Subcellular Localization of APE1/Ref-1 in Human Hepatocellular Carcinoma: Possible Prognostic Significance

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APE1/Ref-1, normally localized in the nucleus, is a regulator of the cellular response to oxidative stress. Cytoplasmic localization has been observed in several tumors and correlates with a poor prognosis. Because no data are available on liver tumors, we investigated APE1/Ref-1 subcellular localization and its correlation with survival in 47 consecutive patients undergoing hepatocellular carcinoma (HCC) resection. APE1/Ref-1 expression was determined by immunohistochemistry in HCC and surrounding liver cirrhosis (SLC) and compared with normal liver tissue. Survival probability was evaluated using Kaplan-Meier curves (log-rank test) and Cox regression. Cytoplasmic expression of APE1/Ref-1 was significantly higher in HCC than in SLC ($P = 0.00001$); normal liver showed only nuclear reactivity. Patients with poorly differentiated HCC showed a cytoplasmic expression three times higher than those with well-differentiated HCC ($P = 0.03$). Cytoplasmic localization was associated with a median survival time shorter than those with negative cytoplasmic reactivity (0.44 compared with 1.64 years, $P = 0.003$), and multivariable analysis confirmed that cytoplasmic APE1/Ref-1 localization is a predictor of survival. Cytoplasmic expression of APE1/Ref-1 is increased in HCC and is associated with a lower degree of differentiation and a shorter survival time, pointing to the use of the cytoplasmic localization of APE1/Ref-1 as a prognostic marker for HCC.

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INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most frequent neoplasm worldwide, accounting for 5.6% of all human cancers (1), and the third cause of estimated cancer-caused deaths. Approximately 560,000 cases are diagnosed and 550,000 deaths result from liver cancer (2,3) each year. The development of HCC is generally the final event of longstanding liver disease. 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 (4).

The molecular events related to hepatocarcinogenesis are still not well known. HCC is a long step-by-step process (5,6) 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 (7).

The vast majority of HCC develops in a cirrhotic liver (8). Hepatocyte DNA damage based on oxidative ground is observed in chronic inflammatory processes (9,10) and, in the imbalance of an adequate base repair system, can induce genomic and mitochondrial DNA damages (11). This is particularly true during the evolution from hepatitis to cirrhosis and to neoplastic process.

A strong body of 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 (12,13). The increase of ROS, associated with the disease development and induced by developing parenchymal damage, promotes oxidative stress damage to proteins, lipids, and DNA (14), and activation of transcription factors such as NF-κB, STAT3, and AP-1 that are either involved

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in cell-survival pathway (15,16) or specifically activated in hepatocellular carcinoma (17,18). ROS generated by chronic inflammation are closely linked to hepatocellular oxidative DNA damage and may be involved in the process of hepatocarcinogenesis (19).

Apurinic apyrimidinic endonuclease/redox effector factor 1 (APE1/Ref-1) is a master regulator of cellular response to oxidative stress conditions. APE1/Ref-1 is a multifunction protein involved in both transcriptional regulation of gene expression during adaptive cellular response to oxidative stress and in the base excision repair (BER) pathway of DNA lesions generated as a consequence of oxidant-induced base damages, contributing to the maintenance of the genome stability (20). Although its subcellular distribution in different mammalian cell types is mainly nuclear, cytoplasmic localization has also been described (20); interestingly, this particular subcellular distribution has been associated with different tumorigenic processes (20). In particular, in the case of lung (21), ovarian (22), thyroid (23,24), and breast (25) 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 expression of APE1/Ref-1 in hepatocellular carcinoma (HCC) and surrounding liver cirrhosis (SLC). The possible prognostic role of APE1/Ref-1 subcellular localization in HCC was also evaluated.

MATERIALS AND METHODS

Subjects

Forty-seven consecutive patients (thirty-four men and thirteen women) who underwent HCC resection at the departments of surgery of the Universities of Trieste and Udine were retrospectively studied. 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. Fifteen patients who underwent resection of hepatic angioma were used as controls for immunohistochemical analysis.

Sampling Collection, Analysis, and Histological Grading

Samples of HCC, SLC, and liver tissue surrounding the angioma (normal liver) were obtained at the time of the surgical resection, fixed in formalin for a maximum of 24 h, and embedded in paraffin. In HCC cases, each liver sample was sufficiently large to include both the tumor and the surrounding cirrhosis. From each sample, 4-μm sections were cut; 1 slide was stained with hematoxylin-eosin and used for morphological diagnosis, whereas the other slides were used for immunohistochemical analysis. HCC samples were histologically classified according to the Edmondson and Steiner criteria (26). All histological specimens were analyzed by a single operator (C.A.).

