Supplementary material

Materials and Methods

The patient signed consent forms to be registered in the neuroendocrine neoplasm database of the Hospices Civils de Lyon (approved by the national data protection commission (Commission nationale de l'informatique et des libertés, CNIL) on the 6th November 2015 (n°15-111), and for the utilization of its tumour samples by the tissue bank ‘Tumorothéque des Hospices Civils de Lyon’, recognized by the French Ministry of Health (DC 2008–2072 and AC 2008–2073). Blood samples were collected as part of the CIRCAN program, a prospective translational program for the evaluation of tumour biomarkers in liquid biopsy.

Biochemistry

All biochemistry biomarkers were analysed at the Multi-Site Medical Biology Laboratory of the Hospices Civils de Lyon. The determination of UFC was carried out using immunochemiluminescence technique kit (Architect C16000, Abbott), ACTH assay was performed using immunometric technique kit (Cobas, Roche). LDH (ref 2P56-21) and ALP (ref 7D55-21) were carried out using spectrophotometric technique kits (Architect C16000, Abbott).

cfDNA molecular profile

Plasma was prepared from 20-30 mL of blood collected in K2 EDTA tubes (BD, 367525, 18 mg). All blood samples were delivered to the laboratory within 5 hours after collection. cfDNA was extracted from 4 mL of plasma using the QIAamp Circulating Nucleic Acid Kit (Qiagen, Cat No 55114, Valencia, CA, USA), with a Qiagen vacuum manifold following the manufacturers’ instructions. cfDNA was then eluted in a final volume of 60 µL of elution buffer (AVE). cfDNA libraries were created using the capture technology provided by in-
house panel. The assay covered mutations on 78 genes. Fastq files, obtained by the demultiplexing of Base-Call Files (BCL), were analysed using the Sophia DDM Platform version 5.1.9 (Sophia Genetics, Saint-Sulpice, Switzerland). The human genome Hg19 (GRCh37.p5) was used as the reference genome. Detailed analytical considerations have been previously published (Garcia et al. 2021).

**Circulating atypical cell enumeration**

Enrichment and labelling of circulating atypical cells were carried out using an in-house optimized immunostaining assay (Garcia et al. 2019). For performing circulating atypical cell enrichment, a volume of 7.5 mL of blood was lysed in 22.5 mL of red blood cell lysis buffer (CBB-F016003, Biolidics limited®), mixed gently by inverting and incubated stand at room temperature for approximately 10 minutes. Then, cells were centrifuged at 500 g for 10 minutes and the supernatant was removed. The pellet was resuspended in 4 mL of resuspension buffer (786-650, BIOSCIENCES®) and loaded onto the CTC Chip™ FR1 of the microfluidic ClearCell® FX1 system. Cell enrichment was collected and centrifuged at 500 g for 10 minutes prior to cyto-centrifuging on the polylysin sample glass slide for performing immunofluorescence assay.

After incubation with 200 µL of paraformaldehyde 4% for 10 minutes, the cytospot was incubated for 30 minutes in 200 µL of in-house saturation mix (Fetal Bovine Serum 5%, Fc Receptor Blocking Reagent 5%, Bovine serum albumin 1%, and PBS) for blocking non-specific protein binding. Then, it was incubated overnight at 4°C in primary antibody against CD45 (rat anti-human monoclonal antibody, MA5-17687, ThermoFisher) diluted at 1:500 in saturation mix. It was then washed three times in PBS and incubated for 1 hour at room temperature in labelled anti-rat AlexaFluor-647 secondary antibody (dilution 1:500) (VB296618, ThermoFisher) and DAPI (dilution 1:1000) for cell nuclei visualization. The
stained cytospot was washed four times in PBS and air-dried during 5 minutes. Slide was mounted with Fluoromount medium (Fluoromount™ Aqueous Mounting F4680, Sigma®) to prevent photo-bleaching and preserve the fluorescent labelled molecules for long-term storage. Slide was scanned by an automated microscope (LionHeart FX Automated microscope, Biotek, USA) and was analysed by microscopy software (Gen5™ version 3.09, Biotek, USA). The circulating atypical cells were DAPI+ and CD45- and had malignancy characteristic pattern (size, shape, mononuclear, nucleo-cytoplasmic ratio).