Using Digital Droplet Polymerase Chain Reaction to Detect the Mosaic GNAS Mutations in Whole Blood DNA or Circulating Cell-Free DNA in Fibrous Dysplasia and McCune-Albright Syndrome

Pauline Romanet, Pascal Philibert, Frederic Fina, Thomas Cuny, Catherine Roche, L'Houcine Ouafik, Françoise Paris, Rachel Reynaud, Anne A. Barlier

Using digital droplet PCR to detect the mosaic GNAS mutations in whole blood DNA or circulating cell-free DNA in fibrous dysplasia and McCune-Albright syndrome.

Pauline Romanet (MD, PhD)[1], Pascal Philibert (MD, PhD)[2], Frédéric Fina (PhD)[3], Thomas Cuny (MD, PhD)[4], Catherine Roche (PhD)[1], L'houcine Ouafik(Prof, MD, PhD)[5], Françoise Paris (Prof, MD, PhD)[6], Rachel Reynaud(Prof, MD, PhD)[7], Anne Barlier (Prof, MD, PhD)[1]

SHORT TITLE: GNAS mutation detection using digital PCR in FD/MAS

AFFILIATIONS :

1: Aix Marseille Univ, APHM, INSERM, MMG, Hôpital de la Conception, Laboratory of Molecular Biology, Marseille, France ;

2: Département de Biochimie et Hormonologie, Hôpital Lapeyronie, CHU Montpellier and Institut de Génétique Humaine, UMR 9002 CNRS-Université de Montpellier, France ;

3: APHM, Unité de Développement Technologique, LBM, Marseille, France;

4: Aix Marseille Univ, APHM, INSERM, MMG, Hôpital de la Conception, Department of Endocrinology APHM, Marseille, France ;

5: Aix Marseille Univ, APHM, CNRS, INP UMR7259, Service de Transfert d'Oncologie Biologique, Marseille, France ;

6: CHU Montpellier, Molecular Biology Department, Montpellier, FRANCE ; endocrinologie pédiatrique CHU Arnaud de Villeneuve Montpellier, France ;

7: Aix Marseille Univ, APHM, INSERM, MMG, Hôpital La Timone Enfants, Pediatric Department, Marseille, France

CORRESPONDENCE : Dr Pauline ROMANET, MD, PhD

Marseille Medical Genetics, Faculté de médecine de la Timone, 27 Boulevard Jean Moulin, 13 385 Marseille Cedex 05, France ; Tel +33 4 91 38 39 16 ; Fax +33 4 91 38 38 94 ; pauline.romanet@univ-amu.fr

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ABSTRACT (max 50 mots)

The *GNAS* postzygotic mosaic activating mutations involved in fibrous dysplasia and McCune-Albright syndrome (FD/MAS) are not detectable in leukocytes by Sanger sequencing. Digital droplet PCR™ detects *GNAS* mutations in 7/12 (58.3%) FD/MAS-suspected patients from whole blood DNA, and in 4/5 patients (80%) from circulating cell-free DNA.

CLINICAL AND LABORATORY OBSERVATIONS

BACKGROUND

Fibrous dysplasia (FD [MIM 174800]) and McCune-Albright Syndrome (MAS [MIM: 139320]) both are rare diseases caused by postzygotic activating mutations in *GNAS*. FD may affect one bone (monoostotic) or multiple bones (polyostotic), and may occur in isolation (FD disease) or in combination with café-au-lait skin pigmentation (SP) and hyperfunctioning endocrinopathies (1–5). *GNAS* encodes for the G protein alpha subunit (Gsα), which transduces the signal of many hormonal receptors (including the PTH/PTHrp, FSH, TSH, ACTH, and GHRH receptors). In FD/MAS, the activating mutations of *GNAS*, are nearly always substitutions of the arginine 201 by cysteine (NM_000516.5: c.601C>T, p.Arg201Cys, rs11554273) or histidine (NM_000516.5: c.602G>A, p.Arg201His, rs121913495) that constitutively activate Gsα. Here, we have respectively named these mutations R201C and R201H. MAS endocrine lesions include peripheral precocious puberty

(PPP), thyroid abnormalities, growth hormone (GH) excess, hypercortisolism, and hypophosphatemia due to FGF23 overproduction (3,6–9). The acquisition of mutation during early embryogenesis leads to a mosaic distribution of the lesions.

