Determination of Exosome Mitochondrial DNA as a Biomarker of Renal Cancer Aggressiveness

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Research

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Abstract

Background: Components of liquid biopsy are promising non-invasive biomarkers for monitoring the renal cell carcinoma (RCC) status. Present study tries to evaluate the role of exosomes and mitochondrial DNA (mtDNA) as promise, novel and stable biomarkers that could improve current ones in RCC.

Methods: A total of 140 fractions obtained by ultracentrifugations of whole blood samples stored in EDTA anticoagulant from 28 (13 patients and 15 controls) were included in present study. An exosome collection protocol and exhaustive analysis of all phases obtained by ultracentrifugations (named from B to F) was performed. Subsequently, an analysis of dPCR (digital PCR) using the QuantStudio™ 3D Digital PCR platform and the quantification of mtDNA copy number using by QuantStudioTM 12K Flex Real-Time PCR System (qPCR) was developed. Moreover, Next Generation Sequencing (NGS) analyses were included using MiSeq system (Illumina, CA, USA).

Results: F fraction, which contains all exosomes´ data and all mitochondrial markers (hypervariable region 1 (*HV1*) and apocytochrome b of complex III (*MT-CYB*)) were statistically identified in dPCR and qPCR (p values < 0.05) when a comparison between cases and controls was performed. Moreover, the studied mitochondrial genes shown a relevant significance in RCC aggressiveness analyses (metastasis and stages 4).

Conclusions: To sum up, this is the first time a relation between mtDNA genetic markers in exosome and clinical management of RCC is suggested.

Background

Renal cell carcinoma (RCC) is the third most common urologic malignancy, and remains being one of the most lethal among urological ones [1]. The incidence of RCC is increasing globally, with rates varying by country, age, race, and sex [2]. Major troubles and sceneries in managing this disease are mainly two: i) Unspecific symptoms causing diagnosis in high stages; and ii) Incidental detection of RCC by abdominal imaging techniques favours a decrease in the stage of new diagnosed tumours. These both aspects reinforce the need of identifying novel predictive biomarkers for RCC diagnosis, progression and prognosis [3]. RCC is not a single entity, but includes various tumour subtypes that have been identified on the basis of either characteristic pathologic features or distinctive molecular changes [4].

One of the major challenges of personalized oncology lies in identifying predictive biomarkers of response to therapy for their direct use in clinical setting [5]. For that reason, there are many efforts in searching biomarkers for a proper stratification that will help with an accurate treatment by the differentiation of diverse subtypes [6]. There are some data that initiate a correlation between some expression patterns and worse prognosis in kidney renal clear cell carcinoma; such as *AGXT, PTGER3* and *SLC12A3*; or a reduced survival such as *ALOX5* [7]. Or the role, indicating that high expression patterns of MXD3 is an independent risk factor for poor prognosis in ccRCC (clear cell renal cell carcinoma) [8]. There is scarce data about single nucleotide polymorphisms (SNPs) impact as biomarkers in RCC, it is well to

name the role of rs10932384 (*ERBB4*) consistently associated with both recurrence and overall survival in stages I–III in RCC patients [9]. Several genes, such as *PAK1* and *PIK3R1*, have been indicated with a crucial role in cell migration and mobility in RCC pathway by computational analysis data [10]. Others studies, reported that over-expression of miR-15a was strongly associated with poor histological prognostic features of ccRCC, suggesting miR-15a as a potential prognostic molecular biomarker [11]. Target genes of miR-576 (*CUL3* and *RAC1*) have been identified as involved in the regulation of multiple cancer-related biological pathways, and the target gene of miR-616 (*ASB13* and *FBXW2*) has been reported to be associated with the development of other cancers. Finally, these data support that miR-576, miR-616 and miR-133a-2 may have guiding significance for the early diagnosis of RCC [12]. Or in the case of miR-103a-3p, suggested as a reflection of pathology and treatment response in other renal diseases [13].

In relation to the role of non-invasive biomarkers such as exosomes, it has been suggested that increased patterns of Polymerase I and transcript release factor (PTRF)/Cavin1 detected in urine exosomes of ccRCC, are suggested as biomarkers in this tumour [14]. Moreover, urinary exosomal are one of the best options. Recent studies suggest that changes in mRNA levels of urinary nano extracellular vesicles reflect the disease status of kidney tissues and their functional alterations [15]. Others studies correlate the role of miR-30c-5p in urinary exosomal with ccRCC progression by *HSPA5* expression modulation [16]. Furthermore, recent reports indicate the full-length form of (pro)renin receptor ((P)RR), a single transmembrane protein encoded by the *ATP6AP2* gene, as a novel biomarker for cancer diagnosis, severity evaluation and prognosis prediction. This is also a promising therapeutic target for cancer, including RCC [17]. There are others reports that indicated the role of mtDNA (mitochondrial) copy number as biomarker of risk in head and neck squamous cell carcinoma [18].