Sample collection for Western blot analysis was performed as described (27).

Immunohistochemical Analysis

Immunohistochemical detection of APE1/Ref-1 was performed by immunohistochemistry using the anti-APE1/Ref-1 mouse monoclonal antibody, prepared as described (28), as primary antibody and the Super Sensitive TN Polymer HRP IHC (Bio Genex, San Ramon, CA, USA) as the detection system.

The dewaxed sections were treated with H_2O_2 and PBS solution (40 mL H_2O_2, 140 vol., added to 160 mL PBS) for 10 min at room temperature to inhibit endogenous peroxidase, and then immersed in 10 mM citrate buffer (pH 6.0) for 40 min at 98°C. The sections were then washed with PBS and incubated with the primary antibody (1:50 diluted in PBS) at 4°C overnight. The sections were washed twice with PBS and incubated with secondary antibody (Super Sensitive TN Polymer HRP IHC, Bio Genex) for 30 min at room temperature. The peroxidase activity was developed with 3,3′-diaminobenzidine (used as chromogen) for 10 min; Mayer's hematoxylin counterstain for nuclei was subsequently applied.

A positive reactivity for APE1/Ref-1 was indicated by the presence of a dense, homogeneous brown staining for the nucleus and a granular brown staining for the cytoplasm of the hepatocytes.

APE1/Ref-1 expression was evaluated by counting the positive cells on 1000 hepatocytes for each slide and expressed as a percentage. 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.

APE1/Ref-1 immunohistochemical analysis on normal liver was performed with the same protocol used for HCC on the liver tissue surrounding the angioma.

Western Blot Analysis

APE1/Ref-1 protein expression analysis was performed by Western blotting (WB) in five cases of HCC and compared with a similar number of normal liver tissues. Proteins (15 μg) were separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was immunoblotted with the same anti–APE1/Ref-1 antibody used for immunohistochemistry (28). β-Actin was used as housekeeping protein, by using a specific antibody (Sigma, Milan, Italy).

Real-Time RT-PCR (Q-PCR)

APE1/Ref-1 mRNA levels were evaluated on the same samples used for WB analysis. Total RNA was extracted using Tri-Reagent kit (Sigma-Aldrich, St Louis, MO, USA) according to manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Her-

Table 1. Primer sequences

Gene	Accession Number	Forward primer	Reverse primer	Product, bp
APE1/Ref-1	NM_080649	CTGCCTGGACTCTCTCATCAATAC	CCTCATCGCCTATGCCGTAAG	118
18S	X03205	TAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG	150
β-actin	NM_001101	CGCCGCCAGCTCACCATG	CACGATGGAGGGGAAGACGG	120

cules, CA, USA). Real-time quantitative PCR was performed with i-Cycler IQ (Bio-Rad Laboratories); 18S and β-actin were used as housekeeping genes. All primer pairs were synthesized by Sigma Genosys and were designed using the software Beacon Designer 6.0 (Premier Biosoft International, Palo Alto, CA, USA). Primer sequences and references are reported in Table 1. The relative quantification was made using the Pfaffl modification of the ΔCt equation (29,30), taking into account the efficiencies of individual genes. The results were normalized to 18S and β-actin.

Statistical Analysis

Continuous variables, including APE1/Ref-1 expression as detected by immunohistochemistry, are given as median and interquartile ranges (IQR) because of skewed distributions; categorical variables are given as the number and/or percentage of subjects with the feature of interest. Paired and unpaired between-group comparisons were performed with the Wilcoxon and Mann-Whitney tests, respectively. Survival time was defined as the time elapsed between surgical resection of HCC and death. For survival analysis, a positive APE1/Ref-1 expression was defined as greater than or equal to the median value. Survival of patients with and without positive APE1/Ref-1 expression was compared using Kaplan-Meier curves and the log-rank test. Univariable and multivariable Cox regression was used to study the relationship between survival and APE1/Ref-1 expression and to correct for the effect of HCC grading. Statistical significance was set to $P < 0.05$. Sample size was calculated by considering that a Cox regression of the ln hazard ratio on a covariate with a standard deviation of 0.5 (binary variable split at the median) based on a sample of 50 patients followed until death has a 97% power at 0.05 significance level to detect a ln hazard ratio of 1.098 (corresponding to an hazard ratio of 3.0). Statistical analysis was performed using STATA 9.2.