MAS diagnosis is based on clinical features, but the time lag between the first sign (often PPP at an average of 3 to 4 years old) and the others leads to an important delay of clinical diagnosis (10,11). A molecular diagnosis could allow an earlier diagnosis and consequently a follow-up and a therapeutic choice more adapted to the FD/MAS context, reducing the complications and optimizing the quality of life. Moreover, FD requires a molecular diagnosis in case of monolesion. However, the mosaic distribution of the *GNAS* mutations in FD/MAS makes the molecular diagnosis highly challenging. Moreover due to a low level of mosaicism in blood cells, the routine non-invasive methods are insufficiently sensitive.

Consequently, the development of a sensitive and non-invasive test is crucial for an early molecular diagnosis. Digital droplet polymerase chain reaction™ (ddPCR™) is a breakthrough technology of quantitative PCR useful for a targeted detection and quantification of rare events (12,13). Moreover, the emergence of the new non-invasive strategy to detect and monitor mutations in the circulating cell-free DNA (ccfDNA) from patients presented with a cancer, (named “liquid biopsies”), offers new prospects for the molecular diagnosis of FD/MAS (14,15). CcfDNA is fragmented DNA, released in plasma by normal and tumor cells through apoptosis, necrosis, or active secretion, that can be extracted from plasma and detected by sensitive molecular methods (14,15). The objective of this study was to evaluate the digital PCR for a non-invasive molecular diagnosis of FD/MAS. For that, we performed the targeted research of the R201H and R201C *GNAS* mutations by ddPCR™ in whole blood DNA and ccfDNA of 17 patients presenting with at least one sign of FD/MAS.

METHODS:

Patients: After a validation study (Appendix 1; online, Figure 1; online, and Table 1; online), we performed the targeted research of the R201H and R201C *GNAS* mutations by ddPCR™ in the various samples of patients (Table 2). Patients were addressed for FD/MAS genetic testing in the molecular biology laboratory of Conception's Hospital between January 2001 and December 2016. All patients or their parents provided signed informed genetic consent.

DNA extractions: DNA was extracted from whole blood collected in EDT tubes using an automated magnetic-particle technology on the QiaSymphony instrument (Qiagen, Germantown, USA). DNA from tissues was manually extracted using QiAmp DNA mini kits (Qiagen) following the manufacturer's instructions. CcfDNA was extracted from cell stabilizing blood collection tubes (Cell-Free DNA BCT®, Streck, Omara, USA), using the IDXTRACT kit (ID-Solutions, Grabels, France).

R201C and R201H ddPCR^{TM} Mutation Detection Assays: The samples were analyzed using two ddPCR^{TM} Mutation Detection Assays targeting the R201C or the R201H mutations. Each assay used a duplexed Taqman PCR system with two labeled probes: the first, labeled HEX, targeting the wild-type (WT) allele and the second, labeled 6-FAM, targeting the mutant allele. These assays were purchased from Bio-Rad (Hercules, USA; the *GNAS* p.R201C + *GNAS* WT for p.R201C PrimePCR™ ddPCR™ Mutation Detection Assays: named R201C assay in the text and the *GNAS* p.R201H + *GNAS* WT for p.R201H PrimePCR™ ddPCR™ Mutation Detection Assays: named R201H assay in the text). The mixture (reagents + DNA) was emulsified into up to 20,000 oil droplets following the manufacturer's instruction. The droplets were transferred to a PCR plate and amplified by PCR using the following protocol: 10 minutes at 95°C, 40 30-second cycles at 94°C followed by one minute at 55°C. The following 10 minutes at 98°C stop the reaction and clot the

droplets. Following PCR, the fluorescence of each droplet was read on the 200™ Droplet Digital™ PCR System (Bio-Rad).

Data analysis: The results were analyzed using the Quantisoft® software (Bio-Rad) to determine the fraction of PCR-positive droplets in the original sample. Poisson statistical analysis of the number of positive and negative reactions permitted the calculation of the absolute amount of WT or mutated alleles in the starting template without standard curves (12,16,17). The results were reported in fractional abundance (FA) corresponding to the percentage of the mutated alleles out of the total alleles (ratio mutated alleles / mutated + WT alleles).

The statistical comparison between methods was performed using a two-tailed Fisher's exact test performed on the Prism 6 Software (Graphpad, San Diego, USA).

RESULTS

The patients included 16 females and 1 male, comprising 12 children (average age 6.5 ± 4.2 years (1 to 12 years)), 5 adults (average age 43.6 ± 29.3 (18 to 71 years)). Four patients presented with at least 3 FD/MAS symptoms, 9 patients presented with 2 FD/MAS signs, and 4 patients presented with one FD/MAS sign (Table 2). The most common symptom was PPP (14/17). Three patients presented GH excess. Twelve patients were tested only on whole blood DNA. Five patients were tested on both whole blood DNA and ccfDNA.

