecting this locus were present in pituitary adenomas lacking the oncocytic phenotype (two-tailed Fisher’s test; \( P = 8 \times 10^{-4} \)). This copy number gain maps to chr4:3394–3640 kb and encompasses the \textit{RGS12}, \textit{HGFAC}, \textit{DOK7} and \textit{LRPAP1} gene loci. An additional genetic aberration of interest was the gain of 11p15.4, which was significantly more prevalent in oncocytomas (35%) than in non-oncocytic pituitary adenomas (6%) (two-tailed Fisher’s exact test; \( P = 0.03 \)). The chromosomal band 11p15.4 also maps to a known germ-line copy number polymorphisms and is also peri-telomeric. This copy number gain maps to chr11:10 425–10 684 kb and encompasses the \textit{AMPD3}, \textit{RNF141}, \textit{LYVE1} and \textit{MRVI1} gene loci. It has been suggested that genetic lesions in genes with mitochondrial function, but encoded by the nuclear genome, may be called responsible for oncocytic transformation, representing phenocopies of mtDNA mutations (27,45). It should be noted, however, that no known mitochondrial function has been ascribed to the genes found in genomic regions significantly more frequently targeted by copy number aberrations in pituitary oncocytomas than in non-oncocytic pituitary adenomas. To investigate whether oncocytoma samples presented losses of nuclear genes encoding CI subunits and chaperones whose mutations have been reported to cause CI disassembly (46,47), the list of genes located in the regions of recurrent losses in

\textit{Human Molecular Genetics, 2013, Vol. 22, No. 2}
oncocytoma samples was searched for \textit{NDUFAF1}, \textit{NDUFAF2}, \textit{NDUFAF3}, \textit{NDUFAF4}, \textit{FOXRED1}, \textit{NUBPL}, \textit{ACAD9}, \textit{ECSIT}, \textit{NDUFS1}, \textit{NDUFS2}, \textit{NDUFS3}, \textit{NDUFS4}, \textit{NDUFS6}, \textit{NDUFS7}, \textit{NDUFS8}, \textit{NDUFV1}, \textit{NDUFV2}, \textit{NDUFA1}, \textit{NDUFA2}, \textit{NDUFA11}, C2orf56, C6orf66, C8orf38 and C20orf7. None of these genes was specifically lost in the oncocytoma group. For instance, \textit{FOXRED1} was found lost in 12 out of 31 (39\%) pituitary oncocytomas but also in 7 out of 15 (47\%) non-oncocytic pituitary tumours. Moreover, no gains were detected in genomic loci enclosing known mitochondrial biogenesis
factors such as peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (PPARGC1A; ENSG00000109819), which would explain the mitochondrial hyperplasia observed in oncocytomas.

Genomic profiling of pituitary adenomas revealed PTTG1IP gain as a possible oncogenic event in pituitary tumorigenesis

The molecular drivers of pituitary tumorigenesis are still mostly unclear and their thorough identification is out of the scope of the present work. Nonetheless, we investigated common copy number changes in our entire sample set. Regions of recurrent aberrations are shown in Supplementary Material, Figure S7 and summarized in Supplementary Material, Table S4. Recurrent aberrations found in >50% of cases included gains of 7p22.3–q31.1, 8q21.2–q21.3, 10q26.3, 16p13.3, 18q21.1, 19p13.3–p12, 20q13.33 and 22q11.1, whereas the recurrent losses present in >30% of cases mapped to 1p36.32–p36.31, 4q35.2, 9p11.1–q21.11, 11q12.2, 11q23.3–q25, 15q11.2–q13.3, 16q21, 19p13.2 and 22q11.23–q12.1. No recurrent amplification, other than regions of prevalent copy number polymorphisms (e.g. 18q21.1), was identified.

Next, the list of genes located in recurrent regions (i.e. >30%) was analysed in search of factors suggested to be involved in pituitary tumorigenesis, namely GNAS, PRKARIA, PIK3CA, MEN-1, CDKN1B, CCND1, GADD45G, AIP, HMG2 and PTTG1 (32,43,48). The results of the analysis are listed in Supplementary Material, Table S5. Interestingly, PTTG1IP, a pituitary tumour-transforming 1 interacting protein (HGNC: 13524), located at 21q22.3, was found gained in 17 out of 48 (35%) pituitary adenomas, 7 of which were non-oncocytic adenomas (41%) and 10 oncocytomas (32%). A further search revealed gains of regions including the PTTG1 locus in additional two cases, one oncocytoma and one non-oncocytic. The PTTG1 protein is involved in cell-cycle regulation, controlling the segregation of sister chromatids during mitosis (49). It has been found over-expressed in numerous tumours where it is considered to induce cellular transformation by contributing to genomic instability (49,50). Nonetheless, pituitary adenomas revealed to bear relatively few gene copy number aberrations as defined by aCGH, with an average of 9.56% aberrations per sample.

