ISOLATION OF MITOCHONDRIA AND QUANTIFICATION OF MITOCHONDRIAL DNA DAMAGE; ANALYSIS OF THE RELATIONSHIP BETWEEN APE1/REF-1 MITOCHONDRIAL LOCALIZATION AND CLINICAL-PATHOLOGICAL CHARATERISTICS IN HUMAN HEPATOCELLULAR CARCINOMA

1 INTRODUCTION

According to estimates of the International Agency for Research on Cancer, the second leading cause of cancer death worldwide is liver cancer [(1)]. HCC is the most common form of liver cancer and it represents the third cause of cancer mortality worldwide with an estimated half million new cases diagnosed each year [(2)]. HCC is almost always (80%) associated with cirrhosis, at least in developed countries, and chronic hepatitis C and B infection, alcoholic cirrhosis and haemocromatosis are some of the established risk factors [(3)]. The metabolic syndrome related to hypertension, central obesity, diabetes and obesity has been identified as a new risk factor. As a result, screening programs have developed, with the use of ultrasound and α-fetoprotein (AFP), with a hope to increase the chances of diagnosing small HCC and ultimately increase the rate of curability but an early diagnosis is of the utmost importance to improve prognosis and therapeutic efficacy.

The molecular mechanisms mediating carcinogenesis in HCC have not been fully elucidated and represent the final event of a long-standing liver disease that usually begins with an acute hepatic insult which progresses to fibrosis and cirrhosis, finally leading to hepatocyte transformation [(4)]. Any agent leading to chronic liver injury and cirrhosis constitutes a possible risk factor for HCC, the most relevant being viral infection (hepatitis B or C virus), alcohol intake, and other diseases such as nonalcoholic steatohepatitis (NASH), iron or copper deposition, and primary biliary cirrhosis [(5)]. The molecular events related to hepatocarcinogenesis are still not well known.

Several laboratories are actively working on the characterization of the molecular mechanisms responsible for hepatocyte transformation and on the role of mitochondria in tumorigenesis. Mitochondria play multiple roles in energy metabolism and cellular homeostasis, including: the generation of ATP via respiration and oxidative phosphorylation (OXPHOS), the production of reactive oxygen species (ROS), metabolic homeostasis, and the initiation and execution of apoptosis [(6)]. In the past decade, somatic mitochondrial DNA (mtDNA) mutations have been identified in several types of cancer [(7)], including HCC [(8,9)], and are suggested to cause mitochondrial dysfunction, increase the production of ROS, and promote tumor growth contributing to the onset of a vicious cycle [(10,11)]. This accounts for the renewed interest in mitochondrial biology and in methods for studying mtDNA damage and the mechanisms of repair.

Here we describe a protocol for the isolation of intact nuclei and mitochondria of high purity, which may be further used for to prepare protein extracts, isolate nuclear DNA (nDNA) and mtDNA starting from a single mouse liver sample (~1 g) or an equivalent mass of human liver biopsy from healthy or HCC tissue.

The genomic material isolated through this protocol is suitable for PCR amplification and mtDNA could be used to evaluate its level of damage in tumor sample respect to healthy tissue. In this regard, the most widely used method to measure nuclear and mtDNA damage in mammalian cells is a quantitative-PCR (Q-PCR) [(12)]. The Q-PCR assay of DNA damage is based on the principle that many kinds of DNA lesions can slow down or block the progression of DNA polymerase [(13)]. Therefore, if equal amounts of DNA from differently treated samples are Q-PCR-amplified under identical conditions, DNA with fewer lesions will amplify to a greater extent than more damaged DNA. In the protocol

proposed by Furda et al. [12] total DNA isolated from cells or tissue is used as template to measure mtDNA damage. For this reason, the amplification of a short mitochondrial fragment (~100 bp), where the probability of base damage is very low, is used to calculate the relative amount of mtDNA copies between different samples and to normalize the lesion frequencies calculated with the long mitochondrial fragment (~10 kbp). However, we noticed that through this approach high levels of mtDNA damage could be underestimated because also the short fragment contains damaged bases that may slow down or block the action of the polymerase altering the quantification of the mtDNA. Here we demonstrated that to prevent this problem, it is necessary to isolate and use mtDNA as template therefore avoiding the need to amplify a short fragment to normalize the amount of mtDNA.

In addition, to investigate the possible role of APE1/Ref-1 expression in the progression of hepatocellular carcinoma, we evaluated the presence and subcellular localization of APE1/Ref-1 according to HCC and mtDNA damage. In fact, HCC is a long step-by-step process [14,15] leading from a normal hepatocyte to a frankly cancerous cell via a preneoplastic state. This process shows several molecular changes with different phenotypes that suggest different genetic and epigenetic alterations during carcinogenesis such as the occurrence of point mutations, oncogene activation, or tumor suppressor gene inactivation [16]. Hepatocyte DNA damage based on oxidative ground is observed in chronic inflammatory processes [17,18] and, in the imbalance of an adequate base repair system, can induce genomic and mitochondrial DNA damages [19]. This is particularly true during the evolution from hepatitis to cirrhosis and to neoplastic process. A strong evidence suggests a crucial role of reactive oxygen species (ROS) in development of chronic liver disease, and evidence of oxidative stress has been detected in almost all clinical and experimental conditions of chronic liver diseases with different etiology and progression rate of fibrosis [20,21]. The increase of ROS, associated with the disease development and induced by developing parenchymal damage, promotes oxidative stress damage to proteins, lipids, and DNA [22], and activation of transcription factors such as NF-κB, STAT3, and AP-1 that are either involved in cell-survival pathway [23,24] or specifically activated in hepatocellular carcinoma [25,26]. ROS generated by chronic inflammation are closely linked to hepatocellular oxidative DNA damage and may be involved in the process of hepatocarcinogenesis [27]. Apurinic apyrimidinic endonuclease/ redox effector factor 1 (APE1/Ref-1) is a master regulator of cellular response to oxidative stress conditions. Because of its role in DNA repair, APE-1 is likely to protect DNA and transcription factors from oxidative damage and to repair damaged DNA in hepatocytes under conditions of enhanced oxidative stress. Thus APE-1 is a key enzyme of DNA repair that is distributed in a wide range of normal tissues including the liver [28] and may play an important role in the prevention of carcinogenesis and as we assume in hepatocarcinogenes.

