***ATRX* driver mutation in a composite malignant pheochromocytoma**

Iñaki Comino-Méndez [1], Águeda M Tejera[2], María Currás-Freixes[1], Pablo Gonzalvo[3], Raúl Tonda[4,5], Rocío Letón[1], María A Blasco[2], Mercedes Robledo[1,6], and Alberto Cascón[1,6]*

[1]Hereditary Endocrine Cancer Group, and [2]Telomeres and Telomerase Group, Spanish National Cancer Research Centre (CNIO), Madrid, Spain.

[3]Department of Anatomical Pathology, Cabueñes Hospital, Gijón, Spain.

[4]CNAG-CRG, Centre for Genomic Regulation (CRG), Barcelona Institute of Science and Technology (BIST), Barcelona, Spain.

[5]Universitat Pompeu Fabra (UPF), Barcelona, Spain

[6]Centro de Investigación Biomédica en Red de Enfermedades Raras (CIBERER), Madrid, Spain

*Address correspondence to: Alberto Cascón

Hereditary Endocrine Cancer Group, Human Cancer Genetics Programme, Spanish National Cancer Research Centre (CNIO), Melchor Fernández Almagro 3, 28029 Madrid, Spain

Phone: +34 91 224 69 47; Fax: +34 91 224 69 23

e-mail: acascon@cnio.es

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ABSTRACT

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are tumors arising from the adrenal medulla and sympathetic/parasympathetic paraganglia, respectively. Approximately 40% of PCCs/PGLs are due to germline mutations in one of 16 susceptibility genes, and a further 30% are due to somatic alterations in 5 main genes. Recently, somatic *ATRX* mutations have been found in succinate dehydrogenase (SDH)-associated hereditary PCCs/PGLs. In the present study we applied whole-exome sequencing to the germline and tumor DNA of a patient with metastatic composite PCC and no alterations in known PCC/PGL susceptibility genes. Transcriptome, methylome, and copy number variations, and alternative lengthening of telomeres (ALT), were studied in the tumor. A somatic loss-of-function mutation affecting *ATRX* was identified in tumor DNA. Transcriptional profiling analysis classified the tumor within cluster 2 of PCCs/PGLs (without SDH gene mutations), and identified downregulation of genes involved in neuronal development and homeostasis (*NLGN4, CD99* and *CSF2RA*). CpG island methylator phenotype typical of SDH gene-mutated tumors was ruled out, and SNP array data revealed a particular profile of gains and losses. Finally, we demonstrated the presence of ALT in the tumor, probably associated with the failure of ATRX functions. In conclusion, somatic variants affecting *ATRX* may play a driver role in sporadic PCC/PGL.

Key words: pheochromocytoma, *ATRX*, exome sequencing, ALT

SHORT REPORT

Pheochromocytomas (PCCs) and paragangliomas (PGLs) are neural crest-derived catecholamine-secreting tumors arising from the adrenal medulla and from sympathetic/parasympathetic paraganglia, respectively. While initially it was thought that only 10% of cases were caused by germline mutations, the discovery of mutations in several additional susceptibility genes during the last fifteen years has brought the percentage of hereditary cases up to approximately 40%. These mutations affect 16 genes: *VHL*, *RET*, *NF1*, *SDHA*, *SDHB*, *SDHC*, *SDHD*, *SDHAF2*, *MEN1*, *KIF1β*, *EGLN1, EGLN2*, *TMEM127*, *MAX*, *FH* and *MDH2*[1-3]. On the other hand, the presence of somatic events in PCC/PGL has been largely uncharacterized and underestimated because of the lack of studies based on tumoral DNA. Nowadays, it is widely accepted that some of the genes involved in hereditary PCC/PGL also play a role in sporadic disease (especially *NF1*, *VHL* and *RET*), while mutations in *EPAS1*, *HRAS*, *ATRX*, *BRAF* and *TP53* occur in non-hereditary tumors[1,4,5]. Thus, somatic mutations can be detected in an additional 25-30% of tumors[1]. Of note, though PCC/PGL patients rarely develop distant metastases, those that do are usually associated with the presence of *SDHB* germline mutations, with 5-year overall survival less than 60%[6].

