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CtDNA is a prognostic marker in pancreatic adenocarcinoma

Key words: Pancreatic Adenocarcinoma, Circulating tumor DNA, Prognostic, *KRAS*, NGS, digital PCR

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Translational relevance (150 words)

The possibilities of translational research in pancreatic adenocarcinoma (PAC) have been limited during recent years by the difficulty in obtaining sufficient tumor tissue, in quantity and quality. The identification of biological markers from blood could help to overcome this issue. Circulating tumor DNA (ctDNA) is one of the most promising blood biomarkers, providing information about molecular abnormalities, and is associated with a prognostic and/or predictive value in others malignancies. Our study is the first to show that targeted NGS can be used for the detection of ctDNA in a routine procedure, across a large gene panel, and with concordant results obtained with picoliter droplet based digital PCR. Our work highlights that the presence of ctDNA appears as an independent prognostic factor at all stages of PAC, in both advanced diseases and after curative-intent resection. The described procedure offers great potential as a new, simple and non-invasive strategy for patients' care and follow-up.

Abstract: (249 words)

Purpose

Despite recent therapeutic advances, prognosis of patients with pancreatic adenocarcinoma (PAC) remains poor. Analyses from tumor tissues present limitations, identification of informative marker from blood might be a promising alternative.

The aim of this study was to assess the feasibility and the prognostic value of circulating tumor DNA (ctDNA) in PAC.

Experimental Design

From 2011 to 2015, blood samples were prospectively collected from all consecutive patients with PAC treated in our center. Identification of ctDNA was done with Next Generation Sequencing targeted on referenced mutations in PAC and with picoliter droplet digital PCR.

Results

A total of 135 patients with resectable (n=31; 23%), locally advanced (n=36; 27%) or metastatic (n=68; 50%) PAC was included. In patients with advanced PAC (n=104), 48% (n=50) had ctDNA detectable with a median mutation allelic frequency (MAF) of 6.1%. Presence of ctDNA was strongly correlated with poor overall survival (OS) (6.5 vs. 19.0 months; P<0.001) in univariate and multivariate analysis (HR=1.96; P=0.007). To evaluate the impact of ctDNA level, patients were grouped according to MAF tertiles: OS were 18.9, 7.8 and 4.9 months (P<0.001). Among patients who had curative intent resection (n=31), 6 had ctDNA detectable after surgery, with a MAF of 4.4%. Presence of ctDNA was associated with a shorter disease free survival (4.6 vs.17.6 months; P=0.03) and shorter OS (19.3 vs 32.2 months; P=0.027).

Conclusions

CtDNA is an independent prognostic marker in advanced PAC. Furthermore, it arises as an indicator of shorter disease free survival in resected patients when detected after surgery.

INTRODUCTION

Pancreatic Adenocarcinoma (PAC) is a leading cause of cancer-related mortality in western countries and is predicted to become the second leading cause of cancer death in 2020.(1,2) Surgery remains the cornerstone of treatment for patients with resectable PAC and the only curative treatment. The consequence of aggressive growth, early dissemination and lack of early symptoms is that 80% of patients are diagnosed at late clinical stages.(3) Despite recent improvements with new chemotherapy protocols such as FOLFIRINOX or gemcitabine plus *nab*-paclitaxel,(4,5) patients prognosis remains very poor. Many serological markers have been tested like Carbohydrate Antigen 19-9 (CA 19-9) but none is highly prognostic in PAC.(6) These tumors are characterized by tumor-specific genetic and epigenetic changes in DNA, including frequent mutations in *CDKN2A, SMAD4*, *TP53* or *KRAS* genes (7-9), which can be used as potential markers. However, their widespread use is limited by the difficulty in obtaining tissues from patients using endoscopic technics and because only 20% of patients present a resectable tumor. In this context a prognostic non-invasive blood test for PAC would be very valuable.

Measuring tumor-specific alterations in blood nucleic acids offers an interesting approach. In this context, circulating tumor DNA (ctDNA) has produced interesting results for a wide range of cancers.(10,11). The fraction of patients with detectable plasmatic ctDNA as well as its concentration increased with tumor stage.(12) Recent studies demonstrated the prognostic impact of ctDNA in metastatic colorectal cancer.(13,14) In fact, early changes in ctDNA during first line chemotherapy have been shown to predict the later radiologic response.(15)

Recent studies demonstrated a strong correlation between genomic alterations found in pancreatic tumors by sequencing and those found after characterization of DNA extracted from plasma of the same patients.(16). ctDNA has also been evaluated as a diagnostic marker to improve CA 19-9 value.(17) By combining *KRAS* mutations in ctDNA with CA 19–9 levels, Dabritz *et al.* were able to diagnose PAC with a sensitivity of 91%.(18) Despite these results, little information exists on the prognostic value of ctDNA.(16,19,20)

The aim of the present study was to evaluate the feasibility of the detection of ctDNA and the prognostic value of ctDNA in patients with PAC.

