RESEARCH LETTER

Detection and monitoring of circulating tumor DNA in adrenocortical carcinoma

Dear Editor,

Adrenocortical carcinomas (ACC) are rare but aggressive tumors. Currently, the single curative approach is complete surgery. Prognosis, response to treatment and recurrence remain unpredictable, which stresses the need for new biomarkers (Else *et al.* 2014). Recent exome sequencing approaches of tumors identified in 60% of ACC recurrent somatic mutations in 20 genes (Assié *et al.* 2014*a*,*b*, Juhlin *et al.* 2015, Zheng *et al.* 2016). Somatic mutations can be used as surrogate biomarkers for detecting circulating tumor DNA (ctDNA) in blood. This ctDNA corresponds to fragments of DNA released directly by tumor cells into the blood stream among the circulating cell-free DNA (ccfDNA). Discrimination of ctDNA from ccfDNA of non-tumoral origin is based on the detection of somatic mutations, specific of cancer cells. The amount of ccfDNA and the detection of ctDNA largely depend on tumor type and disease stage (Bettegowda *et al.* 2014). In ACC, ctDNA detection has been recently reported in one patient (Creemers *et al.* 2017). However, beyond this proof of concept, the proportion of ACC patients with detectable ctDNA is not established.

The aim of this study was to assess to which extent ctDNA can be detected in ACC patients, using two highly sensitive techniques: deep NGS and droplet digital PCR (ddPCR). We also assessed the evolution of ctDNA during the course of the disease.

We prospectively and randomly included 11 patients with ACC. For four patients, blood was sampled before primary surgery, for three patients at the time of a small relapse or metastasis occurring <2 years after primary surgery, and for four patients in the setting of a rapidly growing metastatic disease. Median age was 48 (range 31–81 years). Six patients had glucocorticoids hypersecretion. Two patients had androgen hypersecretion, while two patients presented no hypersecretion (Table 1). All patients were informed of the project, and signed a written consent for the genetic study of the tumor. A prior agreement from the local ethics committee was obtained, under the COMETE–TACTIC framework.

Tumor and leukocytes DNA were extracted for each patient. Next-generation sequencing (NGS) workflow based on a custom AmpliSeq (Thermofisher) panel was designed for sequencing the 20 genes known to be frequently mutated in ACCs, using a Ion Torrent PGM Sequencer (Life Technologies). Seventeen somatic mutations were detected in eight patients (Table 1). *TP53* (5 hits) and *CTNNB1* (3 hits) were the most affected genes, followed by *NF1* (2 hits) and single-hit mutations in *TERT*, *RPL22*, *ATRX*, *MED12* and *MEN1*. Four patients had more than one somatic mutation, ranging from 2 to 6. Tumor cellularity, clonality and heterozygosity status were assessed with our R algorithm TARGOMICS (Garinet *et al.* 2017, data not shown). Three patients presented no mutations in the genes studied.

For the eight patients with at least one somatic mutation identified in the tumor, ccfDNA was extracted from plasma collected either before surgery or at the time of relapse, metastases or follow-up obtained after double centrifugation of 20 mL of blood (BCT Cell-Free DNA Blood Collection Tube). Concentrations were variable, from 3 to 422 ng/mL of plasma (Table 1). For each patient, tumor mutations were searched in ccfDNA either by deep sequencing or by ddPCR. A library with a unique amplicon harboring the mutation was subsequently prepared for each patient relative to the mutation identified in the tumor DNA and sequenced with an expected depth of 100,000×.

Four mutations were detected by NGS, in two patients, 1 and 7 (Table 1). Notably, these patients displayed massively metastatic diseases, with a rapid evolution. Allelic ratios in tumors indicated that these mutations were present in all tumor cells. Concentrations of ccfDNA were high –17 and 422 ng/mL. For patient 1, the three somatic mutations – in *RB1*, *TP53* and *CTNNB1* genes – were

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Table 1 Patients main clinical features.