DISCUSSION

In this work, we elucidated the role of CI disruptive mtDNA mutations in pituitary tumorigenesis, and showed that tumours carrying such mutations have significantly fewer copy number aberrations than those without these mtDNA lesions.

In addition to mutations in tumour suppressors and oncogenes, neoplastic development involves many additional genetic events, which may either have negligible functional significance under a set of selective pressure forces (i.e. passenger mutations) or modify tumour progression in a positive or a negative manner. These factors may emerge somatically in a stochastic fashion or exist prior to tumour initiation as predisposing elements, providing a permissive ground for secondary transformation. The role of mtDNA mutations in this context has yet to be elucidated. There is evidence that mtDNA mutations may induce either pro- or anti-tumorigenic effects (2).

It is not clear, however, whether they emerge after the initial oncogenic hit as somatic events, or if they exist as low mutant loads in germ-line tissue. In the latter case, they may be subsequently positively selected as it has been described in oncogenic neoplasms (9,24,25), either randomly as passengers in a rapidly proliferating cell (51), or due to their positive effects on tumour progression under selective pressure (52,53). mtDNA mutations causing respiratory CI disassembly are of particular interest, as they have been shown to induce block of aggressive osteosarcoma tumour growth in mice and were functionally implicated in driving the oncogenic transformation in human neoplasia (4,24,54). To investigate whether such mutations are predisposing elements or modifiers, the mitochondrial genome was screened in a set of pituitary oncocytomas. Our results confirmed the high occurrence of CI disruptive mutations in these tumours, revealing their somatic nature as no low-level heteroplasmy was found in the corresponding blood tissue. Therefore, conversely from what has been observed in single-oncocytoma cases of other tissues (24,25), CI mutations in pituitary adenomas are not germ-line predisposing elements (54).

Certain modifying roles of CI disruptive mtDNA mutations have already been described, such as promotion of apoptosis (3) and hampering of hypoxic adaptation (19). In line with the data presented on the anti-tumorigenic effect of CI mtDNA mutations, here we demonstrated that they are also associated with tumours bearing higher levels of genomic stability (P = 0.011), at least in pituitary adenomas. CI disruptive mutations were found to significantly correlate with genomic profiles where <5% of the genome was affected by copy number aberrations, regardless of the tumour size. Interestingly, lower levels of copy number aberrations were found in tumours carrying CI disruptive mtDNA mutations despite the frequent gains of the PTTG1IP gene, which has been associated with the deregulation of genomic architecture in pituitary tumours (49,50).

From the functional point of view, the CI bioenergetic deficit already shown to be a hallmark of oncocytoma appears to lead cancer cells to arrest in a senescence-like status. Such deficit is considered to be the distinctive feature of most oncocyctic tumours regardless of the co-occurrence of CI-specific mutations, although there is evidence that the latter may be underestimated, especially since nuclear CI mutations in these tumours have been seldom investigated (2). In this frame, the significant association between oncocyctic phenotype and low genomic instability may be envisioned as an association between CI dysfunction and a more stable genomic complement, although this warrants further investigation corroborated with biochemical data on mitochondrial respiratory competence of such tumours. Overall, however, the absence of mitotic figures in our oncocyctic samples, as well as in other oncocytomas (6), along with low levels of Ki67, strengthens the observation that oncocytes are stalled into a low-replicative state. Hence, upon the occurrence of replication arrest, the chromosomal complement would not undergo
further re-arrangements and accumulation of aberrations. Since genomic instability is one of the major driving forces of tumorigenesis (55), the here observed higher genomic stability associated with the CI disruptive mutations and with the oncocytic phenotype may therefore be considered a consequence of an early occurrence of mtDNA disruptive mutations driving the arrest of tumour progression.

We provide direct evidence that low levels of chromosomal copy number aberrations are preferentially a feature of pituitary oncocytomas, whereas their non-oncocytic counterparts displayed a significantly higher number of genomic aberrations. From the perspective of genomic instability, therefore, pituitary oncocytomas may be considered analogous to renal oncocytomas, which have been shown to harbour more stable genomes than their non-oncocytic counterparts (12–14) and to carry CI disruptive mutations (10,11,27). Likewise, the genomic architecture of mitochondrial-rich breast cancers has also been demonstrated to be more stable than that of breast carcinomas lacking oncocytic features, although no difference in clinical features was observed (41,56). At variance with oncocytic tumours of other tissues, those of the thyroid were previously shown to display higher levels of chromosomal aberrations when compared with their non-oncocytic counterparts (5,57). Furthermore, conversely from oncocytomas of other tissues, oncotypic thyroid lesions not uncommonly have a more aggressive behaviour than their non-oncocytic counterparts (58), but also have the lowest prevalence of CI mutations among all other types of oncocytic tumours (54,59).