The R201C or R201H ddPCR™ analysis on whole blood DNA was positive in 7/12 patients (58.3%; Table 2). For 3 patients, (# 2, 5 and 8), a second blood sample or a sample from a FD/MAS lesion (FD) was available. The ddPCR™ analysis of this second sample confirmed the result of the blood one. The FA of the mutated allele ranged from 0.03 to 20.87% in whole blood DNA. No correlation was found between the FA or the rate of detection of *GNAS* mutants and the number of MAS signs (Table 2 and Table 3; online).

The combined analyses on both whole blood DNA and ccfDNA led to a FD/MAS molecular diagnosis in 4/5 patients (80%; Table 2, and Table 3; online). In one patient (#7), the test was positive on both ccfDNA and whole blood DNA. The FA increased from 0.08% in the whole blood sample to 1.7% in ccfDNA. In three patients (#3, #6 and #17), the test was positive only on ccfDNA. These patients corresponded to 3 girls, a 6-year-old, presenting with SP and PPP, a 12-year-old, presenting the classic MAS triad, and a 2-year-old with PPP. For child #6, a skin lesion was obtained at the same time. DdPCR™ analysis run on it found the R201C mutant allele, confirming the results obtained on ccfDNA. The test of the fifth patient (#14) was negative on both whole blood DNA and ccfDNA.

We compared the performance of the 2 strategies, one using ddPCR™ on whole blood DNA alone and the other using ddPCR™ on whole blood DNA plus ccfDNA, with the performance of the four published methods (Table 4; 11,18–22). Despite the small number of patients in our series, the number of ddPCR™ diagnoses, was higher than those using nested-PCR ($p<0.05$; Table 4). The ddPCR performance was also higher than those of mutant enrichment with 3 -modified oligonucleotides (MEMO)-PCR and peptide nucleic acid clamping (PNA) clamping but without reaching the threshold of significance and was similar to the PNA-next generation sequencing (NGS, Table 4).

DISCUSSION

FD/MAS results from post-zygotic *GNAS* mutations are not detectable in peripheral blood leukocytes by Sanger sequencing. Biopsies in café-au-lait spots or bone lesions are rarely available (10). Currently, a definitive diagnosis of FD/MAS is often supported by the clinical triad of FD/MAS signs and less frequently by a molecular analysis of bone biopsies. When the triad is incomplete, it is difficult to recommend the performance of an invasive and painful medical act on children with no direct benefits. Moreover, the molecular analysis of

skin biopsies generally turns out negative (23). The early diagnosis of FD/MAS patients should allow an early treatment of lesions and to prevent complications, especially for somatotroph tumors, for which the treatment is often exclusively medical, due to frequent invasive pituitary adenomas and to the presence of FD at the skull base (9,24).

A non-invasive molecular diagnosis of FD/MAS was an unmet medical need that required new more sensitive and cost-effective tools. In this study, we validated the ddPCR™ as an inexpensive method to perform a non-invasive molecular diagnosis of FD/MAS during early childhood. Firstly, we validated ddPCR™ as an accurate and reliable method to carry out a positive diagnosis of FD/MAS, following international guidelines (Appendix 1; online, 25). We assessed the specificity of both assays using limit of blank (LOB) tests, which showed no false positive (Appendix 1; online, and Figure 1; online) (18).

In our series of 12 patients for which FD/MAS was suspected, ddPCR™ from whole blood DNA detected an R201H or R201C mutation in 58.3% (7/12 patients, Table 2). For several years, teams have been developing molecular methods more sensitive than Sanger sequencing to detect rare molecular events in peripheral blood leukocytes DNA, such as the R201H and R201C *GNAS* mutant alleles in FD/MAS. These methods, nested-PCR, PNA clamping, PCR and MEMO-PCR are based on the enrichment of the mutant allele vs. the WT by enzymatic digestion or by masking the WT allele (11,20,21,26). In a series of 88 patients, including 26% of patients with 3 signs of FD/MAS, Kalfa et al obtained a detection rate in blood leukocytes of 35.2% using PNA clamping and 36.6% using nested-PCR (18). The MEMO-PCR, published recently, succeeded in finding genetic abnormality in blood DNA of only 2 out of 7 patients (11). The ddPCR™ displayed the highest detection rate in whole blood DNA with 58.3% of molecular proof of MAS regardless of the number of MAS signs and 67% of patients with the 2 or 3 DF/MAS signs (Table 4). The ddPCR™ performance in patients with only 1 or 2 signs of DF/MAS is particularly promising in comparison to the other methods.