Circulating cell-free tumour DNA (ccfDNA) is an interesting tool in the field of oncology. ccfDNA has been investigated as a potential biomarker in non-invasive diagnosis and prognosis, as well as disease monitoring. It can be easily isolated from body fluids or blood. Its origin is thought to be mainly from apoptotic or necrotic cell death, although active release mechanisms have also been suggested [19]. Numerous studies have confirmed that there is an elevated level of ccfDNA in blood stream of cancer patients in comparison with healthy controls. These findings have opened up new possibilities for the development of clinical applications; Non-Invasive Prenatal Testing (NIPT), cancer diagnosis, transplantation medicine, and virology [20, 21]. Valpione et al. [22] demonstrated the potential role of ccfDNA as a biomarker of tumour burden in metastatic melanoma patients, and that is a prognostic factor for overall survival. It is good to mention that; ccfDNA includes nuclear DNA (nDNA) and mtDNA.

mtDNA exists as a circular, double-stranded nucleic acid with a high copy number. Variations in the copy number of circulating cell-free mtDNA (ccf-mtDNA) have been found in plasma and serum of patients with various tumours like breast, renal, ovarian or lung one [23, 24]. It is known the role of mtDNA and its susceptibility to oxidative stress and mutation [25], for that reason, there are many new research focused on this molecule. There are some studies that found that mtDNA copy number in peripheral blood is associated with risk of developing several cancers. That is the case of the relationship between mtDNA

copy number (mtDNAcn) and the risk of colorectal cancer [26]. Recent data suggested mtDNA as a molecule with relevant prognostic value and staging in cancer [27].

To sum up, even there are many biomarkers in clinical practice such as, imaging molecular, magnetic resonance imaging, texture analysis and radiomics or tissue biomarkers (Immunohistochemistry, mainly by the analysis of expression of *PAX8* and *PAX2*). There is much interest in determining serum (most of them proved in VEGF pathways or *VHL* gene) and urine biomarkers (two urinary biomarkers that have shown promise are the exosomal proteins aquaporin-1 (AQP-1) and perilipin-2 (PLIN2)) [6]. Here we reinforce the role of non-invasive biomarkers in liquid biopsy, mainly in exosomes and mtDNA as promise, novel and stable biomarkers that could improve current ones in RCC.

Methods

Patient selection and sample collection

Participants enrolled in this study and comprised the patient group (n = 13) were recruited by urologists of “Virgen de las Nieves University Hospital”, Granada, Spain. People whose blood samples were obtained from clinics “Gran Capitán, Salvador Caballero and Caseria de Montijo” of Granada, were considered as controls (n = 15). For more details see Table 1. Samples were processed 4 hours later from the collection at collaborators hospitals. To obtain plasma, peripheral whole blood samples stored in ethylenediaminetetraacetic acid anticoagulant (EDTA) were centrifuged for 10 minutes at 1400g and at 4°C (more details in Supplementary Figure S1). After separation, plasma samples were frozen at -80°C until future analysis. The study protocol was approved by the Ethics Committee (CEI) with internal code 0165-N-19. Informed written consent from all participants was obtained in accordance with the tenets of the Declaration of Helsinki.

Table 1
Characteristics of the study population

Characteristic	Patients (n = 13)	Controls (n = 15)
Age (yr)		
Median (range) - yr	68 (47–88)	67 (44–93)
< 65 yr	5	6
> 65 yr	8	9
Sex		
Male	11	7
Female	2	8
Histology		
Papillary	1	NA
Clear cell	12	NA
Size tumour (cm)	9 (5–18)	NA
Stage		
Stage I	1	
Stage II	0	
Stage III	7	
Stage IV	5	
TNM		NA
T1	1	
T2	2	
T3	9	
T4	1	
Fuhrman nuclear grade		NA
G2	3	
G3-4	7	
G4	3	

Abbreviations: *NA* Not applicable, *yr* years

Characteristic	Patients (n = 13)	Controls (n = 15)
Metastasis		NA
No	7	
Yes	6	
Abbreviations: *NA* Not applicable, *yr* years		

Isolation and extraction of exosomes from plasma samples

To carry out exosomes collection we started with 1 ml of plasma that has been previously frozen at -80°C. In order to obtain different of interest plasma fractions, successive centrifugations were carried out; with the main aim of eliminating free part of exosomes containing apoptotic bodies and microvesicles (Table 2). Afterwards, an ultracentrifugation was performed (160,000g at 4°C for 2h) for obtaining rich in exosomes purified fraction (phase F).

Table 2
Fractions obtained during exosomes isolation

Fraction	Sample	Obtaining
B	200 µl plasma	Plasma obtained after centrifugation (1,400 g, 4°C, 10 min)
C	Pellet	Pellet obtained after centrifugation with DTT + PBS (16,000 g, 4°C, 20 min)
D	200 µl supernatant	Supernatant obtained after centrifugation (15,000 g, 4°C, 30 min)
E	6 ml supernatant	Supernatant obtained after ultracentrifugation (160,000 g, 4°C, 2 h)
F	Pellet	Pellet obtained after ultracentrifugation (160,000 g, 4°C, 2 h)

With the purpose of analyzing different plasma fractions, aliquots of 200 µL were taken from supernatants obtained after centrifugations (phases B and D). Sediment from the second centrifugation (phase C) was also analyzed, as well as the supernatant after ultracentrifugation (phase E), see details in Supplementary Figure S1.