Although its subcellular distribution in different mammalian cell types is mainly nuclear, cytoplasmic localization has also been described; interestingly, this particular subcellular distribution has been associated with different tumorigenic processes[29]. In particular, in the case of lung [30], ovarian [31], thyroid [32,33], and breast [34] cancers, cytoplasmic distribution has been associated with an higher aggressiveness of the tumor. The possible causal role played by this particular distribution in tumor progression is, at present, completely unknown. Genomic cellular changes and oxidative stress may trigger APE1/Ref-1 response.

The aim of this study was to assess the mutual relationships between expression of APE1/Ref-1 in tumoral mitochondria and nuclei, mtDNA damage and clinical- pathological characteristics in patients with hepatocellular carcinoma (HCC), looking for a possible prognostic role of APE1/Ref-1 subcellular localization in HCC tumors. Collectively, these data suggest a possible role of APE1/Ref-1 overexpression in the development of the HCC and indicate that the subcellular

localization of APE1/Ref-1 in HCC tissue might be used as a prognostic marker for this worldwide tumor and index of sensitivity to surgical, ablative or chemotherapy treatments.

2 MATERIALS AND METHODS

2.1 Subjets

Ten patients that underwent to hepatic surgery at the departments of surgery of the University of Udine, between October 2015 and January 2017, were retrospectively studied (fig. 1). No patient had undergone chemotherapy before surgery. The diagnosis of HCC was confirmed by histological analysis at the time of surgery. All the patients had been followed up from the time of surgical intervention to death. The population was divided in 2 groups based on mtDNA, higher or lower than normal tissue surrounding the tumor, testing the correlation with other factors such as patient's sex, history of HBV or HCV chronic infection or alcohol abuse, presence of cirrhosis, alpha-fetoprotein (AFP) serum levels (considering the following ranges: <200 ng/mL, 200-400 ng/mL and >400 ng/mL), tumor histotype (classic, fibrolamellar or mixed), multifocality, tumor diameter >5 cm, presence of vascular invasion, subjection to previous treatments, either percutaneous, endovascular or systemic chemotherapy, major hepatic surgery (defined as resection of more than 3 hepatic segments), presence of postoperative complications (distinguishing wound dehiscence and development of either bilioma or hematoma), and tumor recurrence during the follow-up.

2.2 Mouse and human liver samples

The Italian Ministry of Health and the Institutional Ethics Committee of the University of Udine, Italy, approved the use of the mouse model in the experiments and procedures described here. The use of human derived samples from HCC patients was approved by Ethical Committee of the Friuli Venezia Giulia Region, Italy (C.E.R.U. N. 50/2014/ Os del 09.09.2014). The study was conducted using tissues biopsies from four patients affected by primary HCC and not previously treated to avoid the presence of necrotic area and interference with mtDNA damage measurements. Samples of HCC and the surrounding area were removed during surgical liver resection, conserved on ice in IBc buffer (Tris-MOPS 10 mM, EGTATris 1 mM, Sucrose 0.2 M) and immediately processed. The diagnosis of HCC was confirmed by histological analysis at the time of surgery. Nuclei where washed with T1 solution (KCl 10 mM, MgCl2 0.1 mM, HEPES pH 7.9 10mM, EDTA pH 8 0.1 mM) and lysed with T2 solution (NaCl 420 mM, MgCl2 1.5 mM, HEPES pH 7.9 20 mM, EDTA pH 8 0.1 mM, glycerol 5%).

2.3 Cell culture and treatments

HeLa cells were grown as reported in Barchiesi et al. [35]. For mtDNA damage measurements, 4x106 cells/plate were treated with the reported amount of H2O2 in medium without serum for 15 minutes, washed with PBS (phosphate buffer saline) and then cultured for one additional hour in the presence of 10% FBS before harvesting and DNA isolation.

2.4 Western blotting analysis

APE1/Ref-1 protein expression analysis was performed by Western blotting (WB) in five cases of HCC as reported in Barchiesi et al. [35]. Subcellular localization of APE1/Ref-1 was classified as nuclear or cytoplasmic on the basis of the

median expression value. The median was used as cutoff to define the positive cases, considering as positive nucleus or cytoplasm when the APE1/Ref-1 expression was greater than or equal to the median. Reactivity observed in both nucleus and cytoplasm was considered cytoplasmic [36].

2.5 Total DNA extraction from HeLa cells

Total DNA form HeLa cells was extracted as reported in Barchiesi et al. [35] . After purification, DNA was quantified with Quant.iT-Picogreen dsDNA reagent (Thermo Fisher Scientific, Monza) according to the manufacturer's instructions and the concentration was adjusted to 10 ng/µl.