The combined real-time coamplification at lower denaturation temperature (COLD)- and mismatch amplification mutation assay (MAMA)-PCR techniques showed a sensitivity of 0.05% for the detection of the R201C and R201H *GNAS* mutations, which is similar to that of the nested-PCR method but lower than ddPCR^{TM} evaluated at 0.01% (Appendix 1; online, 22,26). This robust method used inexpensive techniques but remains to be clinically evaluated on FD/MAS suspected patients.

For routine use, the increase in the sensitivity of the method should not be obtained due a strong increase in detection lab-costs as for PNA-NGS. The PNA-NGS technique combines an ultra-deep NGS with the PNA clamping (for this method, see legend of Table 4 (22)). In the clinical evaluation study in MAS-suspected patients, the rate of *GNAS* mutant detection was 75% (12/16) including 8 out of the 8 patients with the MAS triad and 4 out of the 8 patients with two major FD/MAS signs. NGS-PNA was a method as sensitive as ddPCR™, but the authors disclosed that they could not discriminate true positives from false positives, which required analysis of paired peripheral blood leukocyte-affected tissues samples. Our validation study of ddPCR™ displayed a specificity at 100%, offering a more robust diagnosis. Additionally, ddPCR™ offers a one-day analysis after DNA extraction for acceptable reagent cost (10€ per PCR sample).

The origin of the GNAS mutated DNA in blood remains unclear. Three possibilities can be hypothesized considering that the routine DNA extraction from whole blood samples collects all DNA types (from nuclear cells or free-cell DNA). DNA should originate from leukocytes bearing a mosaic GNA mutation, ccfDNA from FD/MAS lesions, or circulating tumor cells. In this context, we performed GNAS analysis on both blood DNA and ccfDNA. We found R201H and R201C mutants in 4 out of the 5 analyzed patients (80%). For 3 of these 4 patients, whole blood analysis was negative by ddPCR™, and for the fourth patient, the analysis was positive. For this patient (patient #7), the FA of the mutant in ccfDNA was

20-fold higher than the FA in whole blood DNA (0.08% to 1.7%), suggesting a concentration of genetic information in ccfDNA thanks to the removal of the leukocyte genomic DNA (Table 2 and Table 3; online). By performing a combined test on whole blood DNA and on ccfDNA, we successfully increased the sensitivity of genetic screening from 58.3 to 80%. This combined strategy allows a diagnostic performance similar to that of PNA-NGS (respectively 12/16 and 4/5). However, ccfDNA testing requires removing a blood sample from a specific tube (cell-free DNA tube) not available for all clinical centers. That explained why we prefer to recommend a whole blood DNA ddPCR^{TM} testing in the first step and if negative, to test on ccfDNA. This strategy should be further evaluated on a larger series of FD/MAS patients.

CONCLUSION:

Overall, these results open up new diagnostic and medical care possibilities for FD/MAS patients. In this study, we demonstrated the relevance of a targeted screening of R201C or R201H *GNAS* mutations by ddPCR™ in patients displaying at least 1 feature of FD/MAS. This technology presents considerable benefits in terms of robustness, acceptability and cost-effectiveness. We have shown that the *GNAS* mosaic mutations can be found in ccfDNA. Used together, this breakthrough ultrasensitive quantitative PCR technology and ccfDNA targeting should drastically increase the rate of FD/MAS diagnosis, and thus modify the care of patients, particularly in pediatric populations.

Ethics approval and consent to participate: All patients or their parents provided signed consent for genetic testing.

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LIST OF ABBREVIATIONS:

ccfDNA: circulating cell-free DNA

ddPCR™: digital droplet polymerase chain reaction™

FA: fractional abundance

FD: Fibrous dysplasia

GH: growth hormone

LOB: limit of blank

MAS: McCune-Albright Syndrome

MEMO-PCR: mutant enrichment with 3 -modified oligonucleotides –PCR

NGS: next generation sequencing

PCR: polymerase chain reaction

PNA: peptide nucleic acid clamping

PPP: peripheral precocious puberty

SP: café-au-lait skin pigmentation

WT: wild-type

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FIGURE:

Figure 1; online: Plot representation of the LOB test for the R201C assay using 1 DNA not bearing GNAS mutant (patient X).

Left Y axis: R201H mutated copies concentrations (copies/µL +/- standard deviation; full open square). Right X axis: WT copies concentrations (copies/µL +/- standard deviation; hollow open square). Zero copies/µL of the mutant were detectable in the 40 ddPCR™ wells, whereas 544 to 771 copies/µL of the WT allele were found. Concentrations (Conc, copies/µL) were calculated with Poisson statistical analysis. NEG: No template control.