Therefore, a total of 5 aliquots corresponding to phases B to F were taken for each subject (n = 28), obtaining a total of 140 samples. All of them were kept at -20°C until their subsequent extraction. All these phases were concentrated at 43°C (Thermo Scientific™ Savant™ DNA 120SpeedVac™ Concentrator), and then DNA extraction was carried out according to the method detailed by Freeman B et al. [28], a non-organic (proteinase K and salting out) protocol with some modifications described in Gomez-Martín et al. [29]; subsequently quantified by Qubit Fluorometer and NanoDrop2000c systems (Thermofisher Scientific, MA, USA).

Quantification absolute of mtDNA in a control sample by Digital PCR (dPCR)

To determine the number of mtDNA copies in our quantified control DNA (qcDNA), we used two TaqMan® probes (Thermofisher Scientific): TaqMan-FAM™ (target gene, *mtDNA_LP*) and TaqMan-VIC™ (endogenous gene, *RNAse P*). Probe sequence used for *mtDNA_LP* was 5'-TCGGCAAATCTTACCCC-3'. *RNase P* gene was run in the same PCR as an endogenous gene to determinate the target gene in each sample.

A five-fold dilution series were prepared to calculate the number of copies/µl in the control sample. Reactions were incubated at 96°C for 10 min, followed by 39 cycles of 60°C for 2 min; 98°C for 30 s, and 60°C for 2 min with a hold of 10°C. A 9800 dual PCR System (ThermoScientific) was used for the amplification. The QuantStudio™ 3D Digital PCR (dPCR) instrument was used to analyze the chips following the instructions in the "QuantStudio ™ 3D Digital PCR System User Guide" [30].

Then the control sample was used as template for qPCR. A standard curve was constructed by plotting Ct (cycle threshold) values against the concentration of the mtDNA with different concentrations. The amount of mtDNA of patients and controls groups was quantified by interpolating the Ct values in the standard curve.

Determination of the relative concentration of mtDNA by Real Time PCR (qPCR)

Quantification of mtDNA copy number was performed by QuantStudioTM 12K Flex Real-Time PCR System (qPCR) (ThermoScientific) according to the manufacturer's protocol (iTaq™ Universal SYBR® Green One-Step Kit, Bio-Rad) [31].

Primers were designed for three different regions: mitochondrial hypervariable region 1 (*HV1*), apocytochrome b of complex III (*MT-CYB*) and the hemoglobin subunit beta (*HBB*) gene as reference gene. In addition, two fragments of different length were designed; one of short size between 75–100 bp and another one amplicon with range from 175–200 bp for long one. Primer sequences used for the different regions are shown in the Supplementary Table S1. All these primers were used at a final concentration of 10 µM.

All reactions were incubated in a 96-well plate at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, and 60°C for 1 min. All qPCR reactions were done in triplicate and the negative controls (NTC) were included with every qPCR assay. To increase the statistical power, each replicate was deemed as an individual value. No amplification of the signal was observed when water was added instead of cDNA sample.

Next Generation Sequencing (NGS) analyses and data processing

DNA-sequencing analysis was performed for all patients and control samples. Previously extracted DNA was pooled from each of the phases belonging to both groups; and concentrated using the Concentrator plus (Eppendorf AG, Hamburgo, Germany). Libraries from DNA were prepared using between 0.5–20 ng of starting material and the KAPA HyperPrep Kit (Roche, Pleasanton, CA USA) according to the manufacturer's protocol until "hybridization of the amplified sample libraries and the SeqCap EZ probe pool" step. Concentration and quality of the Amplified Sample Library were measured using the Qubit 4 Fluorometer (Thermo Fisher Scientific, MA, USA) and the 2100 Bio-Analyzer Instrument (Agilent Technologies, CA, USA).

Libraries were pooled in equal molar concentrations and sequenced on the MiSeq system (Illumina, CA, USA) using MiSeq Reagent Kit v2 and paired-end 150 bp read lengths.

Raw and processed data quality controls were performed using FastQC and Qualimap tools. Reads were aligned using BWA v0.7.15 and Bowtiev2. Sam and Bam files were manipulated with Samtools v1.3.1.

Reads per chromosome normalization were calculated, the formula of Reads per Kilobase Million (RPKM) is as follows:

$$RPKM = \frac{ER \times 10^9}{EL \times MR}$$

We adapted RPKM formula to provide a normalized measure of the number of reads that align with each chromosome based on their size: ER equals to the number of mapped read in each chromosome, EL to the chromosome length and MR to the total number of mapped reads.

Statistical analysis

SPSS v.26.0 (SPSS Inc., Chicago, IL) was used for statistical analysis. Shapiro–Wilk's test was performed to check the normality of the variables. Mann-Whitney test or Students t-tests analysis were used to check the differences in distribution of continuous variables. For all the statistical tests, the significance level was set at 0.05.