Noticeably, no common copy number aberration was detected between genomic profiles from our sample set of oncocytomas and the regions previously associated with the development of oncocytic phenotype (5,14,41,60), suggesting that either copy number aberrations are not to be held responsible for oncocytic transformation or that those driving oncocytoma tumorigenesis are site-specific. This finding furthermore establishes CI disruptive mtDNA mutations as the only common genetic hallmark in these lesions. Further studies are necessary, such as parallel massive sequencing analysis of nuclear genes with mitochondrial function to understand other mechanisms involved in the accumulation of mitochondria, especially in those oncocytic tumours which do not harbour mtDNA mutations. Since there are about 1000 genes in the human nuclear genome predicted to have a mitochondrial function (61), it has been suggested that defects in such genes could be called responsible for oncocytic transformation, representing phenocopies of mtDNA mutations (2,27). In fact, our group has recently identified distinct copy number aberrations in mitochondrial-rich breast cancer, which contained diverse nuclear genes with a known mitochondrial role (41). In the present study, however, no annotated mitochondria-related functions were found for the genes located in chromosomal bands 4p16.3 and 11p15.4, the only two regions significantly more frequently altered in oncocytomas than in non-oncocytic pituitary adenomas. Nevertheless, loss-of-function mutations in DOK7 have been reported to cause congenital myasthenia, which is characterized by muscle weakness due to defects in neuromuscular synapse formation (62–64). It has recently been pointed out that similar type of genetic lesions may lead to the oncocytic phenotype in tumour environment, on the one hand, and to the development of neuromuscular mitochondrialopathies, on the other (2). In this context, DOK7 could be a good candidate to investigate its potential involvement in oncocytic transformation.

Taken together, we have confirmed the occurrence of mtDNA mutations in pituitary oncocytomas, revealing their somatic nature and their potential modifying role in the maintenance of higher genomic stability of pituitary adenomas. In the context of anti-tumorigenic effect of CI disruptive mtDNA mutations, together with previously described effects on apoptotic (3) and hypoxic mechanisms (4,19), we here demonstrate that the contribution of such mutations in determining a less aggressive tumour phenotype may, at least in pituitary tumorigenesis, be inversely associated with the presence of chromosomal copy number aberrations, adding up to the previously underlined features of an oncojanus gene (2,4).

**MATERIALS AND METHODS**

**Sample collection**

Samples of 20 sporadic pituitary adenomas (H1–H20) and corresponding blood tissues were collected at Regina Margherita Hospital, Turin, Italy. Moreover, for aCGH analysis, DNA samples from previously described oncocytomas and non-oncocytic pituitary adenomas were included (samples eligible for aCGH analysis are listed in Supplementary Material, Table S3) (4,19). The cases were selected consecutively during the period 2002–2009. All procedures were performed according to internal board review protocols. All cases enrolled in the study were Italians.

**Morphological analysis of pituitary adenomas**

Oncocytic phenotype was evaluated independently by three pathologists (I.M., G.C., C.C.). Samples showing oncocytic phenotype were first selected on the basis of their histological appearance on haematoxylin–eosin-stained sections and then subjected to ultrastructural analysis. Specimens from pituitary adenoma were fixed in 2.5% glutaraldehyde in 0.1% cacodylate buffer, post-fixed in 1% OsO₄, dehydrated in ethanol and embedded in Araldite. Thin sections (1 μm), stained in uranyl acetate and lead citrate, were studied with a Philips 410 transmission electron microscope (Philips, Eindhoven, Holland). Only cases which showed diffuse oncocytic change (>80%) were designated as pituitary oncocytomas, whereas the absence of mitochondrial defects was the criterion for the selection of non-oncocytic pituitary adenomas.