Patients feature at the time of ccfDNA sampling								
No.	Age	Gender	Time to surgery (months)	Primary in place	Metastases	Locoregional recurrence	Tumor burden	Disease progression*
1	44	M	21	No	Liver pancreas lung	Yes	Multiple large metastases	Progression
2	38	F	19	No	Lung	No	Micronodule	Stable
3	48	F	31	No	Lung	Yes	Micronodules	Stable
4	55	F	19	No	Liver lung	No	Multiple large metastases	Progression
5	31	F	Before	Yes	No	No	Small primary	None
6	53	F	3	No	No	Yes	Multiple large nodules	Progression
7	42	F	Before	Yes	Lung	–	Massive primitive and metastases	Progression
8	81	F	21	No	Lung	Yes	35 mm recurrence micrometastasis	Progression
9	21	F	Before	Yes	Lung	–	Large primary multiple micrometastases	Progression
10	53	F	30	No	Bone liver lung	No	Multiple large metastases (bone and liver) micrometastases (lung)	Progression
11	54	M	Before	Yes	No	No	Massive primitive	–

found in ccfDNA. The allelic ratios were close – 11.7, 16.6 and 14.3% respectively. For patient 7, the somatic mutation in *CTNNB1* was found, with an allelic ratio of 13.8%. For both patients, mutations were also detected with ddPCR. Allelic ratios – 15.2% and 15.3% for patient 1 and patient 7, respectively, were comparable to those obtained by NGS.

In contrast with these two patients, no ctDNA mutation was detected for 6 out of 8 patients, neither by deep NGS nor by ddPCR (Table 1). Several parameters may impact detection of ctDNA. First the tumor burden and progression may have a role. Indeed tumor volume was small and slowly/not progressing for three negative patients – patients 2, 3, 5 – and intermediate for one – patient 8. Yet, two of the six negative patients – patients 4 and 6 – also presented with a large and progressing tumor. Tumor burden is not fully associated with ctDNA detection. The clonality of mutations may also have an

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Primary tumor features						CcfDNA				Last follow-up
Steroid secretion	ENSAT stage	Tumor size (cm)	Weiss score	Ki67 (%)	Mutated genes (allelic ratio)	DNA Concentration (ng/mL of plasma)	NGS depth	NGS allelic ratio	ddPCR allelic ratio (positive droplets/total)	Specific death (time)
None	4	8	7	40	*TP53* c.611delT p. Leu204fs (93%) *CTNNB1* c.134C>T pSer45Phe (92%) *RB1* c.1388C>G p. Ser463* (93%)	17.3	301308 155564 117413	11.7% 16.6% 14.3%	– 15.2% (251/1649) –	Yes (28)
A	4	6	7	9	*TERT* c.2657-46C>T (17%) *TERT* c.2677G>C p. Glu893Gln (18%)	3	– 121032	– <0.1%	– 0/101	No (47)
GC	3	20	9	20	*RPL22* c.305T>C p. Val102Ala (5%) *NF1* c.4970A>G p. Tyr1657Cys (5%) *NF1* c.5843_5846del p. Gln1948fs (62%) *TP53* c.146G>A p. Arg49His (64%) *ATRX* c.2542_2545del p.Glu848fs (72%) *MED12* c.5223A>T p. Pro1741Pro (17%)	6	– – – 105972 98586 –	– – – <0.1% <0.1% –	– – – – 0/223 –	No (36)
GC	4	10	8	20	*NF1* c.5017G>T pGlu1673* (75%)	31.5	123524	<0.1%	0/7658	Yes (27)
GC	1	2.5	1	5	*CTNNB1* c.134C>G p. Ser45Cys (13%)	9.4	21574	–	0/1657	No (17)
GC	2	10	9	40	*CTNNB1* c.387A>G p. Ser129Ser (37%)	11	0 (technical error)	<0.1%	0/1199	Yes (9)
GC	4	15	–**	80[$]	*TP53* c.76C>T pArg26Cys (71%)	422	147166	**13.8%**	**15.3% (46/301)**	Yes (2)
A	3	6	9	35	*MEN1* c.779A>G p. Glu260Gly (84%) *TP53* c.229delA pArg77fs (83%)	19	109369 123541	<0.1% <0.1%	0	No[##] (29)
GC MC	4	10	8	35	– (*TP53* germline, 93%)	–	–	–	–	Yes (6)
None	4	18	6	15	–	–	–	–	–	Yes (33)
GC	2	20	8	23	–	–	–	–	–	Yes[#] (0)