PATIENTS & METHODS

Sample extraction and clinicopathological data

From January 2011 to May 2015, plasmas of all consecutive patients with histologically proven PAC, receiving first chemotherapy protocol, were prospectively collected in the Pitié Salpêtière hospital (Paris, France) including resectable, locally advanced and metastatic stages. Blood samples were collected just before: (i) the first cycle of adjuvant treatment, after surgical resection in patients who had curative resection, or (ii) the first cycle of chemotherapy in patients with locally advanced or metastatic disease. All the patients signed an informed consent form, approved by the ethic committee (CPP Ile-de-France 2014/59NICB). The following data were collected in a prospective database: clinical and pathological characteristics (gender, age, medical history, date of diagnosis, location of the primary tumor, primary tumor diameter, tumor differentiation grade, stage of the disease), follow-up data (date of primary resection, date and type of relapse, date of diagnosis of metastatic disease, date and type of chemotherapy regimen, date and type of chemoradiotherapy, date of death or last follow-up) and biologic data (CEA, CA 19-9, albuminemia, bilirubinemia).

DNA extraction from the plasma

Blood samples (9mL) were withdrawn from a central catheter and placed in EDTA tubes. The collected samples were centrifuged at 3,500 rpm for 15 min at 4°C within 3 hours of blood draw. Plasma was stored at -80°C until further use. DNA was extracted from plasma with QIAamp® Circulating Nucleic Acid kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Incubation with proteinase K was performed for 30 min at 68°C. Extracted DNA from 2mL of plasma was eluted with 50µL buffer AVE and stored at -80°C. DNA quantity were assessed using the Qubit™ dsDNA HS (High Sensitivity) Assay kit (Thermo Fisher).

Next Generation Sequencing (NGS)

Sequencing libraries were prepared from circulating-free DNA using Ion AmpliSeq™ Colon and Lung Cancer Research Panel v2 (Thermo Fisher).(21) According to manufacturer's protocols, 10ng of DNA for each sample was used as input for library preparation with the Ion AmpliSeq™ Library Kit 2.0 (Thermo Fisher). The pooled barcoded libraries (max 96) were processed on Ion Chef™ System using the Ion PI Hi-Q Chef Kit (A27198) and sequenced on the Ion Proton™ System using and Ion PI Chip Kit v3 (A26771). The NGS analysis method (see **Supplementary Data File 1**) has been specifically developed to detect low allele frequency mutations, the sensitivity and specificity of which have been validated in positive and negative controls (manuscript submitted).

Droplet-based digital PCR

All plasma samples were screened for the 3 most frequent *KRAS* mutations in PAC (ie. p.G12V, p.G12D, p.G12R) by picoliter droplet-based digital PCR using RainDrop® system (RainDance Technologies). In addition, all additional *KRAS* mutations detected by NGS sequencing were analyzed. This system is based on the use of aqueous picoliter-size droplets separated by oil in microfluidic systems acting as independent PCR reactors.(22) Using this system, single target DNA molecules were compartmentalized in picoliter droplets together with validated fluorogenic TaqMan™ probes specific for mutated and wild-type *KRAS* and all reagents needed for PCR amplification.(23,24) After thermocycling, the fluorescence of each droplet was measured. The amplification of mutant DNA gives a green-fluorescent droplet while the amplification of wild-type DNA gives a red-fluorescent droplet. The ratio of mutant to wild-type DNA was determined from the ratio of green to red droplets. This strategy is both highly quantitative and highly sensitive.

Analyses were performed as described previously.(24) Briefly, after testing all samples using the RainDrop system, data from cluster plots were analyzed with RainDrop Analyst software following standard procedures. Positive control DNA from cell-line bearing the mutant allele was used as a control sample to set the gates.(22) These gates were applied across all samples evaluated with each assay.

CtDNA monitoring

To evaluate the predictive value of ctDNA, we monitored a subgroup of patients (n=8), at the time of inclusion and at different time points during first-line treatment. Then, we compared ctDNA levels and radiological findings during oncological follow-up.

Statistical analysis

The demographic, pre-, and perioperative characteristics of patients were compared by the Chi-square or Fisher's exact test. Continuous data were analyzed with the independent-samples t-test. The cut-off date for analysis was October 2015. Survival rates were calculated using the Kaplan-Meier method.(25) Overall survival (OS) was calculated from the date of diagnosis until death from any cause. Disease free survival (DFS) was calculated from the date of surgery until first recurrence or death.

The Cox proportional-hazards regression model was used to perform univariate and multivariate analyses with 95% confidence interval (CI). Multivariate analysis was performed with variables associated with the outcome in univariate analysis at a P value of < 0.1. All statistical analyses were performed using SPSS software version 21.0 (SPSS Inc., Chicago, IL). A *P* value ≤ 0.05 was considered as significant.

RESULTS

Study population

One hundred and thirty-five patients with resectable (n=31; 23%), locally advanced (n=36; 27%) or metastatic (n=68; 50%) PAC were included in this prospective study (**Table 1**). Median age of patients was 65.6 years (range 39.2-87.3). There was no difference between the 3 groups in demographic and biological characteristics excepted for baseline CA 19-9 (P<0.001). Median coverage depth of sequencing was 5,813 reads (range 842-15,803). Mean and standard deviation of free plasma DNA concentration was 92 ± 201 ng/mL, with 52.5 ± 79.5 ng/mL in patients with resectable tumor, and 105.8 ± 227.25 ng/mL in advanced PAC (P=0.05).