RESULTS

The median age of the forty-seven patients was sixty-six (range 39 to 78) years, with no sex difference ($P = 0.2297$). Seven patients were HBsAg-positive and fifteen anti-HCV positive; seventeen patients had an alcohol consumption > 30 g/day for more than ten years (31); eight patients had no clear predisposing factors for HCC. Eighty-one percent of patients were in stage A and 19% in stage B of liver cirrhosis according to Child-Pugh classification (32). Tumor grading was G1 in nine, G2 in nineteen, G3 in fourteen, and G4 in five patients. Tumor staging, assessed using the Cancer Liver Italian Program score (CLIP) (33), was as follows: 18% CLIP 0; 31% CLIP 1; 17% CLIP 2; 30% CLIP 3; 2% CLIP 4; and 2% CLIP 5.

Figure 1 shows the Western Blot analysis performed in five cases of HCC and in a equal number of normal liver tissue samples. An almost two-fold increase was observed in the content of APE1/Ref-1 in HCC ($P < 0.001$). The same was observed for the mRNA APE1/Ref-1 expression (data not shown). These data, obtained in a small number of frozen samples, were ex-

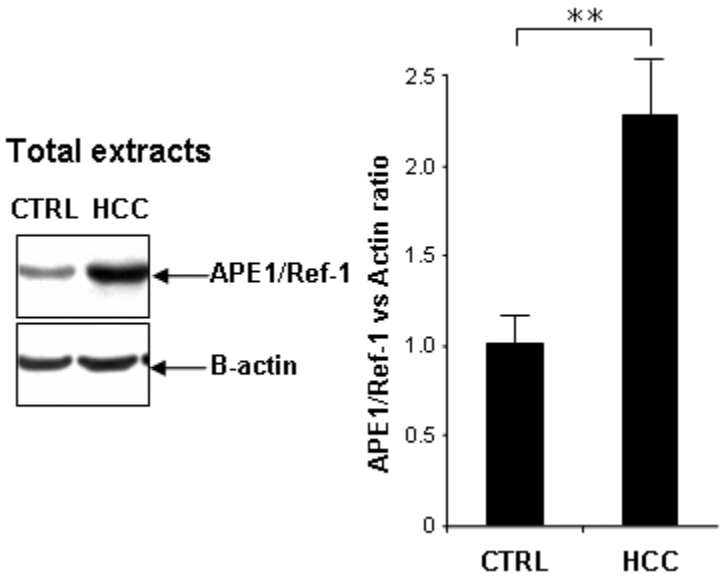

Figure 1. APE1/Ref-1 protein expression analysis. Western blotting in HCC tumor lesions compared with healthy controls (CTRL). A representative Western blot analysis on total extracts from tissue biopsies is shown on the left. The right panel shows expression levels of the two proteins obtained after densitometric analysis of the bands. Values were reported as histograms of the ratio between APE/Ref-1 band intensities and actin (**$P < 0.001$).

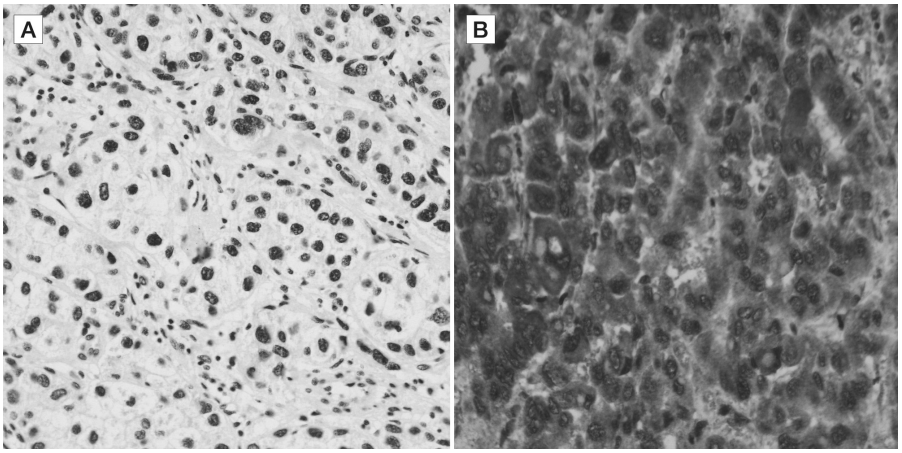

Figure 2. Nuclear and cytoplasmic immunostaining of APE1/Ref-1 in HCC tissue. Nuclear reactivity (*A*) and cytoplasmic reactivity (*B*). Magnification 20×.

panded by immunohistochemistry analysis applied in a much larger cohort of paraffin-embedded cases, allowing us to obtain information on the subcellular distribution of the protein. Positive staining was observed only at the nuclear level of normal hepatocytes and endothelial and biliary duct cells. On the contrary, the expression of APE1/Ref-1 was detected not only in the nucleus (Figure 2A) but also in the cytoplasm (Figure 2B) of HCC cells.