We used germline and somatic whole-exome sequencing (WES) to investigate the genetic etiology for a 66 year-old male who developed a very large malignant composite adrenal tumor, with PCC (90%), ganglioneuroma and ganglioneuroblastoma (10%) components (**figure 1A and B**) and liver metastasis, but no family history of the disease. No germline mutations in any of the major PCC/PGL susceptibility genes had been found. Given the apparently sporadic presentation of the case, we focused our interest on the somatic variations. Somatic variants in 12 genes passed the filtering process (**supplementary table 1**). Three of these (*FSHB*, *DIDO1*, and *ATRX*) were known to be involved in cancer and only *ATRX* had been previously associated with PCC

development. The heterozygous mutation found in *ATRX* was a loss-of-function (LOF) variant, p.E481*, which was subsequently confirmed by Sanger sequencing (**figure 1C**).

ATRX is a large gene located in the X chromosome and is a member of the SWI/SNF family of chromatin remodelers, which play a role in telomere maintenance, chromosome integrity, transcriptional regulation and cancer[7]. In collaboration with death-domain associated protein DAXX, ATRX has an important role in histone regulation by facilitating the incorporation of the histone variant H3.3 into telomeric and pericentromeric chromatin[8]. In addition, ATRX has been described to play a role in the regulation of a range of genes located across the entire genome[9]. Germline *ATRX* alterations cause X-linked alpha thalassemia mental retardation syndrome[10], and somatic mutations in *ATRX* have been found in several non-epithelial tumors such as primitive neuroectodermal tumors, neuroblastomas, gliomas, and very recently, PCC/PGL[5]. Importantly, *ATRX* somatic mutations in gliomas and PCC/PGL have been linked to the presence of alterations in genes encoding Krebs cycle enzymes such as isocitrate dehydrogenase (IDH) 1 and 2 and the succinate dehydrogenase (SDH) genes[5,11], respectively. The role of these somatic *ATRX* mutations is unclear: while defective ATRX has been associated with poor prognosis in several cancers, a better outcome has been well defined in gliomas carrying *IDH1/2-ATRX* mutations[12]. We assessed SDHB expression in the tumor and observed positive immunostaining further confirming that there were no alterations in the SDH genes (**figure 1D**).

Several studies based on genome-wide expression profiling of large series of PCCs/PGLs have described the segregation of tumors into two well-differentiated clusters depending on the genetic alteration they carry[1]. Thus, tumors harboring alterations in Krebs cycle enzymes grouped together in the so-called pseudohypoxic cluster. When we compared the expression profile of the *ATRX*-mutated tumor with those for 83 tumors carrying different somatic or germline alterations (GEO accession: GSE19422), it did not exhibit the characteristic pseudo-

hypoxic transcriptional signature of SDH/FH/MDH2-mutated tumors, which, once again, is consistent with the absence of alterations in Krebs cycle enzymes. Further, we found that the *ATRX*-mutated tumor showed the lowest *ATRX* mRNA expression amongst all tumors analyzed (**figure 1E**), which suggests both the presence of mRNA decay caused by the mutation and the absence of *ATRX* truncating mutations amongst the 83 PCCs/PGLs used as controls. Several studies have reported the role of ATRX in the expression of imprinted regions such as H19/Igf2 and Dlk1/Gtl2, through the maintenance of nucleosome configuration and binding to CTCF factors[9]. Consistent with this latter, the *ATRX*-mutated tumor showed a significant down-regulation of the *DLK1* gene, compared to *ATRX* wild-type PCCs/PGLs (data not shown). This finding is especially important given the correlation of loss of *DLK1-DIO* imprinted locus expression with reduction of the neuronal differentiation in human embryonic stem cells[13]. Moreover, *ATRX*-null mouse cells present with down-regulation of Neuroligin 4 (*Nlgn4*), a very important gene involved in neuronal homeostasis, as well as aPAR genes such as *Dhrsx*, *Asmtl*, *Cd99* and *Csf2ra*[14]. Our data revealed statistically significant downregulation of *NLGN4, CD99* and *CSF2RA* in the *ATRX*-mutated tumor. These findings provide evidence of the lack of expression of important genes directly involved in the development of the central nervous system due to the loss of ATRX functions. Of note, genome-wide overexpression was observed in the *ATRX*-mutated compared to non-mutated tumors (data not shown), further corroborating that ATRX plays an important role in gene regulation. In addition, the analysis of the methylome has recently appeared as another useful method for clustering PCCs/PGLs with respect to their underlying genetic background[15]. Based on the methylome profiling of a large series of PCC/PGL previously assessed by our group[16] (GEO accession: GSE62231), we observed that the *ATRX*-mutated tumor clustered together with tumors presenting low levels of methylation, and not with highly methylated tumors carrying *FH*, *MDH2* or SDH gene mutations[3,15]. Thus, taking into consideration the high reliability of omics-based clustering of PCCs/PGLs, we concluded