DNA Sequencing

In patients with advanced PAC (n=104), 50 (48%) of them had ctDNA detectable by at least one cancer specific gene mutation with a MAF of 6.1% (range 0.1-65.4). Of this group 43 (41.3%) had a *KRAS* mutation (G12D n=18; G12V n=18; G12R n=4; G12C n=1; Q61H n=2). *TP53, SMAD4, NRAS, PIK3CA* and *STK11* gene mutations were detected in 23, 8, 2, 1 and 1 cases, respectively. In the group of patients with detectable ctDNA, 43 (86%) had a *KRAS* mutation, and 27 (54%) had at least 2 mutations detected (**Figure 1**). CtDNA detection was strongly correlated to grade of tumor differentiation: 65% of patients in cases of undifferentiated tumors vs. 58% in moderately differentiated vs. only 30% in well-differentiated tumors (P=0.036 and P=0.042). Median CA19-9 was 380 UI/mL in patients with undetectable and 2748 UI/mL in patients with detectable ctDNA (P=0.015). Forty-four patients (64.7%) with metastatic disease had detectable ctDNA in comparison to only 6 patients (16.6%) with locally advanced PAC (P<0.001). In the subgroup of metastatic patients, no significant correlation was found between presence of ctDNA and the number of metastatic sites (P=0.13).

In patients who had curative resection (n=31), ctDNA was detected in 6 of them (19%), with a median MAF of 4.4% (range 0.7-8.7). *TP53* mutation was observed in 4 cases, *KRAS* and *TP53* mutations in 1 case and ERBB4 mutation in 1 case.

Correlation between NGS and dPCR

All *KRAS* mutations detected by NGS were confirmed by droplet-based dPCR in microfluidics. One patient, considered as negative after NGS was detected positive with dPCR for the *KRAS* G12D mutation, with a MAF of 0.61%. Correlation between the two technics revealed a high concordance with R^2 of 0.94 (**Supplementary Data File 2**).

Prognostic Value of ctDNA in advanced PAC

After a median follow-up of 34.2 months, 76 patients died (73.1%). Presence of ctDNA was strongly correlated with poor OS (6.5 versus 19.0 months; log-rank P<0.001) in patients with advanced PAC (**Figure 2A**).

To evaluate the impact of ctDNA level, patients were grouped according to mutation allelic ratio tertiles. Patients with the higher MAF had the worst OS (**Figure 2B**). The OS decreased from 18.9, 7.8 and 4.9 months (log-rank P<0.001) for the lowest, middle and highest MAF tertiles, respectively. In multivariate analysis, including ctDNA, age, gender and stage disease, ctDNA was independently associated with poor OS (HR=1.96; $CI_{95\%}$ [1.20-3.20]; P=0.007) (**Table 2**).

Twenty-seven patients (54%) had at least 2 mutations detected. To evaluate the impact of presence of multiple mutations, patients were divided in two groups: « *KRAS* only » or « *KRAS* + other » when *KRAS* mutation was associated with at least another mutation. There was a trend illustrative of patients with multiple mutations having a poorer prognosis, but the difference was not statistically significant (median overall survival 3.1 vs. 8.6 months; P=0.128; see **Supplementary Data File 3**).

Similarly, in the subgroup of patients with a *KRAS* mutation, there was no significant difference in overall survival for particular individual *KRAS* mutations: *KRAS* G12V vs.

others: median overall survival 4.9 vs. 9.0 months (P=0.507); *KRAS* G12D vs. others: 4.9 vs. 5.5 months (P=0.594).

Preliminary data from monitoring ctDNA in 8 patients suggest that evolution of ctDNA levels was correlated with chemotherapy efficacy and objective radiological response (see **Supplementary Data File 4**).

Prognostic Value of ctDNA in resected patients

After a median follow-up of 33.3 months, tumor relapse occurred in 23 patients, 13 of them died. Median delay between surgical resection and blood sample collection was 60.5 days (range 37-123). All patients with detectable ctDNA (n=6) present positive lymph nodes and 5 of them had a pT3 tumor.

In this subgroup of resected patients, those with undetectable ctDNA after surgery had a longer DFS (17.6 versus 4.6 months; log-rank P=0.03), and a longer OS (32.2 versus 19.3; P=0.027) than those with detectable ctDNA (**Figure 3A and 3B**). Among 6 patients with detectable ctDNA, tumor relapse occurred in 4 patients. Progression using ctDNA was detected at an average of 2.4 months compared with 4.0 months using standard CT scan (P=0.043).

DISCUSSION

In this series, we showed that ctDNA can improve the prognostic staging of metastatic and locally advanced PAC. Our work highlights that the detection and evaluation of the quantity of ctDNA appears suitable as an independent prognostic factor in stage III or IV PAC, and a prognostic factor of recurrence in resected patients when detected after surgery.

Somatic mutations were analyzed in plasma DNA samples by NGS without any information on primary tumor mutational status. All *KRAS* mutations were also validated with picoliter droplet digital PCR that provides both higher sensitivity and lower cost than NGS but is reduced in the number of mutations that can be interrogated. A strong correlation was observed between the AF measured by both approaches suggesting that our NGS strategy led to quantitative results. In pancreatic cancer patients the diagnostic benefit of optimized NGS compared to dPCR is approximately 15% of the cases (ie. patients with a pancreatic cancer without *KRAS* mutations). The use of one technique or the other is likely to be linked to the workflow of the laboratory. Improvement of sensitivity is likely to be linked to testing of an increased quantity of plasma DNA. In dPCR higher DNA input amounts could be used with an associated increase in sensitivity being achievable. We indeed previously described sensitivity of up to 0.0005%. However, only 6µL of DNA was available for droplet based digital PCR analysis in this particular study. The use of a higher quantity of plasma DNA could have allowed the detection of a higher fraction of patients with *KRAS* mutated ctDNA.