2.6 nDNA extraction Genomic

DNA was extracted from isolated human or mouse nuclei using TRI Reagent (Sigma-Aldrich, Milano) according to the manufacturer's instructions. Briefly, isolated nuclei were lysed in 1 ml of TRI-Reagent, and samples were allowed to stand for 5 minutes at RT. To separate DNA from RNA and proteins 0.2 ml of chloroform were added to the samples, which were then shaken vigorously for 15 seconds and allowed to stand for 15minutes at RT. Samples were then centrifuged at 12,000 g for 15 minutes at 4°C. Centrifugation separates the mixture into three phases with the white interphase containing the DNA fraction. Protein and RNA containing phases were carefully discarded and DNA was precipitated adding 0.3 ml of 100% ethanol. Samples were mixed by inversion, allowed to stand for 3 minutes at RT and centrifuged at 2,000 g for 5 minutes at 4°C. The resulting supernatant was discarded and the DNA pellet was washed twice with 1 ml of 0.1 M trisodium citrate, 10% ethanol solution and centrifuged at 2,000 g for 5 minutes at 4°C. During each wash DNA was allowed to stand at RT for at least 30 minutes.

The DNA pellet was then resuspended in 75% ethanol, allowed to stand for 10 minutes at RT, and centrifuged at 2,000 g for 5 minutes at 4°C. The resulting supernatant was removed; the DNA pellet was dried and dissolved in 8 mM NaOH. Samples were then centrifuged at 12,000 g for 10 minutes at 4°C to remove any insoluble material and pH of the solution was adjusted to pH 8.4 using 0.1 M HEPES. The DNA concentration was measured spectrophotometrically.

2.7 RT-PCR

RT-PCR was performed with iQ5 multicolor real-time PCR detection system (Bio-Rad,Milano), according to the manufacturer's protocol. The following primers were used: mouseGAPDH_fwd (5'-TCAGGCCAAGTATGATGA-3') and mouseGAPDH_rev (5'-GGAGTTGCTGTTGAAGTC-3'), which amplified a region of 115 bp; humanGAPDH_fwd (5'-CCCAGGTTTACATGTTCCAATATG -3') and humanGAPDH_rev (5'-TGGGATTTCCATTGATGACAAGC-3'), which amplified a region of 96 bp. Genomic DNA was amplified in 96-well plates using primers for mouse GAPDH and human GAPDH in separate wells using the 2X iQ SYBR green supermix (Bio-Rad, Milano) and 300 nM of the specific sense and antisense primers in a final volume of 15 µl for each well. Each sample analysis was performed in triplicate. As negative control, a sample without template was used. The cycling parameters were denaturation at 95°C for 10 seconds and annealing/extension at 60°C for 30 seconds (repeated 40 times). In order to verify the specificity of the amplification, a melting-curve analysis was performed immediately after the amplification protocol and PCR products were separated onto a 2% agarose gel.

2.8 DNA quantification with Quant.iT-Picogreen dsDNA reagent.

Quantification of DNA was performed with Quant.iT-Picogreen dsDNA reagent (Thermo Fisher Scientific, Monza) according to the manufacturer's instructions. To evaluate the absolute quantity of DNA a standard curve was created with the indicated increasing amounts of Lambda/HindIII DNA ladder (0.78 ng; 1.56 ng; 3.13 ng; 6.25 ng; 12.5 ng; 25 ng and 50 ng). Each standard and each sample was quantified in duplicate. For each well a solution containing 95 µl TE (200 mM Tris-HCl, 500 µM EDTA), and 5 µl of the standard point or the sample and 100 µl of Quant.iT-Picogreen dsDNA reagent (diluted 1:200) were mixed together. The evaluation of mtDNA and PCR products amount was performed measuring the fluorescence emission with EnSpire multimode Plate Reader (PerkinElmer, Milano) with an excitation wavelength of 480 nm and an emission wavelength of 528 nm.

2.9 mtDNA extraction and mtDNA damage measurement by Q-PCR

mtDNA was extracted from isolated human or mouse mitochondria using Nucleospin plasmid isolation kit (Macherey-Nagel, Duren). Quantification of DNA was performed with Quant.iT-Picogreen dsDNA reagent. DNA integrity was verified on 2% agarose gels and DNA concentration was adjusted to 0.25 ng/µl. The number of mtDNA lesions was determined by Q-PCR.

DNA was amplified usingElongase enzyme mix (Thermo Fisher Scientific, Monza). The PCR reaction was initiated at 94°C with hot-start for the complete denaturation of DNA and allowed to undergo the following thermocycler profile: an initial denaturation for 1 minute at 94°C followed by 19 cycles of 94°C denaturation for 1 minute and 64°C annealing/extension for 11 minutes for the 8.9/10 kbp fragments and 60°C annealing for 45 seconds and 72°C extension for 45 seconds for the 221/117 bp fragments. A final extension at 72°C was performed for 10 minutes for all fragments. To ensure quantitative conditions a sample with the 50% of template amount was included in each amplification and, as negative control, a sample without the template was used. PCR products were quantified in triplicate by using Quant.iT-Picogreen dsDNA reagent, visualized on agarose gels, and quantified with Gel Doc XR System (Bio-Rad, Milano).

3.0 Statistical analysis

Data were obtained through the R software environment (version 3.3.2), with a significance level of $p<0.05$. Fisher's exact test was used in intergroup comparisons of categorical variables, and categorical variables were expressed as numbers. Welch's t-test was used for for continuous variables. Spearman's correlation was run to test the relationship between mtDNA damage levels and tumoral mitochondrial APE1 expression. Resulting data has been presented through mean (± standard deviation), median and interquartile range (IQR), or raw numbers where appropriate.