that, unlike previous *ATRX*-mutated tumors, the one described herein does not carry any alteration in the SDH genes.

We next characterized the copy number variations occurring in the *ATRX*-mutated tumor, taking advantage of the zygosity of the variants detected by WES in the tumor compared to blood, determined using control-FREEC software and makeGraph.R viewer[17]. Losses affecting chromosomes 1p and 11p, which are frequently found in PCC/PGL, were detected in the *ATRX*-mutated tumor (**figure 2A**). Moreover, other less frequent alterations in these tumors, such as the gain of chromosome 5 and partial gains of chromosomes 11p and 8q, were also detected. Loss of regions where PCC/PGL susceptibility genes are located, (deletion of 1p, harboring the susceptibility genes *SDHB* and *KIF1β*) are known somatic events that provoke partial loss of expression of the corresponding susceptibility gene. In addition, specific alterations such as the partial gain of chromosome 2 found in *EPAS1*-mutated tumors[18], have been reported as reliable somatic markers. The infrequent alterations found in the *ATRX*-mutated tumor could be useful markers to detect tumors carrying alterations in this very large gene, but this requires confirmation in independent studies.

Recent studies have reported associations in tumor tissue between *ATRX* mutations and the presence of alternative lengthening of telomeres (ALT)[19,20]. ALT is a mechanism that uses homologous recombination to maintain telomere length and sustain limitless replicability of cancer cells. ALT is observed in ~5% of all cancers, being more prevalent in osteosarcomas, soft tissue sarcoma subtypes and glial brain tumors[21]. To investigate the presence of ALT in the PCC carrying the *ATRX* LOF variant, we performed a telomere-specific fluorescence in situ hybridization (FISH) assay, as previously described[22]. Ultra-bright signals were detected significantly more frequently in the tumor compared to the adjacent normal tissue (**figure 2B**), with longer telomeres appearing in the tumor area. The presence of ALT-associated ultra-bright signals was ruled out in PCCs/PGLs carrying other known mutations including SDH-mutated

tumors. Thus, as occurred with *SDHB*-mutated PCCs carrying *ATRX* alterations[5], the presence of the mutation is linked to ALT, leading to indefinite cell division and tumor progression.

Finally, the patient reported herein developed distant metastasis and, unlike previous cases with *ATRX* mutations coexisting with known driver mutations in other susceptibility genes[5], the *ATRX* mutation could be the causal event in this case. Taking into consideration the very limited therapeutic options for metastatic PCC/PGL and the recently described sensitivity of ALT-cancer cells to ATR inhibitors[23], this finding opens an interesting new therapeutic option to patients carrying *ATRX* mutations.

Figure legends

Figure 1. (**A**) Computerized axial tomography image showing a solid tumor of size 20x17x15cm within the left abdominal cavity of the left kidney. (**B**) Immunohistochemical staining for S100 protein, revealing both the pheochromocytoma (negative staining) and the ganglioneuroma (positive) component of the composite tumor. (**C**) Sequence including the *ATRX* mutation (c.1441G>T) in the tumoral DNA from the patient compared to the wild-type sequence from blood. (**D**) Positive SDHB immunohistochemical staining of the *ATRX*-mutated tumor. (**E**) mRNA expression of *ATRX* (median value of A_24_P348660 and A_23_P136874 probes from the Agilent Whole Human Genome platform 4x44) in the tumor carrying the c.1441G>T mutation and in 83 PCCs/PGLs (GEO accession: GSE19422) used as controls. The horizontal black line represents the median.