Bettegowda *et al.* reported that ctDNA somatic mutation can be detected in >50% of patients with several cancers, including PAC, even in a localized stage.(12) Moreover, Sausen *et al.* recently demonstrated that mutations detected in tumor specimens from 22 patients were detectable in the plasma at diagnosis with a specificity >99.9% confirming that the mutated fraction of circulating DNA arises from tumor tissues.(16) Although all our patients had histologically proven PAC, only 48% had detectable ctDNA in the advanced subgroup. Focusing on metastatic patients, this rate increased to 64.7%. These results are

consistent with Kinugasa *et al.*'s cohort of 66 PAC patients screened for *KRAS* mutations, reporting 54.5% (n=36) of samples detectable for ctDNA in blood samples despite a significant number of metastatic cases in their cohort (n=57).(26) However, Bettegowda *et al.* reported a higher ctDNA detection rate in metastatic PDAC with nearly 90% of patients with detectable ctDNA. This discrepancy may be explained by methodological differences and, more importanty quantity of DNA sequenced in each assay in their study.(19)

The development and growth of PAC involves oncogene activation or loss of tumor suppressor genes function.(27,28) *KRAS* is the most common of these genes and one of the driver of mutations in PAC. Mutations in *KRAS* was the most frequently detected type in our cohort, in isolation or associated with other genes alterations, and present in 86% of patients with ctDNA detected. Our findings in blood are concordant with those previously published from PAC tissue, which report a rate of *KRAS* mutation in PAC of about 80-90%.(16,26) We retrospectively assessed the *KRAS* and *TP53* tumor status in 20 tumor samples. We found an agreement between tumor tissue and plasma ctDNA in 19 out 20 couples tested (95%) both for *KRAS* and *TP53*. The 2 discordant cases (one for KRAS, one for TP53) were positive in plasma, negative in tumors suggesting a potential sampling problem owing to biopsy from a unique metastatic site (data not shown).

Some authors demonstrated that gender, chronic inflammation, age or tumor heterogeneity could influence the level of ctDNA.(29) In this series, most patients with detectable ctDNA had moderate or undifferentiated tumors (p=0.037). Tumor differentiation thus seems to impact the ctDNA level in PAC. Although we did not observe a significant correlation between the number of metastatic sites and the presence of ctDNA, we cannot exclude a relationship between the tumor mass and the presence of ctDNA. The presence of a pancreatic metastatic disease is not synonymous with to be correlated with differentiation grade and could more reflect tumor aggressiveness than tumor burden.

One of the important results in our study was the prognostic value of the presence and level of ctDNA. In the advanced subgroup, ctDNA was an independent prognostic biomarker of OS (HR=1.94; P=0.007). The presence of ctDNA after surgery appeared to be a

prognostic factor of poor DFS and OS. Chen *et al.* have previously described the prognostic value of this biomarker in PAC.(20) In their series, the presence of *KRAS* mutation in plasma was correlated with poor OS (3.9 vs. 10.2 months; P<0.001) in non-resectable patients. More recently, Sausen *et al.* reported that, in resectable patients, ctDNA was a prognostic factor of early tumor relapse if detected before surgery (Log Rank P=0.015). In this study, in a subgroup of 20 patients collected after surgical resection, detectable ctDNA was also a prognostic biomarker of DFS (9.9 months vs. median not reached; Log Rank P=0.02).(16)

Although, in resected patients, the presence of ctDNA after resection could suggest the existence of a micro-metastasis disease, studies are needed to determine if the presence of ctDNA may be used for early detection of PAC. Based on our results we make the educated guess that the detection of small pancreatic tumors will be difficult without improving the quantity of input DNA and consequently the volume of plasma used for this purpose.

Although the blood samples of our patients are prospectively and consecutively collected the study presents heterogeneity in treatments received owing to difference in age, treatment tolerance, or performance status of patients. This heterogeneity could induce a bias in the results even if multivariate analysis confirmed the prognostic value of ctDNA. Secondly, our monitoring results are preliminary without perfect concordance between sample collection and radiological evaluation of patients for example. In the resectable subgroup, there is a lack of pre-operative ctDNA data. Indeed, patients were collected at the first cycle of adjuvant treatment, and our protocol did not include samples before surgery. Finally, despite careful collection and storage of samples, and the use of highly sensitive methods to detect genetic changes, some alterations may not have been detected due to limited plasmatic DNA amounts or very low mutant allele frequency.

CONCLUSION

This study demonstrates that ctDNA can be detected in peripheral blood in PAC, and that it appears as an independent prognostic marker of overall survival in locally advanced or metastatic diseases. Furthermore, it arises as an indicator of shorter disease free and overall survival in resected patients when detected after surgery. The describe procedure may have great potential as a new simple and non-invasive strategy for patients' care and follow-up. Further investigations are needed to confirm these results and their usefulness in the prognosis and in-risk group screening of patients.

Moreover, ctDNA would be of great interest: 1/ to allow a rapid molecular analysis for inclusion in "molecular" trials with targeted therapies, 2/ for initial diagnosis in the case of difficulty in obtaining histological proof.

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Table 1.