Table 1; online only

Table 1; online: Table of the values of mutated and wild-type (WT) copies obtained using the ddPCR™ R201C and R201H Mutation Detection Assays in the DNA previously analyzed by nested-PCR.

*, ovarian cyst surgery; FA: fractional abundance (in % and corresponding to the percentage of the mutated allele on the total allele), MAS: McCune-Albright Syndrome, FD: fibrous dysplasia, NA: Not Available

						ddPCR™ R201C assay			ddPCR™ R201H assay		
Patient	Gen-der	Age (Years)	Origin of DNA	Nested-PCR results	ddPCR results	Number of R201C copies (per well)	Number of WT copies (per well)	R201C FA (%)	Number of R201H copies (per well)	Number of WT copies (per well)	R201H FA (%)
T1	M	11	Ovarian cyst fluid	R201H	R201H	0	9960	0.00	556	6420	7.97
			Blood	Neg	R201H	0	31020	0.00	82	30340	0.27
			Ovarian cyst wall	R201H	R201H	0	10060	0.00	460	51860	0.88
T2	F	22	Blood	Neg	Neg	0	17920	0.00	0	17860	0.00
			FD	R201H	R201H	0	11480	0.00	4360	8480	33.96
T4	M	17	Blood	R201H	Neg	0	15480	0.00	0	18460	0.00
			Testis tumour	R201H	Neg	0	11340	0.00	0	15040	0.00
			Blood	Neg	R201H	0	17380	0.00	1.8	23940	0.01
T5	F	8	Blood pre-treatment*	R201H	R201H	0	12206	0.00	21	25852	0.08
			Blood post-treatment*	Neg	Neg	0	14740	0.00	0	22160	0.00
T6	F	5	Blood	Neg	Neg	0	13440	0.00	0	11660	0.00
T10	F	12	Blood	R201C	R201C	1486	9043	14.11	0	21453	0.00

T12	F	10	Blood	Neg	Neg	0	11980	0.00	0	2800	0.00
			Ovarian cyst fluid	R201C	R201C	42	2688	1.54	0	737	0.00
			Ovarian cyst wall	R201C	R201C	306	13700	2.18	0	18939	0.00
T13	F	14	Blood	Neg	Neg	0	13540	0.00	0	5440	0.00
			FD	R201H	R201H	0	2600	0.00	28	1214	2.25
			FD	R201H	R201H	0	8200	0.00	80	6980	1.13
			FD	R201H	R201H	0	17940	0.00	526	7216	6.79
T3	NA	25	Blood	Neg	Neg	0	9400	0.00	0	12680	0.00
T7	F	36	Blood	Neg	Neg	0	13460	0.00	0	13220	0.00
T8	F	8	Blood	Neg	Neg	0	11120	0.00	0	10840	0.00
T9	F	7	Ovarian cyst fluid	Neg	Neg	0	12720	0.00	0	16000	0.00
			Blood	Neg	Neg	0	10620	0.00	0	11120	0.00
			Ovarian cyst wall	Neg	Neg	0	13640	0.00	0	16220	0.00
			Ovarian cyst wall	Neg	Neg	0	11860	0.00	0	14780	0.00
T11	F	9	Blood	Neg	Neg	0	6200	0.00	0	8520	0.00
T14	F	12	Blood DNA	Neg	Neg	0	10940	0.00	0	9980	0.00
T15	F	15	Ovarian cyst wall	Neg	Neg	0	108	0.00	0	256	0.00
			Peritoneal fluid	Neg	Neg	0	14840	0.00	0	13920	0.00
			Ovarian cyst fluid	Neg	Neg	0	576	0.00	0	626	0.00
T16	F	4	Blood DNA	Neg	Neg	0	12540	0.00	0	11940	0.00
T17	M	11	Blood DNA	Neg	Neg	0	17860	0.00	0	16680	0.00
T18	F	9	Blood DNA	Neg	Neg	0	17180	0.00	0	20900	0.00

Table 2: Characteristics of the 17 patients, results of the ddPCR™ assays using both strategies on whole blood DNA and whole blood DNA + ccfDNA.