Results

mtDNA in a control sample by dPCR

As can be seen in Table 3, we have compared both the Ct values and the parameters of copy number variation in mitochondrial and nuclear genes. We used dPCR, as a way to see how of the different fractions or genes are the most suitable or stable biomarker in free DNA or extra vesicles fractions. As can be seen, in fraction C both *HV1* fractions, both *CYB* fractions and *HBB-short* gene showed statistical significance in qPCR analysis ($p < 0.001$); and maintained when dPCR analysis were performed. Just *HBB-long* gene has not statistical value in both analyses. Finally, phase F showed also relevant results according to mitochondrial genes as exosomes' biomarkers showing significances by qPCR and dPCR in

all fractions except to *HBB-short* and *-long*. The remaining results of others phases are included in Supplementary Table S2.

Table 3
Ct mean analysis cases vs controls comparing genes and copy number

Phase	Gene	P value (*)	P value(cn)	Ct mean controls ± SD	Ct mean patients ± SD
C	*HV1-short*	*< 0.001*	*0.001*	21.62 ± 2.39	19.48 ± 2.32
	HV1-long	*< 0.001*	*0.001*	20.41 ± 2.55	18.30 ± 2.43
	CYB-short	*0.001*	*0.002*	22.34 ± 2.79	20.28 ± 2.43
	CYB-long	*0.001*	*0.002*	21.69 ± 2.98	19.64 ± 2.52
	HBB-short	*< 0.001*	*< 0.001*	21.74 ± 1.73	20.13 ± 2.12
	HBB-long	0.102	0.163	32.46 ± 2.42	31.58 ± 3.17
F	*HV1-short*	*< 0.001*	*< 0.001*	25.16 ± 1.29	23.79 ± 1.62
	HV1-long	*< 0.001*	*< 0.001*	25.82 ± 1.44	24.35 ± 1.84
	CYB-short	*0.001*	*0.005*	27.95 ± 1.41	26.80 ± 1.73
	CYB-long	*< 0.001*	*< 0.001*	27.49 ± 1.40	26.23 ± 1.66
	HBB-short	0.064	0.120	34.36 ± 2.13	33.46 ± 2.27
	HBB-long	0.098	0.107	33.44 ± 2.28	35.58 ± 1.96

Abbreviations: *Ct* Cycle threshold, *CYB* Apocytochrome B, *HBB* Hemoglobin subunit beta, *HV1* Hypervariable region 1, SD Standard deviation. *HBB* as nuclear marker vs *HV1* and *CYB* as mitochondrial markers.

Just significant or close significant values are shown. (*) Just p values comparing genes in cases vs controls by qPCR analysis. P value(cn) represents values of comparisons cases vs controls in copy number by dPCR analysis.

In Fig. 1 we develop a bar chart representation in which we could clarify that phases C and F are the most representative ones in cases cohort.

Focusing in phase F as shown in **Fig. 2**, it is nicely demonstrated that the most representative markers among cases are mitochondrial genes, such as *HV-short* and *CYB- short.*

mtDNA in relation to aggressiveness

According to aggressiveness we have developed two analyses, first of it is comparing samples with higher aggressiveness in accordance to presence of metastasis among patient group. As can be seen in Table 4, both fragments of *HV1* presented significant values of aggressiveness in phase B by qPCR analysis, whereas copy number variation of *CYB-short* in phase C appears to be a good biomarker for

metastasis (p = 0.037). However, a major significance was observed with *HBB-long* in most fractions in both analysis (qPCR and dPCR).

Table 4
Representation of the values for risk of metastasis

Phase	Gene	P value (*)	P value(cn)	Ct mean no metastasis ± SD	Ct mean metastasis ± SD
B	*HV1-short*	*0.020*	0.069	24.54 ± 4.18	22.06 ± 2.08
	HV1-long	*0.035*	0.133	23.92 ± 4.61	21.35 ± 2.56
	CYB-short	0.078	0.223	25.71 ± 4.29	23.69 ± 2.54
	HBB-long	*0.020*	*0.029*	33.52 ± 2.99	36.09 ± 2.69
C	*CYB-short*	0.359	*0.037*	19.95 ± 2.73	20.67 ± 2.03
	HBB-short	*0.001*	*0.002*	19.18 ± 2.41	21.24 ± 0.89
	HBB-long	*0.006*	*0.012*	30.37 ± 3.71	32.99 ± 1.55
D	*HBB-long*	*0.001*	*0.001*	31.84 ± 3.48	36.03 ± 2.77
F	*HBB-long*	*0.007*	*0.014*	31.72 ± 2.13	33.44 ± 1.34

Abbreviations: *Ct* Cycle threshold, *CYB* Apocytochrome B, *HBB* Hemoglobin subunit beta, *HV1* Hypervariable region 1, SD Standard deviation. HBB as nuclear marker vs HV1 and CYB as mitochondrial markers.

(*) Just p value comparing genes in cases vs controls by qPCR analysis. P value(cn) represents values of comparisons cases vs controls in copy number by dPCR analysis.

