Early Detection of Relapse by ctDNA Sequencing in a Patient with Metastatic Thymic Tumor and MEN1 Mosaicism

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Arnaud Lagarde[1*], Lauriane Le Collen[2,3*], Camille Boulagnon[4,5], Hedia Brixi[6], Anne Durlach[4,7], Grégory Mougel[1], Brigitte Delemer[2], Anne Barlier[1£], Pauline Romanet[1£]

[1] Aix Marseille Univ, APHM, INSERM, MMG, Laboratory of Molecular Biology Hospital La Conception, Marseille, France.

[2] Department of Endocrinology Diabetology, University of Reims, Reims, France.

[3]Inserm/CNRS UMR 1283/8199, Institut Pasteur de Lille, EGID, Lille, France

[4] Biopathology laboratory, Pôle de Biologie Territorial, CHU de Reims, 51092 Reims, France

[5] Université de Reims Champagne Ardenne (URCA), CNRS, UMR 7369 MEDyC, Reims, France.

[6] Department of Gastroenterology and Digestive Oncology, Reims University Hospital, Reims, France.

[7] Inserm UMR-S 1250 51092 REIMS Cedex - france.

* these authors contributed equally to this work

[£] these authors contributed equally to this work

Correspondence:
Dr Pauline ROMANET, MD, PhD
Aix Marseille Univ, INSERM, MMG, UMR 1251
Faculté des sciences médicales et paramédicales,
27, boulevard Jean Moulin 13385 Marseille cedex 5, France.
Tel +33 491 69 87 89
Fax +33 491 69 89 20
pauline.romanet@univ-amu.fr
0000-0003-1775-9569

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Abstract:

Context: Multiple endocrine neoplasia type 1 (MEN1) is an autosomal dominant disease due to inactivating mutations in the *MEN1* gene. In the literature, few cases of MEN1 have been reported due to mosaic *MEN1* mutations. Objective: We performed an extensive molecular characterization in several lesions and blood samples, including plasmatic circulating cell-free DNA (ccfDNA) in an exceptional case of a patient with MEN1 mosaicism causing primary hyperparathyroidism, multiple pancreatic neuroendocrine tumors (NET), and a metastatic thymic NET.

Design: Blood, ccfDNA and multiple tissue analysis were performed by next generation sequencing.

Results: *MEN1* mosaicism was confirmed by the multiple tissues analysis. The somatic analysis of the largest pancreatic NET revealed the same *MEN1* second-hit mutation as found in thymic lesion, demonstrating its metastatic origin from thymic lesion. Moreover, in ccfDNA we found the mosaic *MEN1* mutation but also the somatic second-hit mutation found in the thymic primary tumor, revealing the presence of circulating tumor DNA (ctDNA). After surgical removal of the pancreatic metastasis, the mutated fraction of both mutations decreased, before increasing again several weeks before a new clinical relapse, suggesting that thymic ctDNA may be used as an early tumor biomarker.

Conclusion: This exceptional MEN1 case highlighted (1) the importance of looking for *MEN1* mosaicism (2) that *MEN1* mosaicism can cause very aggressive disease, (3) the interest in analyzing ccfDNA for confirming *MEN1* mosaicism but also a potential tumor biomarker for NET.

Key words: thymic neuroendocrine tumor, circulating cell-free DNA, ccfDNA, mosaic mutation, circulating tumor DNA, biomarker, genetic counselling

ARTICLE

Introduction:

Multiple endocrine neoplasia type 1 (MEN1) is a rare disease caused by heterozygous inactivating mutations in *MEN1*(1). MEN1 is characterized by a broad spectrum of clinical manifestations of which the three cardinal lesions are primary hyperparathyroidism (PHPT), pituitary neuroendocrine tumors (PitNET) and neuroendocrine duodeno-pancreatic tumors (DPNET)(2). Other neuro-endocrine tumors (NET) such as adrenal cortical tumors, bronchopulmonary NETs or thymic NETs may also be associated. Twenty-eight to 70% of patients with MEN1 die as a consequence of the disease, particularly as a result of pancreatic and carcinoid lesions (3). The diagnosis of MEN1 allows patients to benefit from a multidisciplinary follow-up program, based on imagery, biochemical testing and physical examinations, to facilitate early detection and treatment of lesions (4). Relatively few cases of *MEN1* mosaicism have been reported in the literature (5–9). Mosaicism is due to the occurrence of a mutation during postzygotic development, after fertilization. We describe here the exceptional case of a man presenting with MEN1 due to mosaicism and the subsequent molecular characterization of the disease in several lesions and samples, including circulating cell-free DNA (ccfDNA).