DF/MAS major signs: FD, MAS Endoc., SP

Abbreviations: -: Negative result, *confirmed on a second whole blood DNA sample, ** confirmed on a skin biopsy, *** confirmed on a bone biopsy, ccfDNA: circulating cell-free DNA, MAS Endoc.: MAS endocrinopathies, FA: fractional abundance, FD: fibrous dysplasia, GHAd: somatotroph adenoma, HPT: hyperparathyroidism, FD/MAS: fibrous dysplasia and McCune-Albright Syndrome, NA: not available, PPGL: pheochromocytoma/paraganglioma, PPP: peripheral precocious puberty, SP: skin pigmentation

Patient #	Age (yrs)	Gender	Number of FD/MAS major signs#	SP	MAS Endoc.	FD	Others	Whole blood DNA	Mutated FA in blood (%)			Molecular evidence of FD/MAS
1	1	F	1		PPP			R201H	0.04			Yes
2	4	F	2		PPP, GHAd			R201C*	0.03 / 0.06			Yes
4	10	F	2		PPP	+		R201H	0.07			Yes
5	11	F	2		PPP	+		R201C***	5.61			Yes
8	43	F	3	+	PPP, thyroid nodule	+		R201C*	4.59 / 20.87			Yes
9	54	F	2		GHAd,	+	PPGL, HPT	R201H	0.04			Yes
10	71	M	3	+	PPP, hyperthyroidism	+		R201C	2.97			Yes
11	1	F	1		PPP			NEG	-			No
12	2	F	2	+	PPP			NEG	-			No
13	6	F	1		PPP			NEG	-			No
15	11	F	3	+	PPP	+		NEG	-			No
16	32	F	2		GHAd	+		NEG	-			No
Patient N°	**Age (yrs)**	**Gender**	**Number of FD/MAS major signs#**	**SP**	**Endoc.**	**FD**	**Others**	**Whole blood DNA**	**Mutated FA in blood (%)**	**ccfDNA**	**Mutated FA in ccfDNA (%)**	**Molecular evidence of FD/MAS**
3	6	F	3	+	PPP	+		NEG	-	R201C	0.45	Yes
6	12	F	2	+	PPP			NEG	-	R201C**	1.17	Yes
7	18	F	2	+		+		R201H	0.08	R201H	1.70	Yes
14	8	F	2	+	PPP			NEG	-	NEG	-	No
17	5	F	1	+				NEG	-	R201H	0.10	Yes

Table 3; online: Table of the values of mutated and wild-type (WT) copies using the ddPCR™ R201C and R201H Mutation Detection Assays obtained in patients included in the clinical evaluation study.

Description: Blood: whole blood, FA: fractional abundance (%), MAS: McCune-Albright Syndrome, NA: not available.

		ddPCR™ R201C assay			ddPCR™ R201H assay		
Patient	**Origin of DNA**	**Number of WT copies for R201C (copies/ well)**	**Number of mutated copies for R201C (copies/ well)**	**Mutated FA for R201C (%)**	**Number of WT copies for R201H (copies/ well)**	**Number of mutated copies for R201H (copies/ well)**	**Mutated FA for R201H (%)**
1	Blood	16560	0	0	16020	6.8	0.04
2	Blood	5929	2	0.03	1092	0	0
	Blood	23380	13.2	0.06	15460	0	0
3	Blood	30040	0	0	32060	0	0
	Plasma	353	1.6	0.45	NA	NA	NA
4	Blood	23280	0	0	18440	12	0.07
5	Blood	4240	252	5.61	4600	0	0
	Bone	26620	9300	25.89	NA	NA	NA
6	Blood	6020	0	0	NA	NA	NA
	Skin	32560	2	0.01	NA	NA	NA
	Plasma	84	1	1.17	NA	NA	NA
7	Blood	14120	0	0	14340	0.55	0.08
	Plasma	NA	NA	NA	451	7.8	1.70
8	Blood	1748	84	4.59	NA	NA	NA
	Blood	14260	3760	20.87	12800	0	0
9	Blood	6750	0	0	14480	6	0.04

10	Blood	21860	668	2.97	32580	0	0
11	Blood	20980	0	0	22040	0	0
12	Blood	31360	0	0	31540	0	0
13	Blood	16820	0	0	13780	0	0
14	Blood	14969	0	0	27840	0	0
	Plasma	422	0	0	166	0	0
15	Blood	26420	0	0	26460	0	0
16	Blood	22240	0	0	23340	0	0
17	Blood	28000	0	0	20140	0	0
	Plasma	4200	0	0	1619	1.6	0.1

Table 4: Comparison of 5 methods to detect the R201C or R201H mutations in non-invasive specimens of FD/MAS-suspected patients.