When comparing to aggressiveness, we have developed another comparison among patients, clustering those with a more aggressive stage (3 and 4) vs those with a less one (1 and 2). In this case (Table 5), *HV1-short* shown significance in aggressiveness in phase B by qPCR analysis (p = 0.027). Additionally, mitochondrial *HV1-long* (p = 0.042) and *CYB-long* (p = 0.023) fragments extracted from the phase D resulted as good options to distinguish among aggressiveness in dPCR assays. Likewise, according to previous results, *HBB-long* shown significant tendency to aggressiveness in most fractions in both analysis (qPCR and dPCR).

Table 5
Representation of the values for aggressive stages

Phase	Gene	P value (*)	P value(cn)	Ct mean St1 ± SD	Ct mean St4 ± SD
B	*HV1-short*	*0.027*	0.102	24.59 ± 4.18	22.08 ± 2.29
	HV1-long	0.051	0.202	23.92 ± 4.61	21.42 ± 2.82
	HBB-long	*0.001*	*0.001*	33.52 ± 2.99	37.11 ± 1.90
C	*HBB-short*	*< 0.001*	*0.002*	19.18 ± 2.41	21.52 ± 0.70
	HBB-long	*0.001*	*0.002*	30.37 ± 3.71	33.53 ± 1.05
D	*HV1-long*	*0.030*	*0.042*	27.11 ± 3.21	29.35 ± 2.47
	CYB-long	0.069	*0.023*	29.71 ± 3.49	32.03 ± 3.88
	HBB-short	*0.011*	*0.008*	32.49 ± 2.99	35.17 ± 2.65
	HBB-long	*< 0.001*	*< 0.001*	31.84 ± 3.48	36.71 ± 2.51
F	*HBB-short*	*0.004*	*0.006*	32.91 ± 2.49	34.79 ± 1.05
	HBB-long	*0.005*	*0.009*	31.72 ± 2.13	33.59 ± 1.41

Abbreviations: *Ct* Cycle threshold, *CYB* Apocytochrome B, *HBB* Hemoglobin subunit beta, *HV1* Hypervariable region 1, SD Standard deviation, *St1* Stages 1 and 2, *St4* Stages 3 and 4. *HBB* as nuclear marker vs *HV1* and *CYB* as mitochondrial markers.

(*) Just p value comparing genes in cases vs controls by qPCR analysis. P value(cn) represents values of comparisons cases vs controls in copy number by dPCR analysis.

NGS analyses

To evaluate the quality of mitochondrial DNA, NGS analyses were developed in all different samples. As can be seen in Table 6 by comparing data of RPKM and % mtDNA, phase C (0.361%), followed by B (0.038%) and finally F (0.016%) are the ones with higher proportion of mtDNA.

Table 6
NGS analysis of the samples comparing autosomes versus mitochondrial genome

Phase	%Mapped	Mapping Quality	Percentage in genome	RPKM
B	99	28.65	Autosomes chr. 95.575%	0.320
			Sexual Chr. 4.387%	0.173
			mtDNA 0.038%	22.382
C	89	28.575	Autosomes chr. 95.331%	0.316
			Sexual Chr. 4.308%	0.179
			mtDNA 0.361%	212.287
D	98	98	Autosomes chr. 95.647%	0.322
			Sexual Chr. 4.344%	0.176
			mtDNA 0.010%	5.598
E	100	29.65	Autosomes chr. 95.665%	0.319
			Sexual Chr. 4.333%	0.177
			mtDNA 0.002%	0.437
F	94	29.535	Autosomes chr. 95.800%	0.325
			Sexual Chr. 4.184%	0.167
			mtDNA 0.016%	9.303

Abbreviations: *mtDNA* mitochondrial DNA, *RPKM* Reads per Kilobase Million

When comparing reads alignment with Bowtie and BWA, we have obtained the same results. Phase E and B are respectively the ones with most efficient reads lectures, although all of them are about the 85% range recommended for libraries analysis. Moreover, phase C (in cases and controls) is the one with a higher proportion of mtDNA and vesicles (0.361%); following the same patterns as previously described in qPCR analysis. Furthermore, in phase F; we found a cover reads (0.016%) of mtDNA contained in vesicles with a high rate of mapping as it also reported in qPCR.

Discussion

It is well known the continuous improvements developed in clinical management of tumours, mainly focused on minimizing invasiveness techniques like tissue biopsies. There is an increasing focus on research about improving liquid biopsies analysis, most of them centre their attention in the analysis of CTCs (circulating tumour cells), cfDNA (cell free DNA), TEP (tumour educated platelets) or EVs (extracellular vesicles) [32]. Here we have proved that EVs are a good strategy as non-invasive biomarkers in RCC. It is known that EVs are vehicles of intercellular communication involved in many (patho)

physiological processes. Moreover, these qualities join to their molecular composition; have positioned EVs as one of the most stable options for diagnostic and therapeutic purposes [33]. According to our data, phase F, which is the one harbouring all exosomes, contains all mitochondrial markers totally stable in blood with significances among patients and controls which makes them as potential non-invasive biomarkers for RCC diagnosis.

