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

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1 Early detection of relapse by ctDNA sequencing in a patient with
2 metastatic thymic tumor and *MEN1* mosaicism.

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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

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ARTICLE

66

67 **Introduction:**

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

83

84 **Case Report**

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

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

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

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

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

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

124

125 **Genetic investigations:**

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

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

142 The MOSAIC variant was also found in circulating cell-free DNA (ccfDNA), i.e.
143 fragmented DNA released into the circulation by the destruction of normal body cells and
144 tumor cells and extracted from plasma (12). We examined 3 plasma samples before and
145 after the splenopancreatectomy for the presence of the MOSAIC variant during follow-up. In
146 the first plasma sample, taken one month before the splenopancreatectomy, the mutated
147 AF of the MOSAIC variant was 16.2% in ccfDNA (compared to 10.5% in DNA extracted from
148 whole blood collected on the same day, Figure 1B). Surprisingly, we also detected the
149 THYMIC variant in ccfDNA. The AF of the THYMIC variant was 6.5% in ccfDNA, while the
150 THYMIC variant was absent from whole blood DNA. One month after the
151 splenopancreatectomy, the AFs of both the MOSAIC and THYMIC variants decreased in the
152 ccfDNA: from 16.2% to 11.8% for the MOSAIC variant, and from 6.5% to 2.6% for the THYMIC
153 variant (*versus* 10.6% for the MOSAIC variant in the whole blood DNA sample on the same
154 day, Figure 1B). Two months after splenopancreatectomy, the AFs increased again: from
155 11.8% to 19.5% for the MOSAIC variant, and from 2.6% to 13.9% for the THYMIC variant
156 (*versus* 11.3% for the MOSAIC variant in whole blood DNA on the same day, figure 1B). At
157 the same time, the ccfDNA concentration decreased from 14.8 ng/mL before surgery, to 6.8
158 ng/mL one month after surgery, and increased again to 14.1 ng/mL two months after
159 surgery (Figure 1B).

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163 DISCUSSION

164 We report here the case of a patient with aggressive clinical features of MEN1 due to
165 *MEN1* mosaicism. In the case of mosaicism, only some cells in the organism harbor the
166 variation. Here we detected a pathogenic *MEN1* mosaic variant at an AF of 9.6% in blood.
167 The presence of somatic cells harboring this variant excludes a false-positive result. The
168 patient initially presented with hyperparathyroidism and a thymic NET, then later developed
169 multiple pancreatic NETs, including one tumor that was larger than the others. Thymic NETs
170 are rare but represent very aggressive MEN1 lesions. The prevalence of thymic NETs in
171 MEN1 cohorts has been reported as between 2% and 3.4% (13–16). The probability of their
172 occurrence is estimated at 2.6% (range, 1.3-5.5%) at the age of 40 years (16). The median
173 age at diagnosis is approximately 40 years and the overall 10-year survival rate is around 25
174 to 45% (15,16). Thymic NETs can be the first manifestation of the MEN1 disease (16). In our
175 patient, the occurrence of the thymic NET coincided with the occurrence of PHPT,
176 supporting the diagnosis of MEN1 disease and the initiation of the MEN1 follow-up program
177 (4).

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

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

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

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

211

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

217

218

219 Materials & Methods

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

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

233

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241 **Ethics declaration:** Informed consent was obtained from the subject involved in the study.

242 The study was conducted according to the guidelines of the Declaration of Helsinki and
243 approved by the Ethics Committee of Aix Marseille Univ (ref 2018-13-12-004, date of
244 approval: 12/14/2018).

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326

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

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

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

334

335 **Figure 2: Histological characteristics of the thymic NET and the largest pancreatic NET.**

336 **NET: neuroendocrine tumor.**

337 Histological characteristics of the well-differentiated thymic NET (left column) and the
338 largest pancreatic NET (right column). Hematein-eosin-saffron stain (HES, magnificationx20):
339 Tumor cell are monomorphous and arranged in small nests. Below, immunohistochemical
340 characteristics are compared side by side for all markers with positive staining appearing
341 brown. Both tumors showed the same immunohistochemical characteristics with AE1/AE3
342 positivity (magnification x10), Chromogranin A positivity (x10), Synaptophysin positivity
343 (x10), CDX2 negativity (x10), TTF1 negativity (x10), ISL1 negativity (x10) and PAX8 negativity
344 (absence of nuclear positivity, thus considered negative, x20). Details of the antibody used
345 for immunohistochemistry (Antibody, species, clone, dilution, catalog#, company): AE1/AE3,
346 Mouse, AE1/AE3, 1:50, M3515, Agilent; CDX2,Rabbit, EPR2764Y, Ready-to-use (RTU),
347 BRB028 Zytomed Systems; Chromogranin A, Mouse, LK2H10, RTU, BMS018, Zytomed
348 Systems; GATA3, Mouse, L50-823, RTU, 390M-17, Cell Marque; ISL1, Mouse, 1H9, 1:500,
349 ab86472, Abcam; PAX8, Rabbit, polyclonal, 1:50, 10336-1-AP, Proteintech; Synaptophysin,
350 Rabbit, SP11, RTU, RM-9111-R7, Thermo fisher scientific; TTF1, Mouse, 8G7G3/1, 1:50, MS-
351 699-RQ, Thermo fisher scientific.