TABLE 1. Demographic and clinicopathologic characteristics of study population

Variable	**Total n = 135**	**Resectables n = 31**	**LA n = 36**	**Metastatics n = 68**	***p***
Sex					ns
Male	85 (63%)	17 (62%)	24 (66%)	44 (65%)	
Female	50 (37%)	14 (38%)	12 (34%)	24 (35%)	
Age, median (range), yr	65.6 (39.2-87.3)	67.9 (46.0-85.1)	64.7 (40.5-84.6)	66.9 (39.2-87.3)	ns
Body Mass Index, (range), kg/m^2	23.3 (14.5-42.3)	24.3 (19.1-32.5)	24.3 (16.7-38.2)	22.2 (14.5-42.3)	
Tumor location					
Head and isthmus	94 (70%)	24 (77%)	24 (67%)	46 (68%)	
Body	18 (13%)	3 (10%)	5 (14%)	10 (15%)	
Tail	23 (17%)	4 (13%)	7 (19%)	12 (17%)	
Differentiation grade (n=98)					
Well	31 (32%)	11 (35%)	10 (43%)	10 (23%)	
Moderate	39 (40%)	10 (32.5%)	7 (31%)	22 (50%)	
Poor	28 (28%)	10 (32.5%)	6 (26%)	12 (27%)	
Baseline CA 19-9, median (range), UI/mL	238.0 (0.6-636000)	26.0 (0.6-2225)	179.0 (1.0-10970)	2748.0 (1.5-636000)	***<0.001***

LA indicates locally advanced tumors

Table 2. Prognostic factors for overall survival at univariate and multivariate analysis in advanced PAC

TABLE 2. Prognostic factors for disease-free survival at univariate and multivariate analysis in advanced PAC

		Univariate Analysis		**Multivariate Analysis**	
	N° patients N=104	**Hazard ratio (95% CI)**	***P-value***	**Hazard ratio (95% CI)**	***P-value***
Age*		1.05 (0.99 – 1.04)	0.249	1.01 (0.97 – 1.04)	0.608
Gender (Male)	66	0.74 (0.45 – 1.19)	0.207	0,65 (0.38 – 1.16)	0.147
Metastatic disease	68	**3.22 (1.90 – 5.45)**	**<0.001**	**2,87 (1.52 – 5.42)**	**<0.001**
CA 19-9 > 200 UI/mL	59	1.33 (0.78 – 2.27)	0.30	1.14 (0.65 – 2.00)	0.64
ctDNA detectable	50	**2.24 (1.41 – 3.54)**	**0.001**	**1.99 (1.13 – 3.50)**	**0.016**

* Continuous variable
PAC indicates Pancreatic Adenocarcinoma
ctDNA indicates circulating tumor DNA

Figure legends:

Figure 1. Mutated genes distribution in advanced patients with detectable ctDNA.

Figure 2. Survival curves according to detectable ctDNA in advanced patients (A) Overall survival (B) Overall survival according to ctDNA tertile

Figure 3. Survival curves according to detectable ctDNA in resected patients. (A) Disease Free Survival (B) Overall survival

Figure 1. Mutated genes distribution in advanced patients with detectable ctDNA.

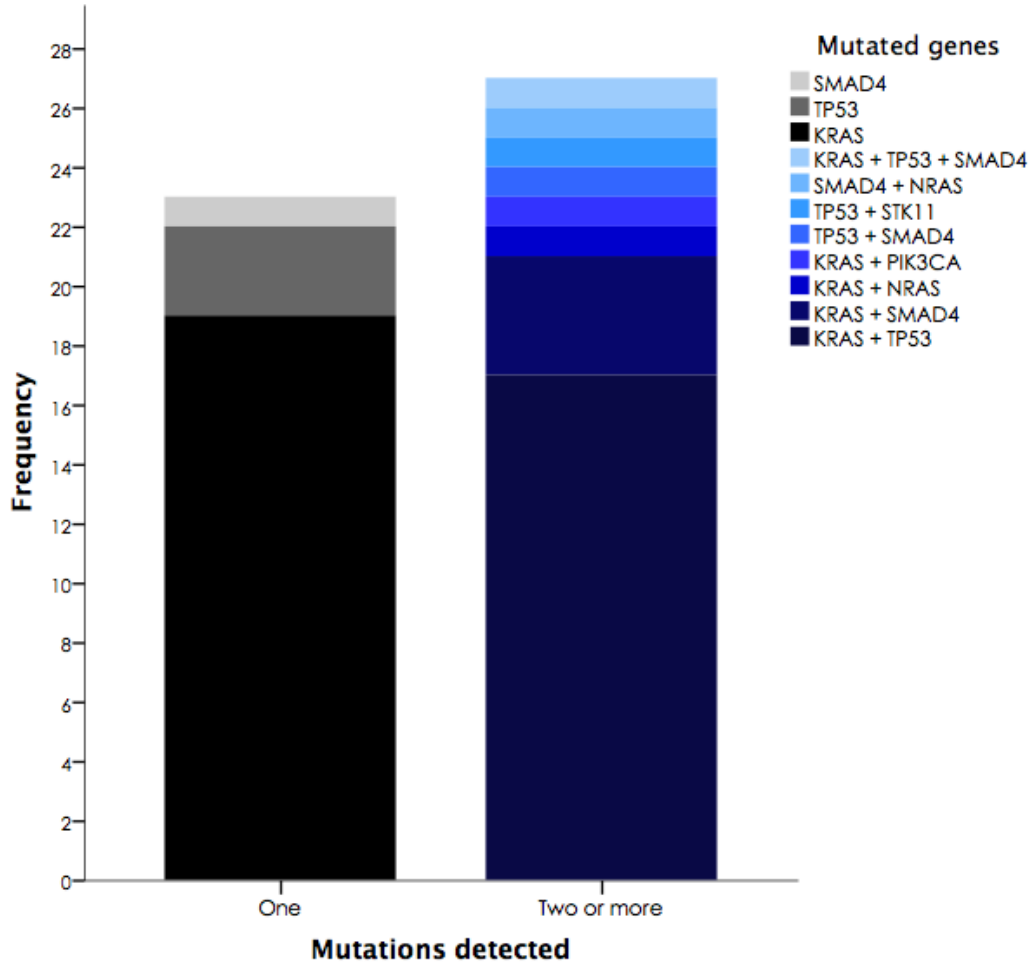
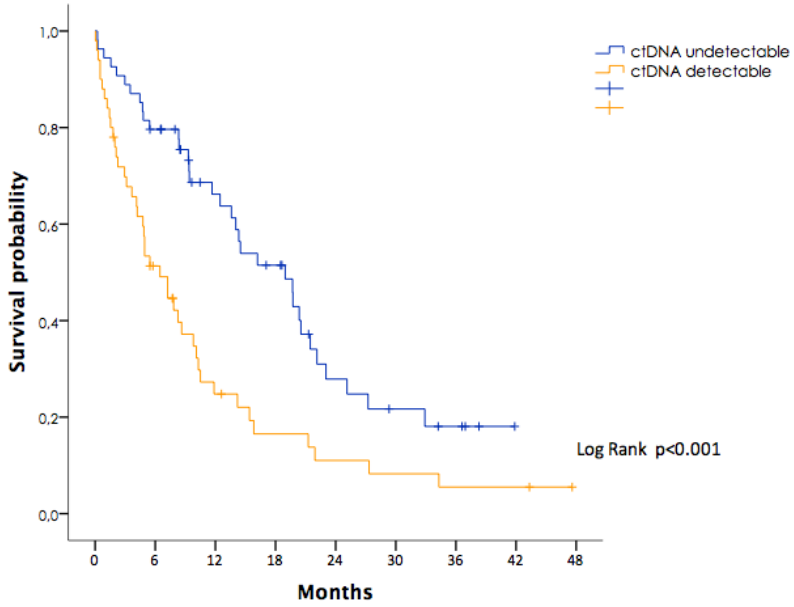