Case Report

A 43-year-old patient presented with cervicobrachial neuralgia, revealing a thymic mass on CT scan. The diagnosis of MEN1 was suspected because of its association with a concomitant primary hyperparathyroidism (See timeline in Figure 1A). The initial biochemical evaluation found an increased level of parathyroid hormone of 235 pg/mL (normal: 12-65), hypercalcemia of 3 mmol/L (normal: 2.10-2.55), with a slightly increased plasma chromogranin A level and normal pituitary function. The patient underwent thymic resection after control of hypercalcemia with calcimimetics. Histopathology on the thymic tumor showed a well-differentiated grade 3 NET with vascular and lymphatic emboli. The tumor had a Ki67 proliferation index of 30%. Immunolabelling with CDX2, ISL1, TTF1, PAX8 and GATA3 antibodies were all negative. Thereafter surgery on two of the hyperplastic parathyroid glands was performed (located at each lower extremity of the thyroid lobes) as

the other two glands were not localized during the procedure. Fortunately, plasma calcium was normalized, and the patient has not presented any recurrence of hyperparathyroidism to date.

Four months later, the patient developed lymph node recurrence of the thymic cancer and first line chemotherapy, using 5 FU-Oxaliplatin was commenced (Figure 1A). This treatment was effective at the beginning of his disease, allowing alternating chemotherapy and therapeutic pause until the age of 47. Unfortunately, the disease evolution was marked by recurrence of metastasis in the mediastinal area. Radiotherapy of the cervico-mediastinal region was then performed, which stabilized the disease for several years.

Bone metastases were discovered at the age of 51 years in the sternum, T10, L1 and S1, and in the left acetabulum, justifying the resumption of new lines of chemotherapy. In addition, mediastinal nodes with subcarinal and paraaortic metastases developed, which were treated locally with radiotherapy.

At 53 years, a 3 cm caudal pancreatic mass was discovered via CT scan monitoring and which appeared in only four months. Half a dozen hypervascular tissue lesions in the head, body, and tail of the pancreas were then detected on magnetic resonance imaging (MRI), without associated liver metastases. A pancreatic biopsy of the largest lesion (longest axis 6.5 cm) was performed and suggested the presence of a NET. A left splenopancreatectomy, removing the largest lesion, was then performed after at least two episodes of pancreatitis at the age of 55 years. Histological examination confirmed the presence of a grade 3 well-differentiated pancreatic NET. This tumor had a Ki67 proliferation index of 25% and had metastasized to lymph nodes. As with the thymic NET, immunohistochemistry for CDX2, ISL1, PAX8, TTF1 and GATA3 were all negative in the pancreatic NET (Figure 2).

Four months after splenopancreatectomy, the patient relapsed, presenting a dysphonia secondary to a recurrent paralysis. The latter was caused by a left mediastinal lymph node recurrence of his thymic tumor. The patient is currently undergoing repeat chemotherapy for this recurrence.

Genetic investigations:

The initial diagnosis of MEN1 was made on clinical grounds. Ten years later, at the age of 54 years, *MEN1* was sequenced using next generation sequencing (NGS). This analysis

revealed a mosaic variant in exon 3 of *MEN1* (NM_130799): c.496=/C>T, p.(Gln166=/*) already known to be pathogenic (10). For clarity, we will refer to this variation as "MOSAIC variant" in this paper. The mutated allelic frequency (AF) of the MOSAIC variant on the first whole blood sample was 9.6%. The mutation was not found in the left parathyroid gland but was found in the thymus, the right parathyroid, and the pancreatic NET, confirming *MEN1* mosaicism in this patient. In somatic tissues we identified *MEN1* second hits (Figure 1B). In the right parathyroid tumor, the second hit was a loss of the normal allele, leading to a loss of heterozygosity. In the thymic NET, the second hit was a well-known *MEN1* pathogenic variant in intron 4: c.784-9G>A, which results in altered splicing (11). For clarity, we will refer to this variation as: "THYMIC variant" in this paper. Surprisingly, we found the THYMIC variant in the largest pancreatic NET. The same 2nd hit being found in both lesions, the size and the progression of this pancreatic lesion, and the identical immunohistochemistry staining, strongly suggested that this pancreatic NET was in fact a metastasis from the thymic NET, which appeared 10 years after discovery of the primary site.