Figure 2. **(A)** MakeGraph.R representation, of control-FREEC analysis of WES data from tumoral and blood DNA from the patient carrying the *ATRX* mutation, showing tumoral regions of copy number gain (shown in red) and loss (shown in blue). (**B**) Telomere FISH shows large ultra bright telomere signals indicative of alternative lengthening of telomeres (denoted by the yellow arrowheads) in the *ATRX*-mutated tumor cells (lower panel) compared to adjacent normal cells (upper panel). Scale bars, 25 µm. (**C**) Distribution of telomere spot size as determined by quantitative-FISH in tumor and adjacent non-tumor areas showing an increase of telomere signal size in tumor cells. Mean is indicated in red.

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Contributors

The project was conceived by AC, MR and MAB. Samples and clinical information were collected by MCF and PG. Whole-exome sequencing data analysis and filtering was performed by RT, ICM and AC. Genome expression profiling was performed by AC and ICM and analysis of gains and losses was performed by AC, ICM and RT. Fluorescence in situ hybridization assay was performed by AMT. Additional experiments and data analysis were performed by ICM, AC, RL and MCF. The manuscript was written and revised by AC, ICM, MR, MAB and AMT. All authors approved the final version.

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Patient consent

Obtained.

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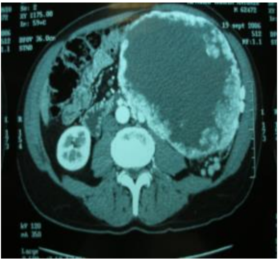
Figure 1

A



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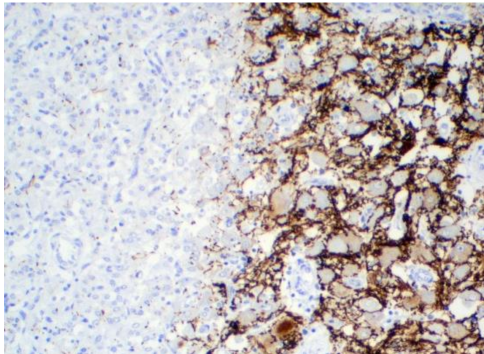

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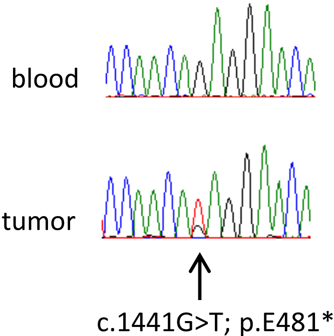

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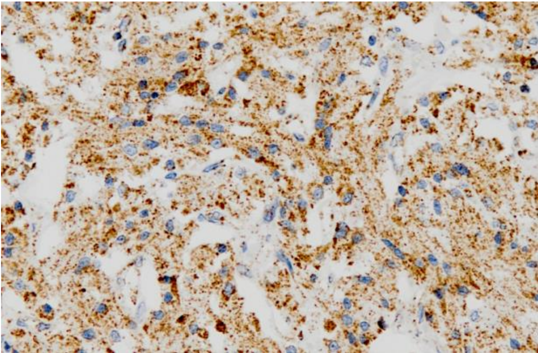

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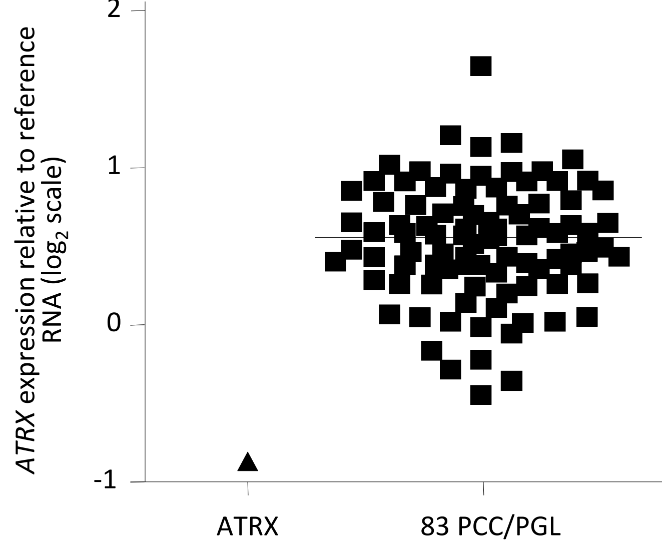