The methods are ddPCR™ on whole blood DNA and ddPCR™ on whole blood DNA and circulating cell-free DNA (ccfDNA), MEMO-PCR (Cho et al. (11)), nested-PCR (Kalfa et al., Lumbroso et al. (18,23)), PNA-Clamping (Kalfa et al. (18)); and PNA-NGS (Narumi et al., (22)). The results are expressed in percentage of positive patients (n of positive/n of total tested patients). Statistical analyses: 2-tailed Fisher's exact test versus nested-PCR; *: $p<0.05$. Notes: MEMO-PCR (mutant enrichment with 3'-modified oligonucleotides - polymerase chain reaction) is enrichment PCR method based on the use of a 3'-modified oligonucleotide primer that blocks extension of the normal allele but enables extension of the mutated allele. PNA-clamping (peptide nucleic acid clamping) is based on the inhibition of the WT allele amplification. The PNA primer that is complementary to the WT sequence overlaps the binding site of the PCR primer and prevents amplification of the WT allele. The PNA primer will not bind to the mutant sequence, and the mutant allele is thus amplified during PCR. NGS-PNA combined PNA-clamping and ultra-deep next generation sequencing (NGS).

Method	ddPCR™ (whole blood DNA and ccfDNA)	ddPCR™ (whole blood DNA)	Nested-PCR [13,20]	MEMO-PCR [10]	PNA clamping [20]	PNA-NGS [22]
Overall detection rate (all patients)	80% (4/5) *	58.3% (7/12)*	28% (47/168)	29% (2/7)	35% (25/71)	75% (12/16)
Detection rate in FD/MAS patients with three signs	100% (1/1)	67% (2/3)	46% (11/24)	0% (0/3)	NA	100% (8/8)
Detection rate in FD/MAS patients with two signs	67% (2/3)	67% (4/6)*	21% (7/33)	50% (2/4)	NA	50% (4/8)
Detection rate in patients with one FD/MAS sign	100% (1/1)	33% (1/3)	8% (3/40)	- (0/0)	NA	- (0/0)

Figure 1; online only

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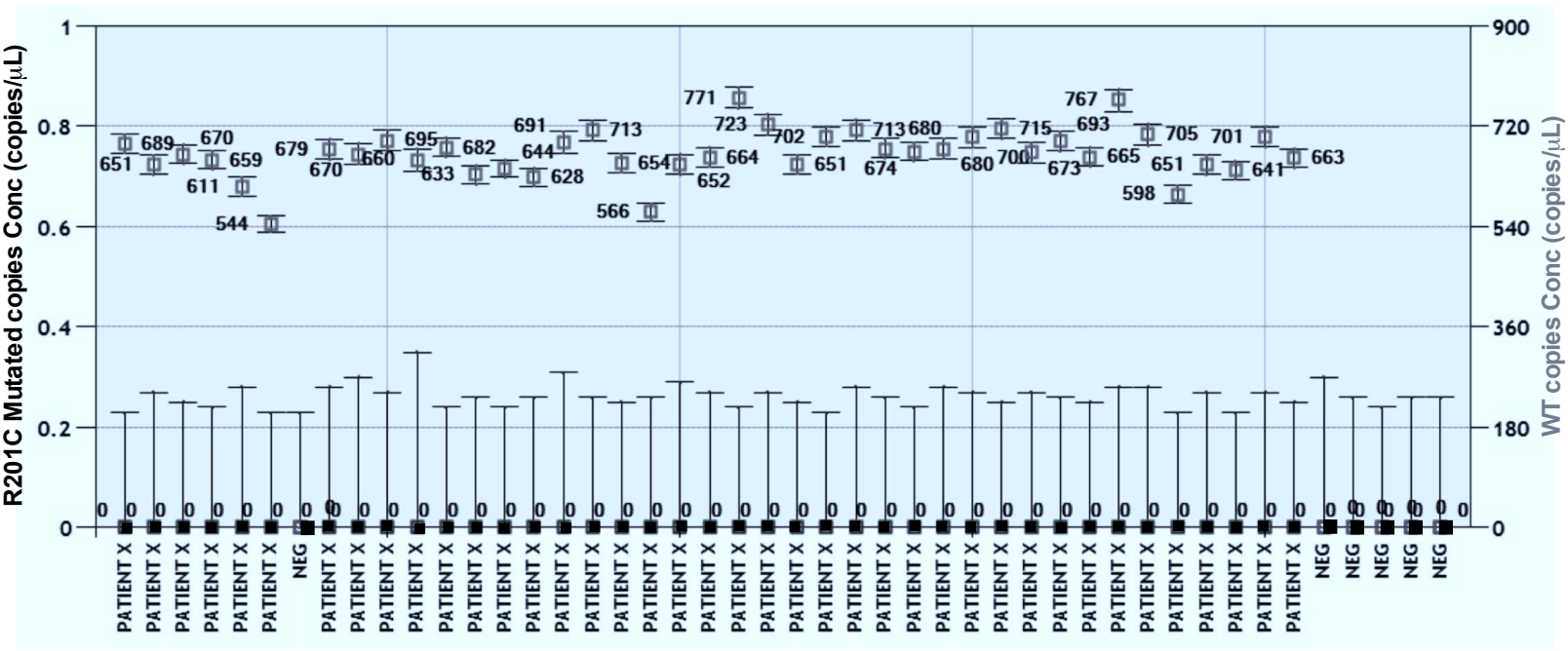

Appendix; online

We performed a validation study following international guidelines for digital PCR experiments (1).