3.1 RESULTS

3.2 Isolation of nuclei and mitochondria from mouse liver and HCC samples

Mouse liver from an adult mouse Balb/c strain and human biopsies derived from the tumoral liver mass (HCC), and from the non-tumoral (Distal) section were processed as previously described by Frezza et al. 2007 [(54)]. The protocol was modified to obtain, in addition to mitochondrial, also the nuclear fraction. Liver tissue was immersed in 50 ml of ice-cold IBc buffer, rinsed two times to completely remove blood, cut into small pieces, and transferred to a glass potter.

Next, 7 ml of IBc buffer were added and the liver was homogenized using a Teflon pestle at the minimum speed until the suspension was homogeneous. This process leads at the disruption of liver tissue and the lysis of cells. The homogenate was then transferred to 15 ml falcon tube and centrifuged at 70 g for 3 minutes at 4°C to separate non-homogenized tissue pieces. Supernatant was transferred to a 15 ml falcon tube and centrifuged at 600 g for 10 minutes at 4°C to pellet intact nuclei that were than treated separately. The supernatant, containing mitochondria, was transferred to a 15 ml glass centrifuge tube and centrifuged at 7,000 g for 10 minutes at 4°C. Supernatant was discarded and the pellet, containing mitochondria, was washed with 5 ml of IBc buffer, centrifuged at 7,000 g for 10 minutes at 4°C, and resuspended in 800 µlof IBc buffer and represented the mitochondrial fraction (MF). Mitochondria were finally washed with IBc buffer containing 1 M KCl to eliminate cytoplasmic proteins and contaminant nuclear DNA (nDNA). The pellet of nuclei was washed by gentle resuspension in T1 solution and centrifuged at 600 g for 10 minutes at 4°C. To obtain nuclear protein extract, the pellet was lysed in T2 solution and a after incubation on ice for 30 minutes, centrifuged at 14,000 g for 20 minutes to remove membranes. The supernatant was collected and represented the nuclear protein fraction (NF). For the isolation of nDNA, after the wash with T1 solution the pellet was lysed with TRI Reagent and nDNA isolated as reported in the Material and Methods section. Western blot analysis of mitochondrial (MF) and nuclear (NF) fractions from mouse liver and human HCC biopsies confirmed the possibility of isolating the two subcellular compartments. Mitochondrial membrane ATP synthase Complex V (ATP5A) and lysine (K)-specific demethylase 1A (LSD1) where used as mitochondrial and nuclear markers, respectively. Notably, we did not observe any cross-contamination between the mitochondrial and nuclear compartments.

3.3 Murine and human nDNA and mtDNA

Genomic DNA was isolated from nuclei (~250 µg of nDNA per g of liver tissue), and quantified. To examine the quality of genomic material, 100 ng were used as template to amplify a portion of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene by RT-PCR. The nDNA amplified with a Ct value of 13.33 while the control sample without template DNA amplified with a Ct value of 30.98. The amplification reaction was analyzed using agarose gel electrophoresis and showed a single band of the expected size while in the control reaction only a faint signal corresponding to dimer of primers was visible. Due to its circular structure of approximately 16 kbp, we used a plasmid isolation kit to purify the mtDNA [55]. Three milligrams of isolated mitochondria were used to extract mtDNA (~1.3 µg of mtDNA per g of liver tissue) which was then quantified with Picogreen reagent. Afterwards, 150 ng of the mtDNA samples were separated on agarose gel confirming the integrity of the mtDNA. Moreover, to prove the absence of contamination of nDNA in the preparation we repeated the RT-PCR for GAPDH using 100 ng of nDNA as positive control, 10 pg of mtDNA, which corresponds to 10 time the number of base pairs of nDNA (considering the nDNA as a unique molecule), and a reaction without template as negative control. As previously reported, the nDNA amplified with a Ct value of 13.56, while the mtDNA and the control reaction amplified with a Ct value of 30.17 and 31.29, respectively. In conclusion, we demonstrated that through this approach it is possible to purify nuclei and mitochondria and isolate high quality mtDNA without the contamination of nDNA. Genomic DNA from HCC samples was isolated from nuclei (~200 µg of nDNA per g of liver tissue) and 100 ng were used to amplify by RT-PCR a region of the human GAPDH gene. The nDNA amplified with a Ct value of 18.72 and 18.89 for the distal and tumoral tissues respectively, while the amplification of the control was not detectable. mtDNA from HCC and non-tumoral tissues was purified (~450 ng of mtDNA per g of liver

tissue) and quantified with Picogreen reagent. Figure 2 H shows the image of an agarose gel where 150 ng of mtDNA from the distal area and HCC tissue of Patient 1 were separated showing the presence of a single band of approximately 16 kbp. Then, the degree of nDNA contamination was evaluated by RT-PCR. Samples containing 100 ng of nDNA as positive control, 10 pg of mtDNA, and a control without template were amplified using specific primers for GAPDH. The Ct of nDNA was of 20.15 cycles, while those of mtDNA of the distal and tumoral regions were 31.15 and 29.70, espectively. As in the case of mouse mtDNA, also the quality and purity of mtDNA isolated from human tissues was confirmed.