Figure 2. Survival curves according to detectable ctDNA in advanced patients (A) Overall survival (B) Overall survival according to ctDNA tertile

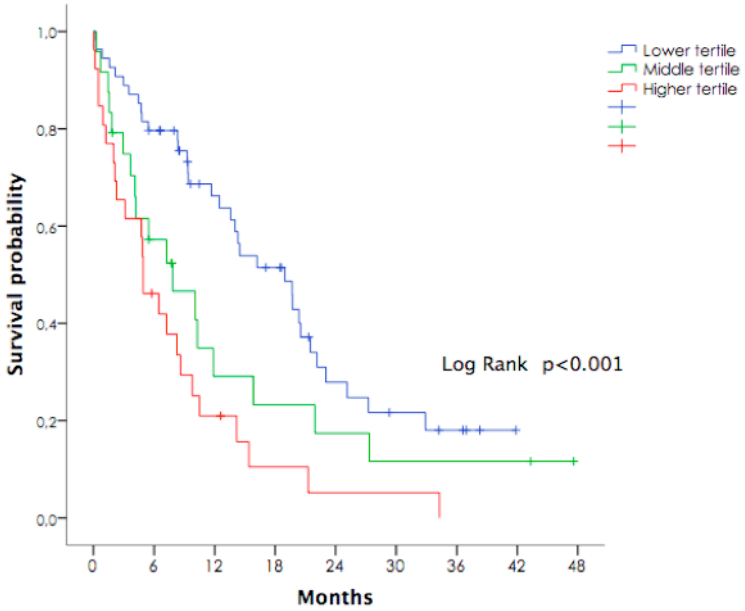
A



Number of patients at risk

	0 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo
ctDNA undectectble	54	38	27	18	8	5	2	0
ctDNA detectable	50	22	10	6	4	3	2	1

B

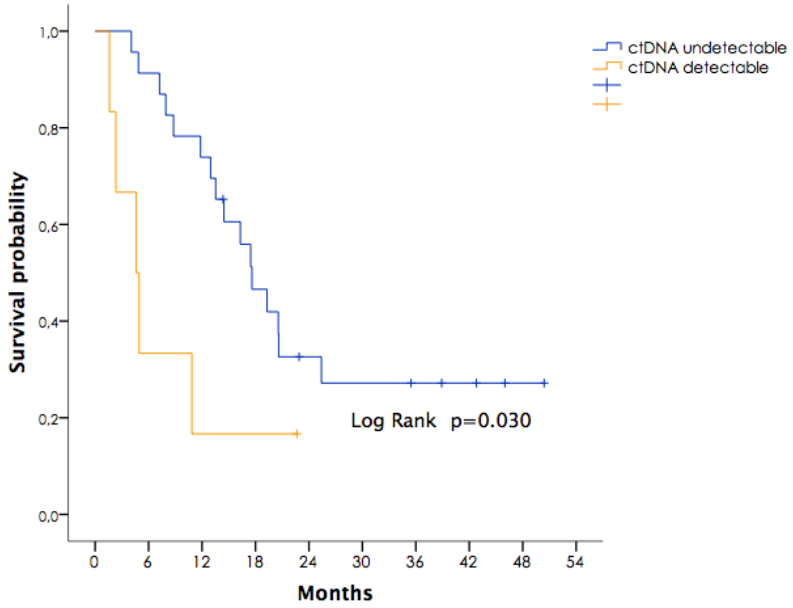


Number of patients at risk

	0 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo
Lower tertile	54	38	27	18	8	5	2	0
Middle tertile	25	11	5	4	3	2	2	1
Higher tertile	25	11	5	2	1	1	0	0