*RECIST criteria; **no Weiss score could be determined since only a biopsy was performed; ##palliative care only at this time; #deceased from pulmonary infection after surgery; [$]determined on a lung metastases.
A, androgens; GC, glucocorticoids; MC, minerlocorticoïds.

impact. Indeed, no ctDNA could be detected for the two patients with subclonal mutations – patients 2 and 5. One tumor – patient 5 – was of borderline malignancy (Weiss score of 2), and it remains to be checked whether ctDNA can be detected in benign adrenal tumor.

It remains challenging to conclude whether ctDNA detection was negative because of a limited sensitivity. Indeed, one theoretical limitation of the sensitivity remains the initial quantity of DNA in the test sample. In our study, quantities of ccfDNA were low (<10 ng/mL) for three patients – patients 2, 3 and 5 – and the number of DNA copies analyzed in ddPCR remained <300 for patients 2, 3 and 8. The minimum detectable ratio is thus lowered, and this is a detection limit *de facto*. However, it might be possible that some ACC do not release any ctDNA. This is the case for some tumor types, like glioma or renal cell carcinoma for which ctDNA is detected in less than 50% of patients, with currently no clear explanation

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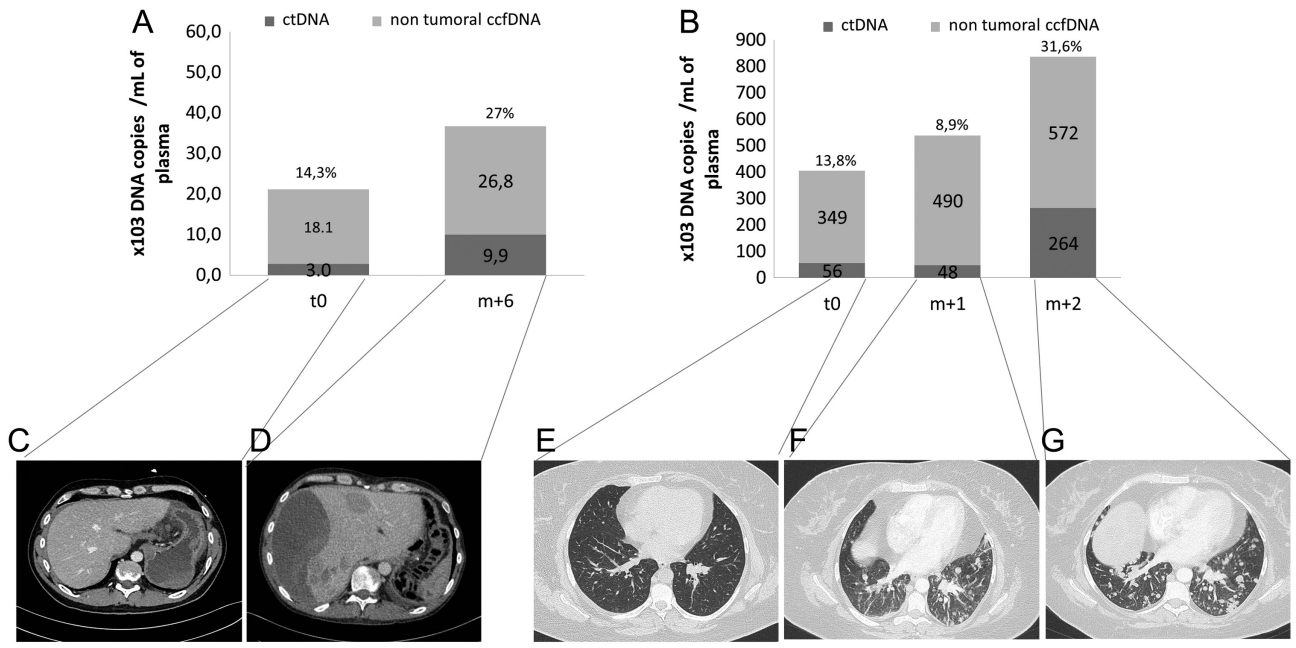