**IHC for CI subunits**

Immunohistochemical analysis with antibodies against NDUFb6 (MIM: 603322) and NDUFb8 (MIM: 602140) subunit of CI and ATP5B (MIM: 102910) subunit of Complex V (Invitrogen, Milan, Italy) was performed on paraffin-embedded sections as previously described (24).
DNA extraction

Extraction of DNA from formalin-fixed, paraffin-embedded (FFPE) tissue was performed using the DNeasy Blood & Tissue Kit (Qiagen, Milan, Italy), following the manufacturer’s instructions. All patient tumour samples were microdissected to ensure >70% of purity of neoplastic cells. Microdissection was performed with a sterile needle under a stereomicroscope (Olympus SZ61, Tokyo, Japan) from 6–10 consecutive 8 μm thick sections stained with nuclear fast red as previously described (65). DNA concentration was measured with the PicoGreen® assay (Invitrogen, Monza, Italy). The DNA quality was assessed by multiplex PCR following the protocol developed by van Beers et al. (66). Blood samples were extracted using the GeneElute Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich, Milan, Italy) and quantified by NanoDrop2000 (Thermo Scientific, France).

mtDNA sequencing

Whole mtDNA resequencing was performed with MitoAll (Applied Biosystems, Foster City, CA, USA) as previously described by our group (67), using the SeqScape v.2.5 software for electrophrogram analysis (Applied Biosystems). The pathogenic effect of mutations causing amino acid substitutions was predicted with PolyPhen1 server, which is based on sequence comparison with homologous proteins. Profile scores (PSIC) are generated for the allelic variants and represent the logarithmic ratio of the likelihood of a given amino acid occurring at a particular site relative to the likelihood of this amino acid occurring at any site (background frequency). Generally, PSIC score differences >2 indicate a damaging effect, scores between 1.5 and 2 suggest that the variant is possibly damaging, and scores <1.5 indicate that the variant is benign. Amino acid conservation analysis was performed with Clustalw2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/), retrieving the FASTA sequences from http://www.ncbi.nlm.nih.gov. The effect of the mutations on tRNA secondary structure was estimated using the RNA fold server (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi) and the tRNAscan-SE Search Server (http://lowelab.ucsc.edu/tRNAscan-SE). RNA fold predicts the stability of the RNA, considering the minimal free energy (∆G), whereas tRNAscan considers the cloverleaf structural constraints which do not imply that the structure is the most energetically stable. Nucleotide and amino acid variability data for known mutations were retrieved from the Hmtdb database (www.hmtdb.uniba.it), where the human mtDNA site-specific variability is estimated and annotated based on 8300 and 1500 deposited mitochondrial genomes from ‘normal’ individuals and ‘disease’ samples, respectively.

Haplogroup prediction

The haplogroup prediction has been performed by applying the ‘classify your genome’ option available within Hmtdb (31). The comparison of each complete mtDNA sequence, based on mutational events along the human mtDNA phylogeny, has been carried out by taking into account the ‘Coper- nican’ reassessment (68), and using as reference the Reconstructed Sapiens Reference Sequence (RSRS) implemented in Phylotree (http://www.phylotree.org) (69), rather than the revised Cambridge Reference Sequence (rCRS).

Fluorescent PCR

PCR reactions were performed using AmpliTaq Gold polymerase (Applied Biosystems) in the presence of a primer with a 5’ fluorescent tag and analysed as previously described (29). Primers were designed following the instructions for preventing nuclear mitochondrial sequence (NumtS) co-amplification. Primer sequences and amplification conditions are detailed in Supplementary Material.

Allele-specific locked nucleic acid real-time PCR

PCR reactions were performed using SYBR Green chemistry (Applied Biosystems) in 15 μl final reaction volume, with 0.4 μM of each primer, one of which was allele specific for the mutation and labelled with an LNA base on its 3’ end (Exiqon, Milan, Italy). Germ-line samples were run together with known standards (stock, 1:10, 1:100, 1:1000 and 1:10 000), following previously described protocol (70). Primers were designed following the instructions for preventing NumtS co-amplification. Primer sequences and amplification conditions are listed in Supplementary Material.

aCGH analysis

The aCGH platform was constructed at the Breakthrough Breast Cancer Research Centre and comprises approximately 32 000 BAC clones tiled across the genome. This type of BAC array platform has been shown to be suitable for the analysis of DNA samples extracted from FFPE tissue; it has a resolution comparable with that of high-density oligonucleotide arrays (71,72). Labelling, hybridization, washes, image acquisition and aCGH data analysis were carried out as previously described (65,73). Low-level gain was defined as a circular binary segmentation (CBS)-smoothed log2 ratio of between 0.12 and 0.45, corresponding to approximately three to five copies of the locus, whereas gain amplification was defined as having a log2 ratio of >0.45, corresponding to more than five copies. Hierarchical cluster analysis was performed as previously described (41). Briefly, CBS-smoothed log2 ratio values and categorical data were used for clustering, considering Euclidean distance and using Ward’s clustering algorithm. To identify statistically significant differences between the genomic profiles of oncocytomas versus non-oncocytic pituitary tumour 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. Statistical power to address the hypothesis that oncocytomas versus non-oncocytic pituitary adenomas would be distinct entities at the genomic level was calculated by applying Walters normal approximation, which showed that with n = 17 and α = 0.05 we were able to detect differences between 5 and 50% with the power of 0.783.
aCGH data on copy number changes affecting sex chromosomes were excluded from the final analysis given that female DNA was used as a reference for both male and female cases.