Moreover, we also discovered that phase C, which contains all mitochondrial fractions, is also another interesting marker for RCC diagnosis between cases and controls. Previous machine learning analysis developed in RCC based on RNA-sequencing found mitochondrial and angiogenesis-related genes signatures, to be the most predictive ones within clustering approaches in clear cell, papillary and chromophobe RCC. This analysis identified a high risk ccRCC subgroup which is the best described by a mitochondrial signature and a down-regulation of angiogenesis-related genes, not exclusive to one RCC subgroup [34]. Despite the fact that nuclear *HBB* region shown remarkable significance in aggressiveness analyses, our data reinforce the power of mtDNA as an aggressiveness biomarker in RCC both in metastasis and in most advanced stages.

Others metabolic genes, including *VHL, MTOR, ELOC, TSC1/2, FH, SDH*, as well as mtDNA, revealed that the vast majority of RCC histology in the last 30 years [35]. On the other side, there are also nuclear markers such as *FOXD1* that has been suggested as a potent driver of tumour growth in ccRCC. *FOXD1* expression inversely correlated with patient outcome and was also shown to be grade and stage dependent [36]. The role of mitochondrial damage in tumour, is not new. For example, in ovarian cancer, MRPL15 (mitochondrial ribosomal protein 15) is suggested as a prognostic indicator and therapeutic target [37], or XRCC2 repairing mtDNA damage in hepatocellular carcinoma [38].

It is renowned that the detection of circulating EVs in the plasma of cancer patients represents a promising “liquid biopsy” strategy; exosomes are the EVs in which more research is focused. Due to their multifactorial content, exosomes constitute a unique tool to capture the complexity and enormous heterogeneity of cancer in a longitudinal manner. Moreover, it is also due to molecular features like high nucleic acid concentrations and elevated coverage of genomic driver gene sequences [39]. Furthermore, recent studies developed by Lazar et cols. [40] highlighted the possible role of platelet-derived microvesicles, as previous demonstrated role of platelets in cancer progression. In RCC, there are some studies which include the role of miRNAs in serum EVs such as novel diagnostic markers. miRNA-4525 expression was higher in RCC tissue than in the adjacent normal tissue, suggesting this miRNA in EVs as novel biomarker for RCC [41]. Or exosomal miR-9-5p, which plays an important role in RCC, indicating it may be used as biomarker for diagnosis and monitoring the efficacy of therapy [42]. Concerning to metastatic ccRCC, it is described an increase of EV-derived miR-301a-3p, and decrease of EV-derived miR-1293 [43]. Apart from miRNAs, EVs-derived tissue inhibitors of metalloproteinases (TIMP-1) mRNA are also included as good prognostic biomarker candidate for ccRCC [44]. In present work, we highlight the role of EVs and focus the strength of mtDNA as a relevant marker for both screening and aggressiveness.

The existence of fluctuation of copy number of mtDNA was previously reported in relation to injury and oxidative stress that contributes to the development of the toxicity of dioxin-like compounds [45]; in acute myeloid leukemia (higher in aggressiveness stages) [46], hepatocellular [47] or tissue samples in breast cancer [48].

Mitochondria are considered as the power-generating units of the cell due to their key role in energy metabolism and cell signaling, for that reason many studies concerning angiogenesis or other phases of cancer, analyze them. Here we focus in the findings of the stability of whole functional mitochondria in extracellular fluids like blood. These findings follow the same patterns that previous one reported by Dache et al. [49] who could detect extracellular full length mtDNA in particles over 0.22 μm holding specific mitochondrial membrane proteins in peripheral blood. Current efforts are mainly focus on the role of EVs, however we have demonstrated that mitochondria and mtDNA could also be a stable and potential analysis for cell-cell communication and cancer biomarker, as we have proved in present study in RCC. We believe that circulating cell-free intact mitochondria have crucial biological and physiological roles because their role as cell communication and hereditarian patterns [49]. This study is the second showing free structurally intact mitochondria in plasma, and the first indicating its role as stable molecule in RCC. Previously, Elsayed et al. [50] suggested that an increased peripheral blood mtDNA copy number is associated with increased risk of RCC. Therefore, RCC might be considered in the range of potential tumours in patients with an elevated blood mtDNA copy number. Here we also indicated the most stable centrifugation conditions and phase (C and F) for mtDNA analysis, as well as the most suitable mitochondrial genetic markers for this purpose (*HV1* and *CYB*).