3.3 Analysis of mtDNA damage in HCC

In contrast to commonly used protocols for the evaluation of DNA damage, where mtDNA is not separated from the genomic DNA [(12)], here we used mtDNA as template instead of total DNA extracted from the tissue. Therefore, it was necessary to find out the correct amount of mtDNA to obtain quantitative amplification conditions. To determine the Q-PCR conditions for the measurement of the level of mtDNA damage in HCC samples, first we used the mtDNA from mouse liver to amplify the template in a quantitative manner. For this purpose 2, 1, 0.5 ng of mtDNA were used as template and Q-PCR reactions were separated on an agarose gel showing the presence of a single specific band (fig. 2a right). Then, reactions were quantified with Picogreen reagent and the histogram (fig 2.a left) shows the relative amount of each amplification reaction, clearly demonstrating that reducing the amounts of template (50% and 25% of 2 ng) results in a proportional reduction of the mitochondrial amplicon. We then used the mtDNA of tumoral and distal regions purified from four patients (Pt.) to evaluate the levels of DNA damage in HCC mitochondria with respect to the healthy tissue. The gel image (fig. 2 b) and the quantification of relative band intensity of the Q-PCR analysis of Pt. 1 are reported as example. To this end, the Q-PCR reactions were quantified with Picogreen reagent and the levels of mtDNA damage of each patient were normalized to the amount of amplicons of the mtDNA of the respective healthy tissue that was arbitrary fixed to 1. In all cases, the mtDNA purified from mitochondria of the tumoral tissue was significantly more damaged than that derived from the distal region ($p=0.037$) (fig. 2 c). Moreover, we amplified a short fragment of 221 bp that was typically used to normalize the amount of mtDNA when nDNA and mtDNA are not separated during extraction. In Figure 3d the amplicons of the distal and HCC mtDNA of Pt. 1 are visible and the band analysis shows an unexpected reduction of 38% of the HCC amplicon with respect to the distal area. Next, the Q-PCR reactions of all patients were quantified with Picogreen reagent confirming that the presence of high levels of base damage in the HCC samples ($p=0.04$)(fig. 3 e).

To test the hypothesis that high levels of mtDNA damage could affect the amplification of the short amplicon, therefore altering the quantification of mtDNA damage levels, we treated HeLa cells with increasing amount of H_2O_2 for 15 minutes and measured the levels of mtDNA damage. The histogram in Figure 3f reports the quantification of the long mitochondria Q-PCR amplicons normalized or not-normalized for the short PCR fragment.

Normalized measurements (white bars) show an initial increase of the damage that reaches its maximum after a treatment with 400 µM of H_2O_2 and unexpectedly does not rise with higher concentration of H_2O_2. On the contrary, avoiding the normalization (grey bars) we observe a significant concentration-dependent increase in the level of damage. The reduced amplification efficiency of the short mitochondrial PCR fragment in cells treated with 600 µM and 1 mM of H_2O_2 (fig. 3 g) explains the bias of the measurement of mtDNA base lesions when the sample is normalized for

the short amplicon. These data confirm the hypothesis that in samples with high level of damaged bases, such as tumoral tissues, it is not possible to use a short amplicon to normalize the amount of mtDNA for the calculation of the damage.

3.4 Retrospective study

The population was composed of 7 males and 3 females, with a mean age of 71.3 years (±10.2). Median follow-up was 6.5 months (3.0-7.0). A total of 3 cases showed history of alcohol abuse, and 3 presented HCV chronic infection; no patient showed evidence of HBV infection. Tumor histotype resulted to be in 3 case the classical type and in the others 3 cases mixed type, while no patient had findings of a pure fibrolamellar type; vascular invasion was reported in a total of 5 cases. In 4 cases tumor was multifocal. AFP level resulted to be <200 ng/mL in 8 cases, between 200 and 400 ng/mL in 1 case and >400 ng/mL in another one. In all patients, only one patient had history of pre-operative treatment. In 4 cases major surgery was performed, and 3 out of the total encountered postoperative complications (all related to wound dehiscence). During the available follow-up, 4 cases presented recurrence and 3 deads.

Mean relative mtDNA damage resulted to be 1.64 (±1.22), being lower than 1 in a total of 5 patients (tab.1). Cytoplasmic localization f APE1/Ref-1resulted an average 1.32 (±0.84) times in neoplastic tissue than distal ones (Tab.3) Moreover, it showed a weak, negative monotonic correlation with the levels of tumoral mtDNA damage (rho=0.334), although not statistically significative (p=0.345) (tab. 2). Within our sample, there was no evidence of statistically significant correlation between the level of damage of mtDNA and sex (p=1.000), age (p=0.978), HCV infection (p=1.000), alcohol abuse (p=1.000), presence of cirrhosis (p=1.000), histotype (p=1.000), multifocality (p=0.524), lesion diameter (p=1.000), AFP levels (p=1.000), previous therapy (p=1.000), major surgery (p=0.524), postoperative complications (p=1.000), and recurrence (p=1.000). On the other hand, vascular invasion appeared to be related to mtDNA damage (p=0.008).