Figure 1
Monitoring of ctDNA and tumor evolution in patients with positive detection. (A and B) Relative and absolute concentration of ctDNA among ccfDNA in patients 1 and 7 respectively. (C and D) Growth of pancreatic and hepatic lesions at CT scan in patient 1. (E, F and G) Increase of pulmonary lesions at CT-scan in patient 7.

(Bettegowda *et al.* 2014). Further studies will be needed to test this hypothesis. Compared to the study of Creemers and coworkers, who used a NGS panel (Creemers *et al.* 2017), our study used two of the currently most sensitive molecular biology technologies, including deep NGS and ddPCR. However, though being limited, our series suggest at least that patients with the most aggressive forms of the disease do secrete ctDNA. This subgroup of patients with aggressive disease is certainly the subgroup in which such a tool as ctDNA may have the most obvious applications, both in terms of prognosis and in terms of disease follow-up.

Amounts of ctDNA were monitored during follow-up, and our data suggest that quantity of ctDNA parallels disease evolution (Fig. 1). Patient 1 had a pancreatic recurrence, and no hepatic lesion at the time of first blood sampling. After 6 months, both ctDNA absolute quantity and allelic ratios increased from 2.7 to 9.9×10^3 copies of DNA in a 10 mL sample and from 13.5 to 27% respectively (Fig. 1A). This increase paralleled disease progression, with the appearance of liver metastases and growth of the pancreatic lesion (Fig. 1C and D). Patient 7 had small pulmonary and liver metastases at the time of first blood sampling. After one month, ctDNA absolute quantity remained similar, while allelic ratio decreased – from 56 to 48×10^3 copies of DNA in a 10 mL sample and from 13.8% to 8.9% of ctDNA respectively. Two months later, both ctDNA absolute quantity and allelic ratio increased importantly, reaching 264×10^3 copies and 31.6% of ctDNA. This rapid increase paralleled disease progression, with a rapid and massive growth of metastases (Fig. 1E, F and G), leading to patient's death one month later. For both patients, quantities of non-tumoral ccfDNA were also at a higher level than observed in healthy controls and increased strongly when patient 7 was in terminal phase. This finding has been observed in other studies, and could be explained by an overall inflammation and excessive cell death releasing more ccfDNA (Zhang *et al.* 2017). Therefore, in clinical routine, ctDNA should be monitored with both the ratio relative to ccfDNA, and the absolute quantity. Monitoring ctDNA could help to monitor the response to treatment and disease progression in ACC patients. Further studies are needed, to compare ctDNA with imaging techniques for this purpose, both in terms of sensitivity.

In conclusion, ctDNA is detectable in a subset of ACC patients. When detected, ctDNA can be accurately quantified and seems to follow tumor dynamics. However, ctDNA could not be detected in several patients, including some with large tumor burden, and despite the use of highly sensitive technologies. Thus, despite some promising value, it is not currently possible to foresee the exact place of ctDNA in ACC management. Its applications

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as a potential biomarker remain to be determined on a larger cohort, with a longitudinal monitoring.

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Declaration of interest

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