Conclusions

We have developed a simple and highly sensitive method that will allow detecting mtDNA variants as promise biomarkers in RCC. This experiment allowed us to analyze the fragment size distribution pattern of different regions of interest (as ccfDNA and content in EVs) in each plasma fraction, and we have confirmed the high mtDNA content in exosomes as a powerful biomarker. Therefore, application of liquid biopsy in the clinical scenario is a promising non-invasive technique for prediction, early diagnosis and monitoring of cancer treatment. We affirm that it would be quite interesting to study how the amount of mtDNA varies in controls and patients with RCC in fractions rich in exosomal content, allowing the preservation of mtDNA thanks to its lipid bilayer structure. We have discovered that studied mitochondrial genes (*HV1* and *CYB*) were suitable marker for detecting aggressiveness and metastasis, with a high sensibility detected in qPCR and dPCR analysis and for instance, in liquid biopsy. These new biomarkers will have relevant implications in diagnosis and management of RCC where there are no molecular biomarkers with direct application in clinical scenario. This is the first time that mtDNA is proposed as a stable biomarker in RCC and cancer management.

Abbreviations

bp: base pairs; ccRCC: clear cell renal cell carcinoma; ccfDNA: circulating cell-free tumour DNA; ccf-mtDNA: circulating cell-free mtDNA; CEI: Ethics Committee; Ct: Cycle threshold; CTCs: circulating tumour cells; dPCR: Digital PCR; EDTA: ethylenediaminetetraacetic acid anticoagulant; EVs: Extracellular vesicles; *HBB*: Hemoglobin subunit beta; *HV1*: mitochondrial hypervariable region 1; min: minutes; *MT-CYB*: Apocytochrome b of complex III; mtDNA: mitochondrial DNA; mtDNAcn: mtDNA copy number; NA: Not available; nDNA: nuclear DNA; NGS: Next generation sequencing; qcDNA: quantified control DNA; qPCR: Real Time PCR; RCC: Renal cell carcinoma; RPKM: Reads per Kilobase Million; s : seconds; SD: Standard deviation; yr: years.

Declarations

Ethics and approval and consent to participate

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by CEI-Granada, Ethics Committee for Clinical Research of Granada (Code: 0165-N-19). Informed consent was obtained from all subjects involved in the study.

Consent for publication

Not applicable.

Availability of data and materials

All data generated or analyzed during this study are included in this published article and its supplementary information file.

Competing interests

There is no conflict of interest to declare.

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Authors' contributions

MJAC and LJMG conceived, designed, and supervised the study, wrote the first draft of the manuscript and critically reviewed the paper. CRC collected blood samples. VR and EA developed the experiments and contributed toward data collection. LJMG, ARR and MJAC carried out the analysis and interpretation of the data. FMOP and ARR performed the statistical analyses. All authors read and approved the final manuscript.