Figure 3. Survival curves according to detectable ctDNA in resected patients. (A) Disease Free Survival (B) Overall survival

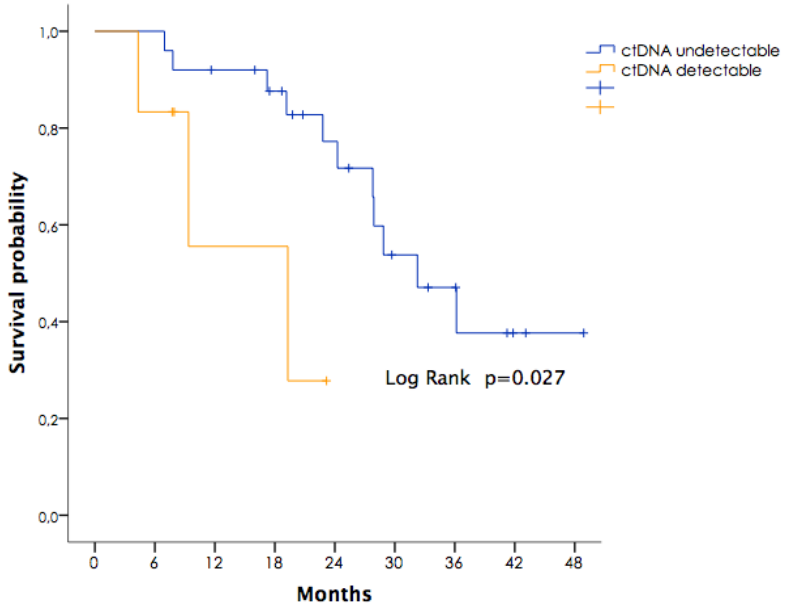
A



Number of patients at risk

	0 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo	48 mo
ctDNA undetectable	25	21	16	9	6	4	3	2	1
ctDNA detectable	6	2	1	1	0	0	0	0	0

B



Number of patients at risk

	0 mo	6 mo	12 mo	18 mo	24 mo	30 mo	36 mo	42 mo	48 mo
ctDNA undetectable	25	24	21	17	13	7	4	1	1
ctDNA detectable	6	4	2	1	0	0	0	0	0

Supplementary Data File 1: Supplementary NGS analysis method specifically developed to detect low allele frequency mutations.

The BAM files produced by IonTorrent® Suite V4.2.1 were processed by GATK 3.4 [1] for realignment around indels and base quality recalibration. The analysis of aligned sequences (BAM) was performed on 8,741 positions (Supplementary Table 1) of the panel after exclusion of eight bases at 5'- and 3'- ends of each amplicon and exclusion of known single nucleotide polymorphism with allele frequency >0.01% in Exome Aggregation Consortium variants v0.3. Nucleotides (A,T,C,G) and INDEL >2 nucleotides counts were obtained from reads with MAPQ>5 and BAQ>20 using Rsamtools [2]. The position-error rate (PER) of the sequencing method was measured on each genomic position in 29 negative controls. Single nucleotide variations (SNV) and insertions/deletions (INDEL) were analyzed separately. PER was defined at each base position as the sum of non-reference alleles in 29 controls/sum of depth in 29 controls. For each tested sample the minor allele frequency (ie, second most frequent allele) at each position was compared to its specific PER using a binomial test. SNV or INDEL strand bias (SB) was evaluated by the Symmetric Odds Ratio (SOR) test developed by GATK [3] and computed as log(ratio*(R+1/R)) where ratio = [min(Ref_plus, Ref_minus)/max(Ref_plus, Ref_minus)] / [min(Alt_plus, Alt_minus)/max(Alt_plus, Alt_minus)] and where R = (Ref_plus * Alt_minus) / (Ref_minus * Alt_plus). SNVs with SOR > 2.5 were excluded if non-hotspot. Less stringent criteria was applied for hotspot positions and INDEL using SOR > 3.1. Hotpost positions are listed in Supplementary Table 1.

Finally, for a given sample, an outlier detection method was applied on all retained p-values of the sample. Only outlier p-values < 10^{-6} were considered as a true mutation.

The complete method has been implemented in a *R* package BPER, available under request. (manuscript submitted)