The MOSAIC variant was also found in circulating cell-free DNA (ccfDNA), i.e. fragmented DNA released into the circulation by the destruction of normal body cells and tumor cells and extracted from plasma (12). We examined 3 plasma samples before and after the splenopancreatectomy for the presence of the MOSAIC variant during follow-up. In the first plasma sample, taken one month before the splenopancreatectomy, the mutated AF of the MOSAIC variant was 16.2% in ccfDNA (compared to 10.5% in DNA extracted from whole blood collected on the same day, Figure 1B). Surprisingly, we also detected the THYMIC variant in ccfDNA. The AF of the THYMIC variant was 6.5% in ccfDNA, while the THYMIC variant was absent from whole blood DNA. One month after the splenopancreatectomy, the AFs of both the MOSAIC and THYMIC variants decreased in the ccfDNA: from 16.2% to 11.8% for the MOSAIC variant, and from 6.5% to 2.6% for the THYMIC variant (*versus* 10.6% for the MOSAIC variant in the whole blood DNA sample on the same day, Figure 1B). Two months after splenopancreatectomy, the AFs increased again: from 11.8% to 19.5% for the MOSAIC variant, and from 2.6% to 13.9% for the THYMIC variant (*versus* 11.3% for the MOSAIC variant in whole blood DNA on the same day, figure 1B). At the same time, the ccfDNA concentration decreased from 14.8 ng/mL before surgery, to 6.8 ng/mL one month after surgery, and increased again to 14.1 ng/mL two months after surgery (Figure 1B).

DISCUSSION

We report here the case of a patient with aggressive clinical features of MEN1 due to *MEN1* mosaicism. In the case of mosaicism, only some cells in the organism harbor the variation. Here we detected a pathogenic *MEN1* mosaic variant at an AF of 9.6% in blood. The presence of somatic cells harboring this variant excludes a false-positive result. The patient initially presented with hyperparathyroidism and a thymic NET, then later developed multiple pancreatic NETs, including one tumor that was larger than the others. Thymic NETs are rare but represent very aggressive MEN1 lesions. The prevalence of thymic NETs in MEN1 cohorts has been reported as between 2% and 3.4% (13–16). The probability of their occurrence is estimated at 2.6% (range, 1.3-5.5%) at the age of 40 years (16). The median age at diagnosis is approximately 40 years and the overall 10-year survival rate is around 25 to 45% (15,16). Thymic NETs can be the first manifestation of the MEN1 disease (16). In our patient, the occurrence of the thymic NET coincided with the occurrence of PHPT, supporting the diagnosis of MEN1 disease and the initiation of the MEN1 follow-up program (4).

MEN1 is an autosomal dominant disease however the tumor suppressor gene is recessive at the somatic level. According to Knudson's theory, a somatic second hit, inactivating the single functional allele, is necessary for development of tumors. In multiorgan diseases, the second hits are specific to each tumor localization, and can consist of various events, ranging from single nucleotide variants to complete chromosomal deletion, leading to a loss of heterozygosity. Here, a second hit was detected in all tumors except in the left parathyroid, suggesting that this tissue was in fact healthy. In the thymic NET and the largest pancreatic NET, the same second hit was present, strongly suggesting that this pancreatic NET was in fact a distant metastasis of the thymic NET. Indeed, this tumor was much larger than the other pancreatic NETs, grew very rapidly, in less than four months, while the other tumors were stable. Pancreatic metastasis of a thymic NET has been previously described in the literature (17). As found in the thymic NET, CDX2, ISL1, PAX8, TTF1 and GATA3 immunostaining was unexpectedly negative. Some thymic NETs have been shown to express PAX8(18). Pancreatic NETs classically express ISL1 and PAX8, but do not

express CDX2, GATA3(19–21). Nevertheless, none of these markers is specific for thymic NETs thus they just allow diagnosis of a NET.

To confirm the *MEN1* mosaicism, we analyzed ccfDNA using NGS and found the MOSAIC variant at a greater AF to that found in whole blood DNA (16.2% vs. 10.2%). Moreover, we detected the THYMIC variant, identified in both the thymus and the biggest pancreatic NET, at an AF of 6.5%, reflecting the presence of circulating tumor DNA (ctDNA) from the thymic NET or from its pancreatic or bone metastasis. The ctDNA AF of the THYMIC variant (6.5%) was approximately equal to the difference in the AFs of the MOSAIC variant between blood and ccfDNA (respectively 10.2% and 16.2%). This suggests that the fraction of MOSAIC variant in ccfDNA may be the result of the release of DNA from both normal and tumoral cells. After the splenopancreatectomy, the AFs of the MOSAIC and THYMIC variants in ccfDNA decreased to 11.8% (compared to 16.2%) and 2.6% (compared to 6.5%) respectively, suggesting that most of this ctDNA was released from the largest pancreatic NET.