Materials and methods:

The ability of both ddPCR™ assays to detect mutations was assessed using the DNA extracted from somatotroph adenomas, previously characterized with the presence of heterozygous R201C or R201H *GNAS* mutations by Sanger sequencing. The accuracies of the R201C and R201H assays were assessed by determining the limit of blank (LOB). For that, regular DNA not harboring the targeted mutant alleles was analyzed through 40 technical replicates. Additionally, the repeatability of the quantitative measurement was evaluated by measuring concentrations of the WT DNA copies in the LOB test.

Subsequently, positive controls provided from two somatotroph tumors were introduced in each run for technical validation and also used to determine the reproducibility and the accuracy of the test.

We performed biological validation by working blindly on the DNA of various samples extracted from patients previously characterized by nested-PCR method for the presence of *GNAS* mutations. Nested-PCR uses a modified primer to obtain a PCR product from normal DNA that can be digested by a restriction enzyme (EagI), whereas the PCR product obtained from mutated DNA is resistant to this enzyme. The successive completion of the PCR steps and enzyme digestion results in an enrichment of the mutated allele (2,3). In this series, DNA was extracted from blood or from fresh tissues.

Results:

Technical validation of ddPCR™

In the two mutated DNA samples from the somatotroph tumors (positive controls), ddPCR™ detected the R201H or R201C mutant alleles (Table 1; online). The specificity of both R201C and R201H assays was considered to be 100% since no positive droplet for the mutant was detected in any of the 40 PCR wells containing only normal genomic DNA (LOB test; Figure 1). The test was therefore considered positive if there was at least 1 positive droplet for the mutant allele and negative if there were only positive droplets for the WT allele. The repeatability of each assay was assessed by measuring the WT DNA concentration in the LOB test with an input of 50 ng of DNA and were respectively 670 +/- 40 copies/µL (mean +/- SD) for the R201C assay and 673 +/- 49 WT copies/µL for the R201H assay. The variation coefficients (VCs) were respectively 6.7 and 5.6%. The inter-run accuracy was determined and monitored using the FA of the somatotroph pituitary adenoma positive controls during the subsequent tests: the VC was 3.2% for the detection of the R201C mutant and 2.5% for the R201H mutant.

Comparison between the ddPCR™ method and the nested-PCR method:

To validate ddPCR™, we compared the results obtained blindly by ddPCR™ to those obtained previously by nested-PCR on 34 samples from 18 patients, published 14 years ago (Table 1; online; 3). DdPCR™ analysis confirmed the molecular diagnosis of MAS given by nested-PCR for all the patients suffering from MAS. The lack of R201H or R201C mutant alleles was confirmed using ddPCR™ in 10 out of 10 patients negative for a GNAS mutation with the nested-PCR method. For 2 patients (T1 and T4) for whom several samples were available, the results from the 2 techniques showed discrepancies depending on the samples. In the DNA from patient T1, ddPCR™ showed an R201H mutation in blood, whereas this result was negative in nested-PCR. Nevertheless an R201C mutation was also carried out by ddPCR™ for this patient. The lowest detectable mutation abundance was 0.01% with ddPCR™.

Discussion-Conclusion:

Our aim was to validate ddPCR™ as an accurate and reliable method to carry out a positive diagnosis of FD/MAS. Firstly, we assessed the specificity of both assays using LOB tests, which showed no false positive. Then, we used a cohort of 18 patients previously independently analyzed by the nested-PCR method. The amount of positive and negative results for patients screened with the two techniques matched well, despite the fact that DNA samples were old and had been previously tested 12 years ago. FD/MAS molecular diagnosis was confirmed by ddPCR™ for the 7 patients previously diagnosed by nested-PCR and fully analyzed by ddPCR™. The lowest detectable mutation abundance was 0.01% with ddPCR™. The main advantage of ddPCR™ in comparison to nested-PCR is a reduced risk of contamination by PCR product and a reduced analysis time, 4 days for nested-PCR versus 1 day for ddPCR™.[4] In patient T1, ddPCR™ gave a positive result for the blood DNA and ovarian DNA samples, but a negative result for the blood DNA sample with nested-PCR, thus suggesting a higher sensitivity of the ddPCR™ assay.

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