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Figures

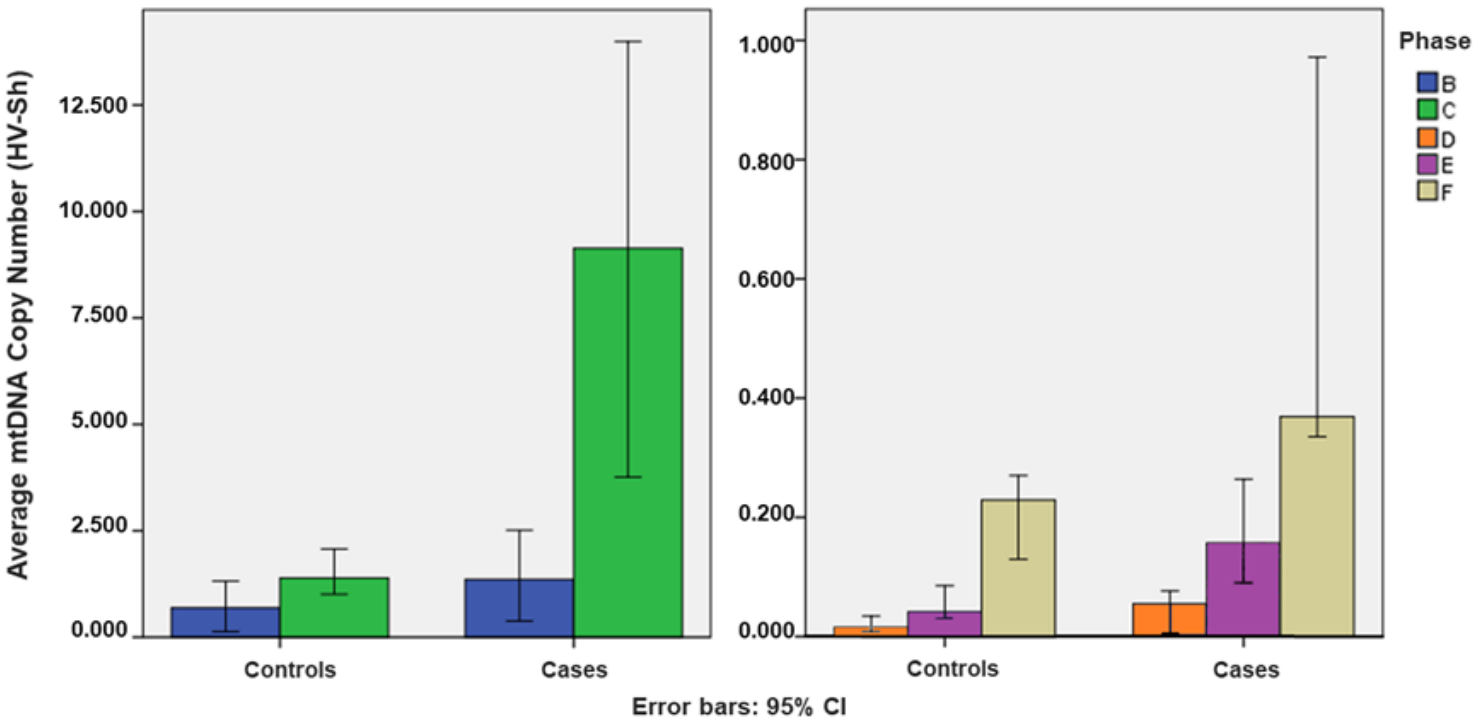

Figure 1

Bar chart representation of mtDNA copy number variation of mitochondrial hypervariable region 1 (HV1) short gene among controls and cases comparing phases.

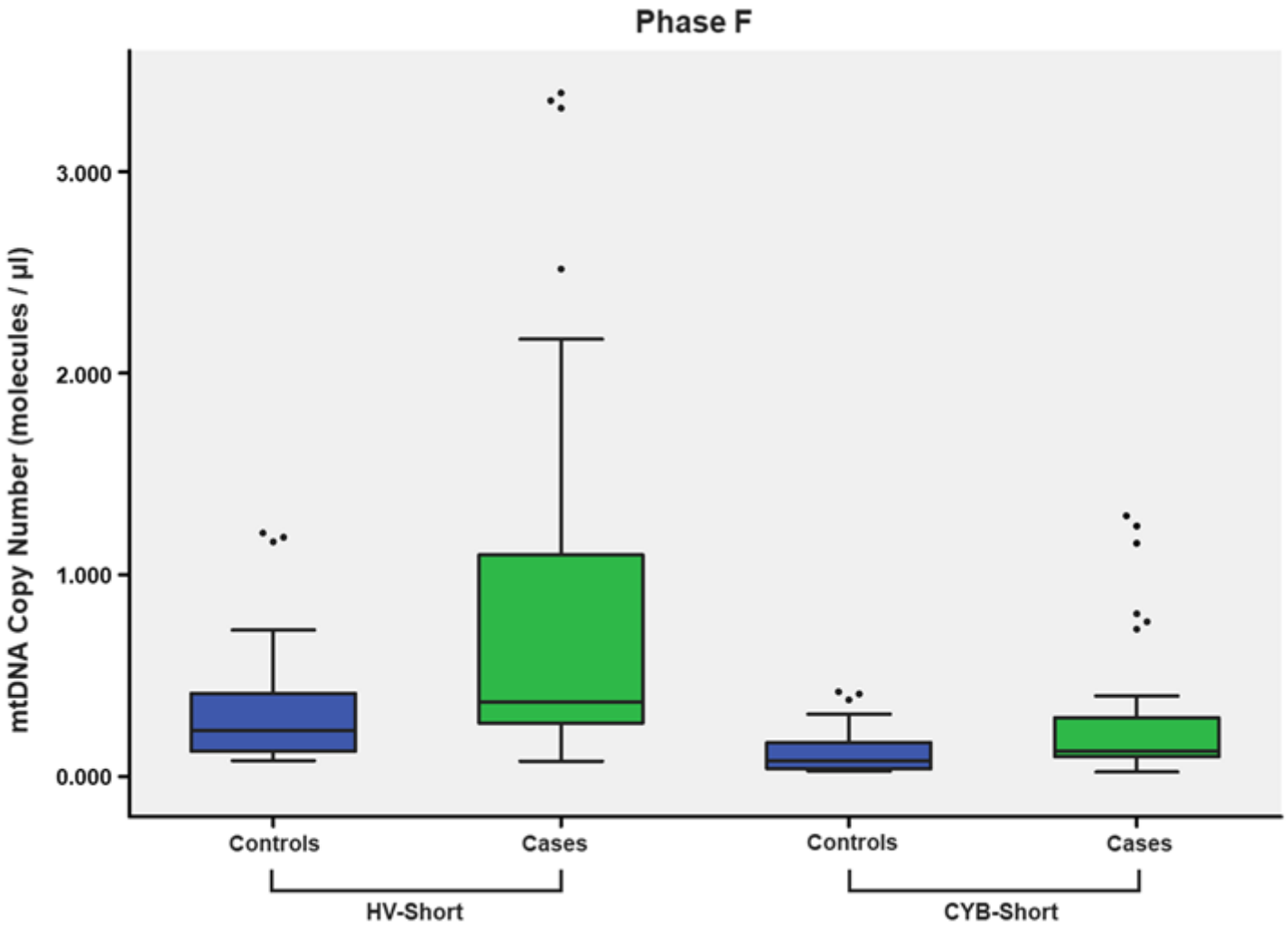

Figure 2

Phase F comparison between cases and controls formitochondrial hypervariable region 1 (HV1) andapocytochrome B (CYB)short regions.

Supplementary Files

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