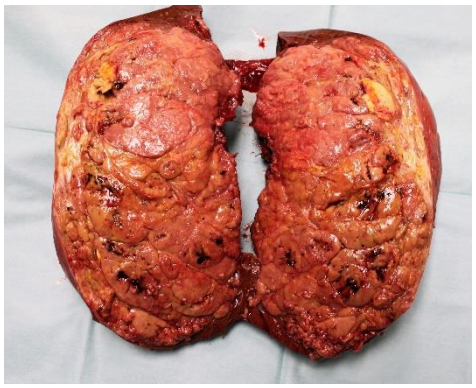

Fig. 1: HCC, surgical resection

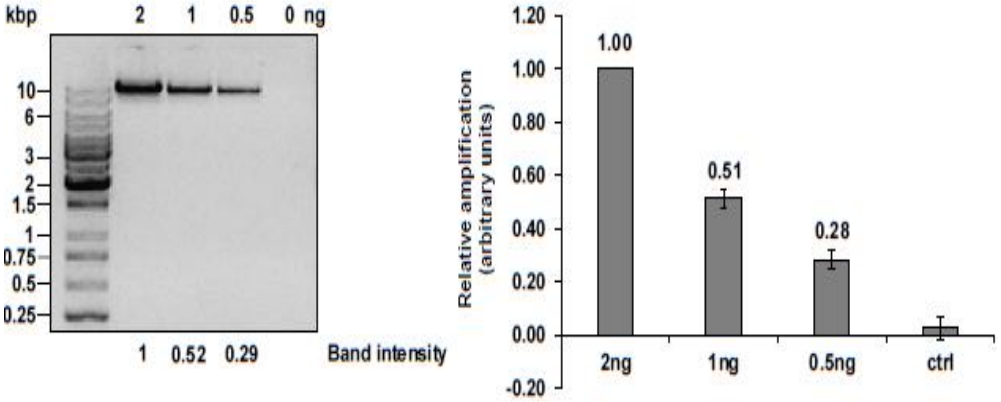

Fig. 2 a

(right and left)

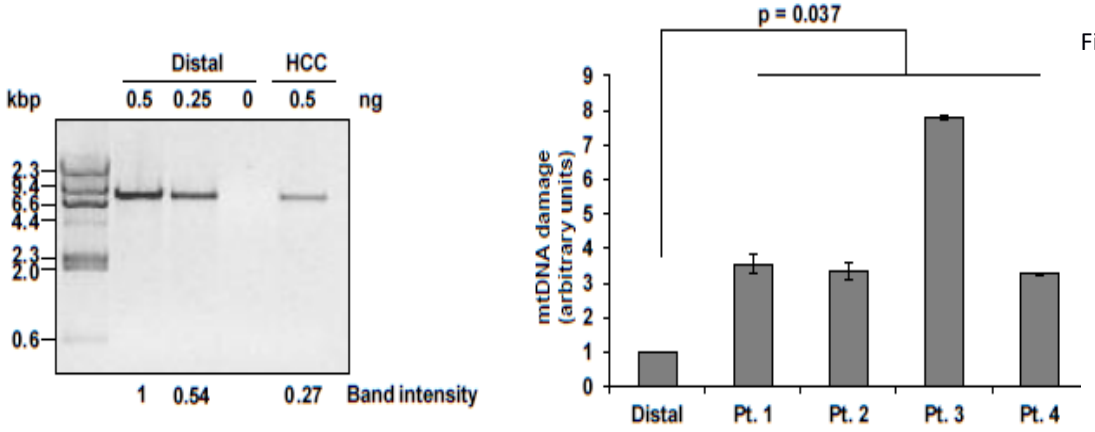

Fig. 2 b - c

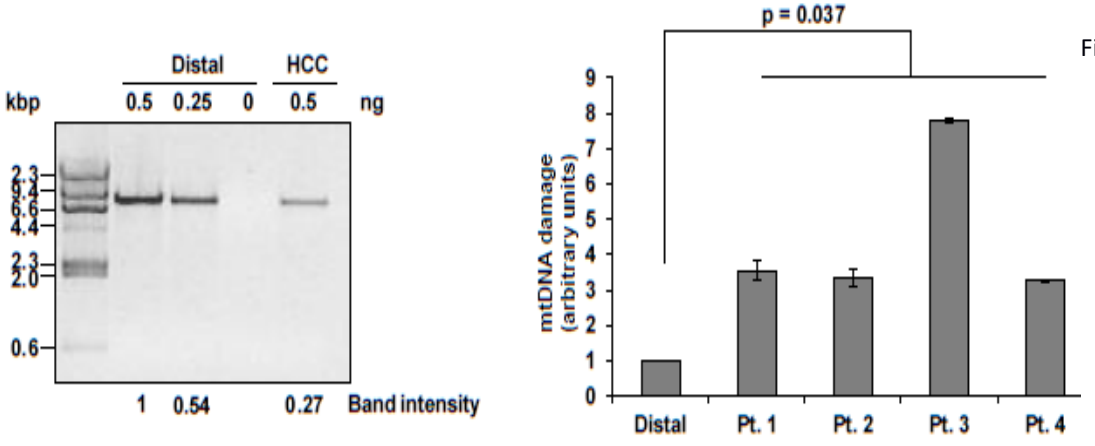

Fig. 2 d - e

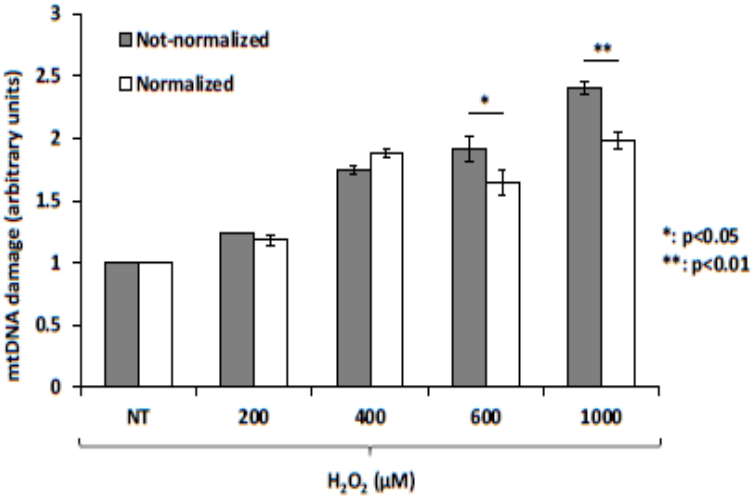
Not-normalized
Normalized
mtDNA damage (arbitrary units)
3
2.5
2
1.5
1
0.5
0
*
**
*: p<0.05
**: p<0.01
NT
200
400
600
1000
H2O2 (µM)