More importantly, an increase in the AFs of MOSAIC and THYMIC variants was detected in ccfDNA two months after the splenopancreatectomy, that is one month before the onset of the relapse in mediastinum symptoms, showing that a thymic NET was growing. Therefore, as is the case in lung cancer, the ctDNA from thymic NETs or their metastases could be an early marker of relapse, preceding clinical symptoms or imaging data.

In conclusion we report here the case of a man with *MEN1* mosaicism bearing primary hyperparathyroidism, a metastatic thymic NET and pancreatic NETs. For the first time to our knowledge, we describe the presence of ctDNA from a MEN1 thymic lesion. Analysis of ccfDNA could be useful in the case of mosaicism to confirm molecular diagnosis but also may detect ctDNA, a potential early tumor marker.

Materials & Methods

Clinical data was collected retrospectively from medical records. Tissue samples from the parathyroid glands, thymic tumors and pancreatic tumors were collected and processed for routine histopathology and immunohistochemistry after formalin fixation. Immunohistochemical analyses were performed on 4µm formalin-fixed paraffin-embedded

(FFPE) sections using anti-AE1/AE3, Chromogranin A, Synaptophysin, CDX2, TTF1, ISL1, PAX8 and GATA3 antibodies, on a Ventana Benchmark XT (Ventana Medical Systems) automated immunostainer. Details on the species, clone, dilution, manufacturers and references are given in table S1 (22). Genetic testing was performed after the patient had given informed consent. Genomic DNA was analyzed by NGS as previously described (23). FFPE tissue DNA was extracted using the QIAamp DNA FFPE tissue kit (Qiagen), ccfDNA from plasma was collected in Cell-Free DNA BCT® tubes (Streck, USA) using the QIAamp Circulating Nucleic Acid Kit (Qiagen), and quantified using a Qubit Fluorometer (Thermo Fisher Scientific Inc., USA).

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Ethics declaration: Informed consent was obtained from the subject involved in the study. The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of Aix Marseille Univ (ref 2018-13-12-004, date of approval: 12/14/2018).

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Figure 1: Timeline of the clinical history and genetic findings in this patient.

A: Timeline of the clinical history of the patient. Clinical events are in bold and therapeutic treatment in italics. NET: neuroendocrine tumor, PHPT: primary hyperparathyroidism, Yrs: years.

B: Genetic data from somatic tissues and blood, and graphical representation of allelic frequencies of mutations in blood before and after the caudal splenectomy. ccfDNA: circulating cell-free DNA, WBC: white blood cell DNA, LOH: loss of heterozygosity.

Figure 2: Histological characteristics of the thymic NET and the largest pancreatic NET. NET: neuroendocrine tumor.

Histological characteristics of the well-differentiated thymic NET (left column) and the largest pancreatic NET (right column). Hematein-eosin-saffron stain (HES, magnificationx20): Tumor cell are monomorphous and arranged in small nests. Below, immunohistochemical characteristics are compared side by side for all markers with positive staining appearing brown. Both tumors showed the same immunohistochemical characteristics with AE1/AE3 positivity (magnification x10), Chromogranin A positivity (x10), Synaptophysin positivity (x10), CDX2 negativity (x10), TTF1 negativity (x10), ISL1 negativity (x10) and PAX8 negativity (absence of nuclear positivity, thus considered negative, x20). Details of the antibody used for immunohistochemistry (Antiboy, species, clone, dilution, catlog#, company): AE1/AE3, Mouse, AE1/AE3, 1:50, M3515, Agilent; CDX2,Rabbit, EPR2764Y, Ready-to-use (RTU), BRB028 Zytomed Systems; Chromogranin A, Mouse, LK2H10, RTU, BMS018, Zytomed Systems; GATA3, Mouse, L50-823, RTU, 390M-17, Cell Marque; ISL1, Mouse, 1H9, 1:500, ab86472, Abcam; PAX8, Rabbit, polyclonal, 1:50, 10336-1-AP, Proteintech; Synaptophysin, Rabbit, SP11, RTU, RM-9111-R7, Thermo fisher scientific; TTF1, Mouse, 8G7G3/1, 1:50, MS-699-RQ, Thermo fisher scientific.

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