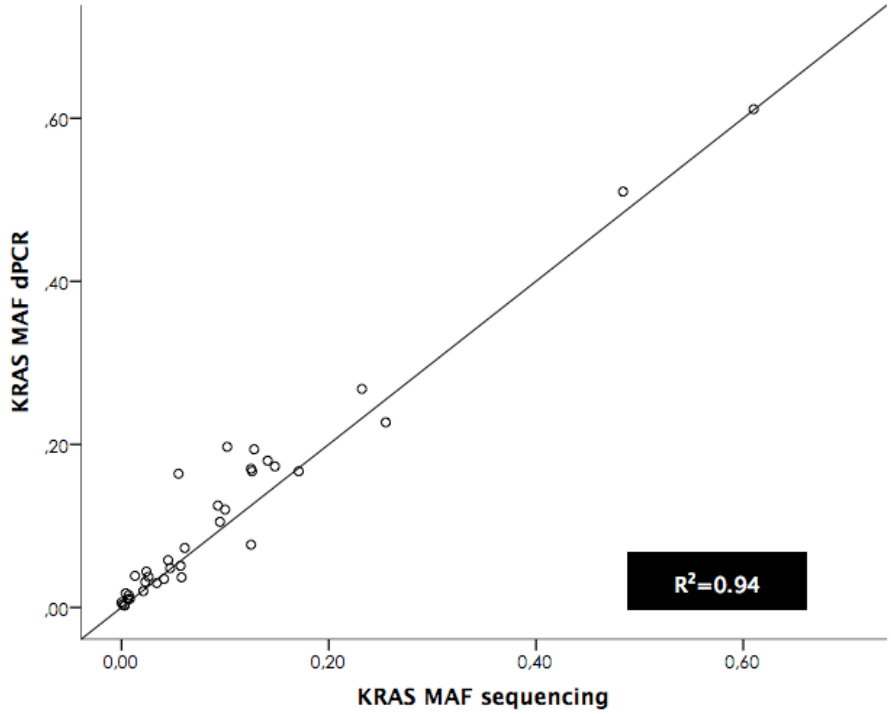
References:

1. McKenna A, Hanna M, Banks E, Sivachenko A, Cibulskis K, Kernytsky A, Garimella K, Altshuler D, Gabriel S, Daly M, DePristo MA (2010). The Genome Analysis Toolkit: a MapReduce framework for analyzing next-generation DNA sequencing data. GENOME RESEARCH 20:1297-303

2. Morgan M, Pagès H, Obenchain V and Hayden N. Rsamtools: Binary alignment (BAM), FASTA, variant call (BCF), and tabix file import. R package version 1.22.0, http://bioconductor.org/packages/release/bioc/html/Rsamtools.html.

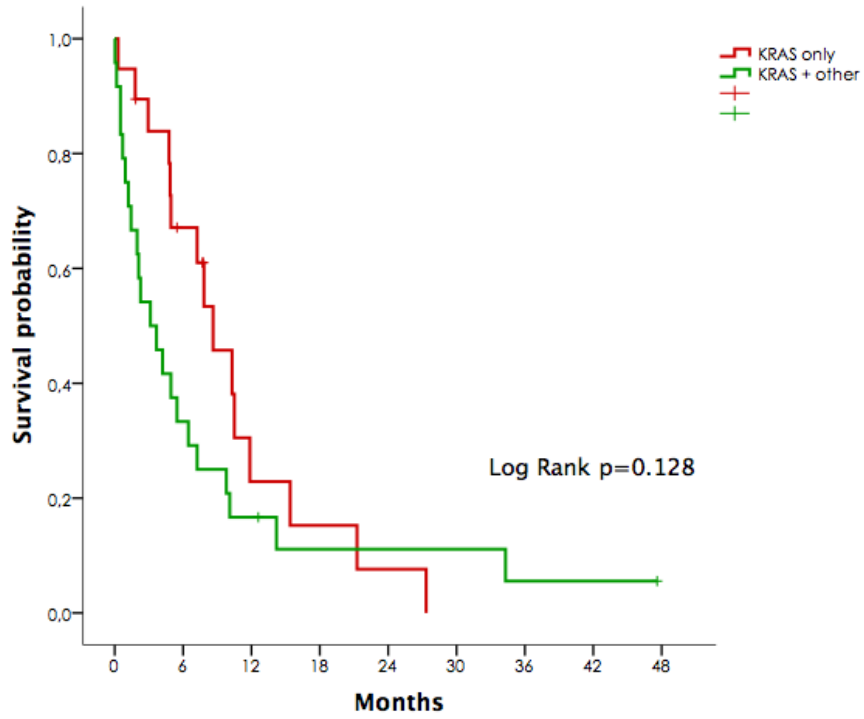
3.https://www.broadinstitute.org/gatk/guide/tooldocs/org_broadinstitute_gatk_tools_walkers_annotator_StrandOddsRatio.php

Supplementary data file 2. Correlation between Mutation Allelic Frequency (MAF) according to Next Generation Sequencing and picoliter droplet based digital PCR



Supplementary data file 3: Survival probability according to KRAS mutation.

Patients divided in « *KRAS* only » or « *KRAS* + other » when *KRAS* mutation was associated with at least another gene mutation.



Supplementary data file 4:

Preliminary data of monitoring ctDNA in 8 patients suggest that evolution of ctDNA levels was correlated with chemotherapy efficacy and objective radiological response.

N° Patient	**Day**	**ctDNA** mutated allelic frequency	**CTScan** RECIST Tumor size
Patient 1	D0	25%	Reference
	D45	7.8%	-3%
	D55	0.5%	
	D65	0.6%	-25%
Patient 2	D0	30%	Reference
	D37	0.2%	
	D67	0%	-57%
Patient 3	D0	25%	Reference
	D30	0%	-23%
	D60	0%	
Patient 4	D0	0.7%	Reference
	D30	0%	
	D60	0%	-30%
Patient 5 (resected)	D0	0.7%	tumor resection
	D30	0.6%	
	D60	0.8%	
	D120		Local progression
Patient 6	D0	0.1%	Reference
	D30	0%	-33%
	D150		-33%
Patient 7	D0	41%	Reference
	D30	33%	
	D50		+50%
Patient 8	D0	0.8%	Reference
	D30	0%	-10%
	D60	0%	-23%