Fig. 2 f

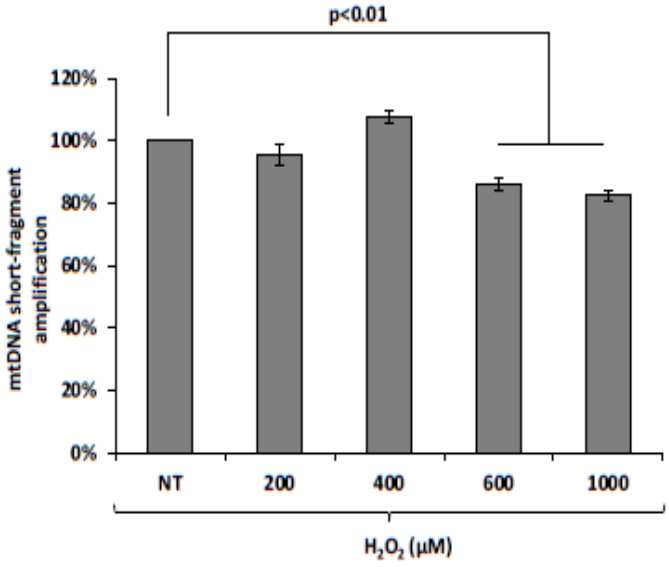
p<0.01
mtDNA short-fragment amplification
120%
100%
80%
60%
40%
20%
0%
NT
200
400
600
1000
H2O2 (µM)

Fig. 2 g

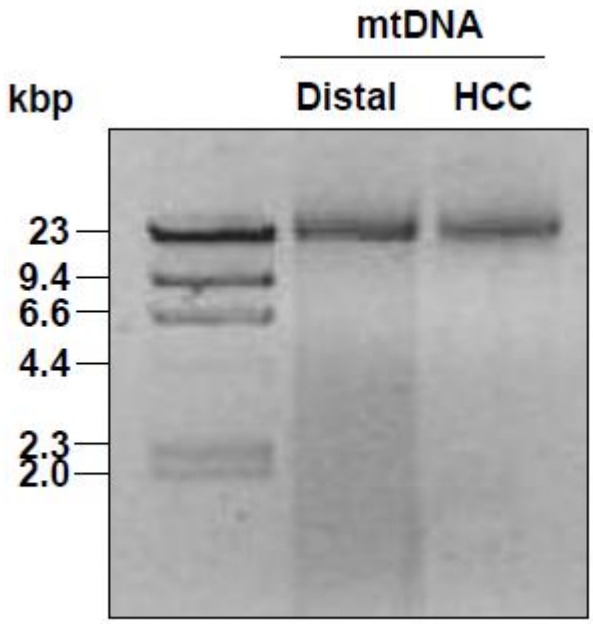

Fig. 2 H

DNA damage relative to distal (1.0)
0,57
0,93
2,71
2,06
0,72
3,82
0,70
0,50
3,21
1,19

Tab. 1: mitochondrial DNA damage was lower in neoplastic tissue compared to surrounding liver tissue

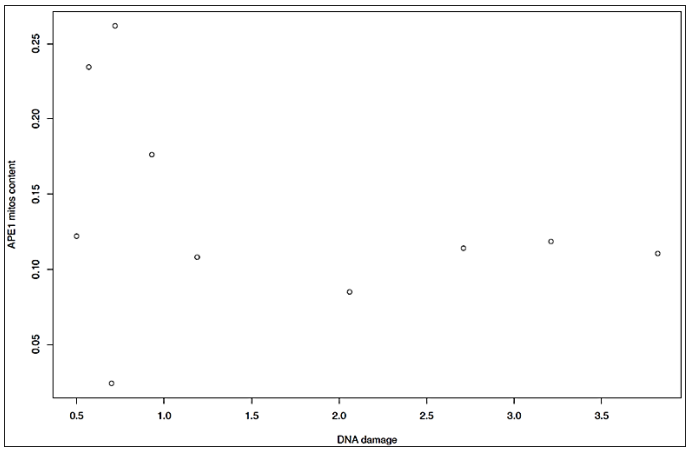

Table 2: relations between APE1 mitochondrial content and mitochondrial DNA damage.