**MSI evaluation**

The presence of MSI was evaluated by the amplification of the mononucleotide repeat markers BAT26 and BAT40 as previously described (74). Briefly, samples were amplified using AmpliTaq polymerase (Applied Biosystems) and primers labelled with a fluorescent tag on their 5’ end. The products were loaded onto 3730 DNA Analyzer (Applied Biosystems) together with GeneScan 500 Liz Size Standard (Applied Biosystems) and analysed using GeneMapper v.3.5 (Applied Biosystems). Tumours were considered MSI-high (MSI-H) when both BAT26 and BAT40 displayed MSI, defined by multiple extra bands above the background. As a control, a blood DNA sample either from the corresponding patient or from a healthy female donor was run in parallel. The microsatellite marker BAT26 has been shown to be reliable in determining the MSI-H phenotype without the requirement for matching normal DNA given that it is ‘quasi-monomorphic’ in DNA from normal individuals or microsatellite-stable tumours compared with MSI-H cancers (74,75).

**TP53 and GNAS mutational screening**

The entire TP53 coding region (transcript ENST00000269305, exons 2–11) was amplified using K2G(2×) polymerase (Kapabiosystems, Rome, Italy) in 10 μl final reaction volume with 0.2 μM of each primer and subsequent thermocycler conditions: 3 h hold at 94°C; 10 cycles at 94°C for 10 s, 62–57°C for 10 s (touch-down protocol with Δ-0.5°C at each cycle), 72°C for 1 s; 25 cycles at 94°C for 10 s, 57°C for 10 s, 72°C for 1 s; 30 s hold at 72°C. Primers are listed in Supplementary Material. GNAS mutation hotspots (transcript ENST00000371100, exons 7–9) were analysed using K2G(2×) polymerase (Kapabiosystems) in 10 μl final reaction volume, with 0.2 μM of each primer (35) and following thermocycler conditions: 3 h hold at 94°C; 30 cycles at 94°C for 10 s, 62°C for 10 s, 72°C for 1 s. Direct sequencing of the PCR amplicons was performed using BigDye v1.1 (Applied Biosystems), following manufacturers’ instructions on a 3730 DNA Analyser (Applied Biosystems). Data were analysed using Sequencher v.4.7 (Gene Codes, Ann Arbor, MI, USA).

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at HMG online.

**Conflict of Interest statement.** None declared.

**FUNDING**

This work was supported by the Associazione Italiana Ricerca sul Cancro - AIRC (IG8810); by the Italian Ministry of University MIUR - Futuro in Ricerca 2008 (J31J10000040001) and Fondazione Umberto Veronesi - DISCO TRIP project to G.G.; by the Breakthrough Breast Cancer Research Centre (A.M., M.B.K.L. and J.S.R.-F.) and by ‘Fondazione della Cassa di Risparmio di Puglia’ to M.A.; I.K. is supported by a triennial fellowship ‘Borromeo’ from AIRC. A.M., M.B.K.L. and J.S.R.-F. acknowledge the NHS support to the NIHR biomedical research centre.

**REFERENCES**

31. Rubino, F., Piredda, R., Calabrese, F.M., Simone, D., Lang, M.,
30. Morandi, L., de Biase, D., Visani, M., Cesari, V., De Maglio, G.,
25. Pradella, L.M., Zuntini, R., Magini, P., Ceccarelli, C., Neri, I., Cerasoli,
26. Alston, C.L., Morak, M., Reid, C., Hargreaves, I.P., Pope, S.A., Land,
23. He, Y., Wu, J., Dressman, D.C., Iacobuzio-Donahue, C., Markowitz, S.D.,
20. Dasgupta, S., Koch, R., Westra, W.H., Califano, J.A., Ha, P.K., Sidransky,
22. Tseng, L.M., Yin, P.H., Chang, C.J., Li, A.F., Chi, C.W., Wei,
19. Rubino, F., Piredda, R., Calabrese, F.M., Simone, D., Lang, M.,
20. Dasgupta, S., Koch, R., Westra, W.H., Califano, J.A., Ha, P.K., Sidransky,
23. He, Y., Wu, J., Dressman, D.C., Iacobuzio-Donahue, C., Markowitz, S.D.,