Figure 2

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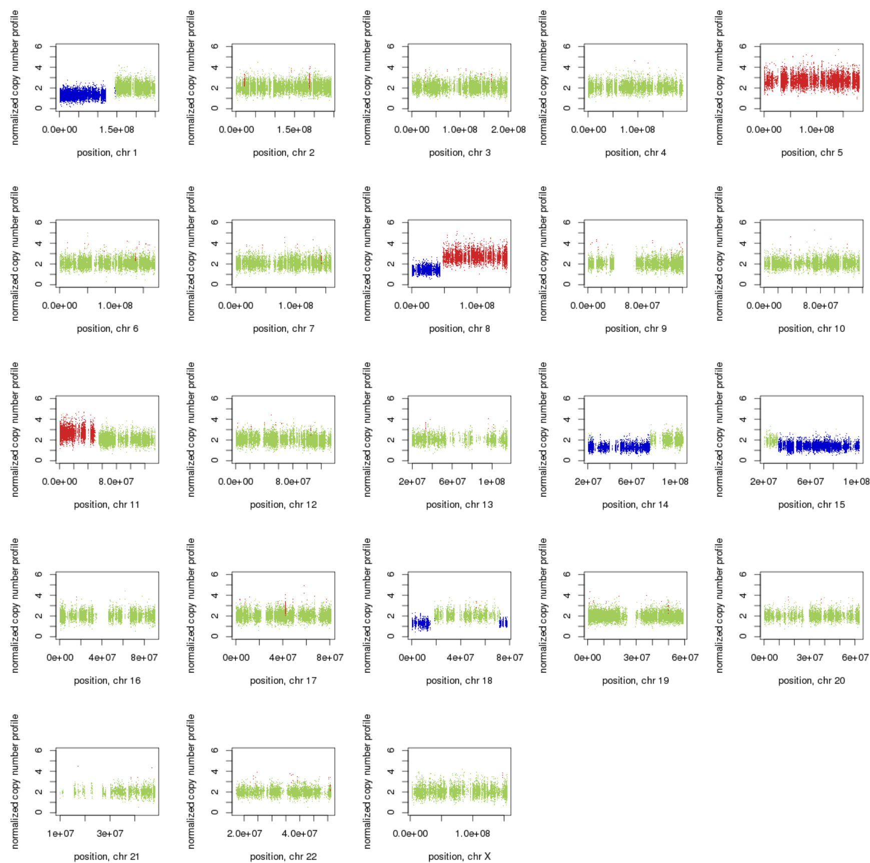

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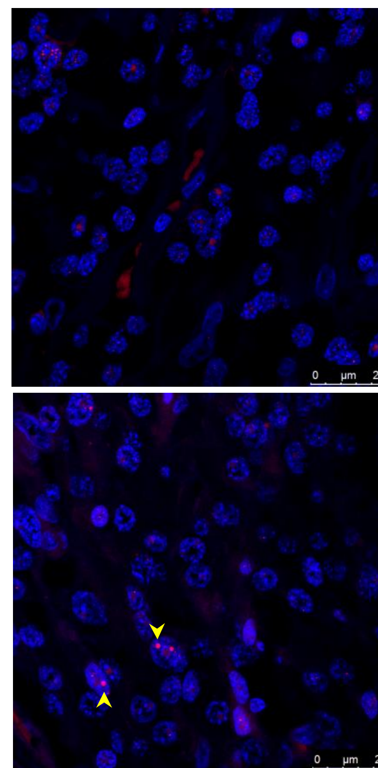

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