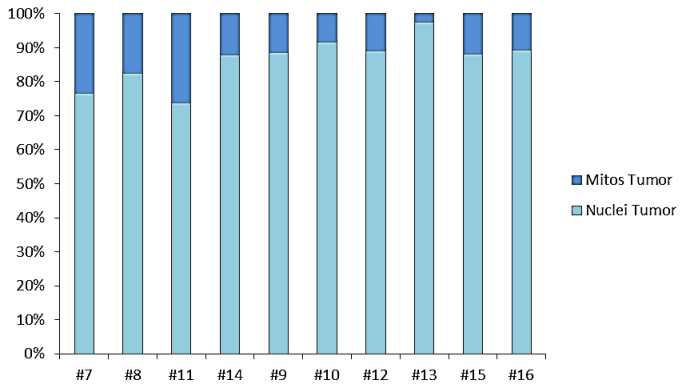

Table 3: nuclear and mitochondrial APE1 distribution in neoplastic tissue

4 DISCUSSION

Hepatocellular carcinoma (HCC) is an aggressive malignant tumor with a high mortality rate and one of the most common neoplasms worldwide [37]. HCC is one of the few cancers for which a number of risk factors are known in great detail [38,39]. HCC is almost always (80%) associated with cirrhosis, at least in developed countries, and chronic hepatitis C and B infection, alcoholic cirrhosis and haemocromatosis are some of the established risk factors [40] . As a result, screening programs have developed, with the use of ultrasound and α-fetoprotein (AFP), with a hope to increase the chances of diagnosing small HCC and unltimately increase the rate of curability but An early diagnosis is of the utmost importance to improve prognosis and therapeutic efficacy [41].

The increased cellular turnover associated with chronic liver injury and HCC occurs in a milieu of chronic inflammation (42) and cellular oxidative stress conditions [43] may increase the intracellular concentration of ROS content. APE1/Ref-1 is a multifunctional protein representing a central factor during cell response to oxidative stress through the modulation of transcriptional activation, as in the case of AP-1 [44], and DNA repair functions. APE1/Ref-1 has been demonstrated to play a prognostic role in a variety of human tumors [22,23,25,26,45].

Several studies demonstrated that different oxidative agents promote an increase of APE1/Ref-1, and that the protein up-regulation is always associated with an increase in both transcriptional and AP endonuclease activity [46,47]. More recently, it was suggested that ROS increase generated by chronic inflammation is closely linked to the occurrence of hepatocellular oxidative damage and may be related to hepatocarcinogenesis [20]. These data point to an important role of oxidative damage, possibly involving APE1/Ref-1 activation in the process linked to the development of HCC.

To investigate the possible role of APE1/Ref-1 expression in the progression of hepatocellular carcinoma, we evaluated the presence and subcellular localization of APE1/Ref-1 according to HCC and mtDNA damage.

It is worth mentioning that in normal liver, APE1/Ref-1 was found to be localized mainly in the nuclei of hepatocytes and endothelial and biliary ductal cells suggesting that a predominantly cytoplasmic localization of this protein may be associated with neoplastic alteration. A similar pattern of APE1/Ref-1 distribution has been described in colorectal cancer [48], breast cancer [26,49], thyroid carcinomas [25], and epithelial ovarian cancers [23]. In each of these tumors, cytoplasmic positivity of APE1/Ref-1 was demonstrated for tumor cells whereas nuclear reactivity was found mostly in the normal cells.

We observed that in 5 HCC cases, mtDNA damage was lower in HCC tissue than in the surrounding liver and a negative (but not statistically significant; p=0.345) correlation with the cytoplasmic APE1/Ref-1 protein localization it's presents. Furthermore we find in those patients only, more elevated cytoplasmic levels of APE1/Ref-1 than nuclear localization. Collectively, we can not yet confirm that, as in other types of cancer, HCC is associated with a significant localization of APE1/Ref-1 in the cytoplasm of tumor cells but additional data are needed to provide an explanation on the molecular events at the basis of these observations.

Factors such as patient age, size and number of tumors, presence of a tumor capsule, vascular invasion, histological grade, pathological TNM stage and surgical resection margins, have all been demonstrated to affect recurrence postoperatively [50–51]. Curently, no data are sufficient to describe an association between cytoplasmatic accumulation of APE1 and those factors or survival time. On the other hand, vascular invasion appears to be related to mtDNA damage (p=0.008). Vascular invasion it's a well know independent factor affecting survival in early HCC reason why as we said, an early diagnosis is so important to improve prognosis and therapeutic efficacy [52] and the improved survival following hepatectomy for HCC has been mainly attributed to the prevention of recurrence.

Finally, a deeper understanding of the molecular events associated with the transformation from normal into neoplastic liver cells is necessary but cellular fractionation and sample preparation represent key processes for both genomic and proteomic analyses. Here we described a procedure that, by starting from a single liver biopsy, allows for: i) the isolation of intact mitochondria and nuclei from liver tissue of human origin; ii) the purification ofnDNA and mtDNA from said tissues for genomic analyses; and iii) the quantification of mtDNA damage. With respect to the latter, few years ago, Authors published an updated quantitative PCR (QPCR)- based method to measure nuclear and mtDNA damage in mammalian cells avoiding the isolation of mtDNA from the genomic [12]. This represents undoubtedly a main strength of the assay because the labor intensive step of mitochondrial isolation is not needed. However, our data demonstrate that through this approach high levels of mtDNA damage may be underestimated because damage to nucleotide bases could occur also in the short amplicon therefore altering the quantification of the relative amount of mtDNA copies (fig. 2.e – g). This data support the hypothesis that in samples with a high level of damaged bases, such as tumoral tissues, it is not possible to use total DNA for the calculation of mtDNA damage [12].

In our study, mtDNA damage is characterized by a lowering localization of cytoplasmic APE1/Ref-1 (even if not so significantly) compared with surrounding liver, contrarily to others authors [23,25,26,48, 53].

Collectively, these data suggest a possible mutual relationship between cytoplasmic APE1/Ref-1 and extension of mtDNA in the development of the HCC. This may indicate that the subcellular localization of APE1/Ref-1 in HCC tissue might be used as a prognostic marker for this worldwide tumor and index of sensitivity to surgical, ablative or chemotherapy treatments.

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