Nuclear and cytoplasmic APE1/Ref-1 expression was significantly higher in HCC than in SLC (Figure 3). The median percentage of positive nuclear staining was almost double in HCC ($P = 0.0004$). This was also the case for cytoplasmic staining, for which the median of APE1/Ref-1 expression was twenty (IQR=40) in HCC and zero (IQR=0) in SLC ($P = 0.00001$).

APE1/Ref-1 expression was also compared between patients with highly differentiated (G1-G2) and poorly-differentiated (G3-G4) HCC. As reported in Table 2, the median value of APE1/Ref-1 cytoplasmic positive staining was almost three-fold higher in poorly differentiated tumors ($P < 0.032$), whereas no difference was found in the nuclear reactivity of APE1/Ref-1. APE1/Ref-1 expression was comparable in the cirrhosis surrounding well and poorly differentiated HCC for both nuclear and cytoplasmic localization.

These findings suggested that the different nuclear and cytoplasmic localization of APE1/Ref-1 may have some relevance to the clinical outcome of the patients, as reported for other types of cancer (21,22,24,25,34). We therefore analyzed whether this was the case for HCC. Because the cutoffs of clinical relevance are not available, we split the value of APE1/Ref-1 expression at the median and defined positive cytoplasm staining as a value of APE1/Ref-1 expression greater than or equal to the median. Figure 4 shows the Kaplan-Meier curves in patients with positive or negative APE1/Ref-1 cytoplasmic expression. Subjects with positive HCC APE1/Ref-1 cytoplasmic reactivity had significantly shorter survival times (median 0.44, 95% CI 0.11–1.46; $P = 0.003$, log-rank test) than those negative for cytoplasmic localization of the protein in HCC (median 1.64, 95% CI 1.09–3.15).

Cox regression was used to study the relationship between survival and APE1/Ref-1 cytoplasmic positivity in HCC tissue and histological grading of the tumor. At invariable Cox regression, age, sex, and Child-Pugh score were not associated with survival time. As shown in Table 3, patients with APE1/Ref-1 cytoplasmic positivity in HCC showed a hazard rate (HR) of 2.5 (95% CI 1.3–4.8, $P = 0.004$), whereas patients with poorly

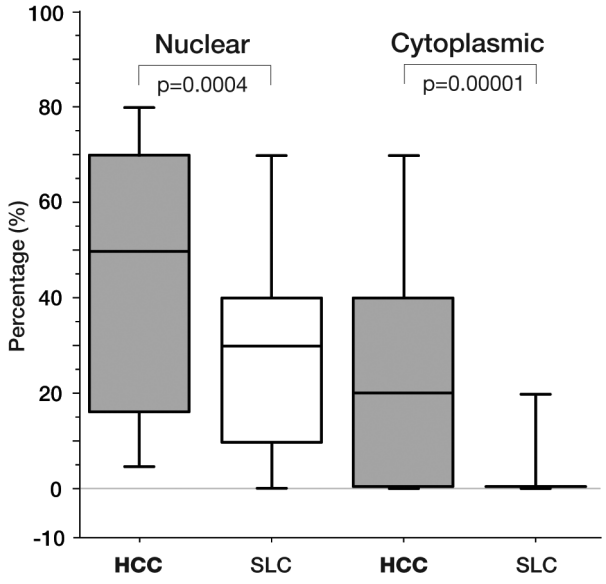

Figure 3. Box plot of the APE1/Ref-1 nuclear and cytoplasmic immunohistochemical reactivity in hepatocellular carcinoma (HCC, gray bars) and surrounding liver cirrhosis (SLC, white bars). Data report the median (expressed in %) of APE1/Ref-1 at both the nuclear and cytoplasmic levels.

Table 2. APE1/Ref-1 subcellular expression according to HCC differentiation grading.

Tissue	Site	G1/G2	G3/G4	*P* Mann-Whitney
HCC	Nucleus	55 (70) (0-85)	45 (35) (0-80)	0.396
HCC	Cytoplasm	10 (28) (0-80)	30 (40) (0-80)	0.032
SLC	Nucleus	20 (38) (0-80)	30 (50) (0-80)	0.335
SLC	Cytoplasm	0 (10) (0-40)	0 (0) (0-40)	0.182

Data are reported as median (range) (min-max) interval. G1/G2, well-differentiated tumors; G3/G4, poorly differentiated tumors.

differentiated HCC (G3-G4 stage) had a HR of 2.0 (95% CI 1.1–3.7, $P = 0.02$).

On multivariable analysis, the HR of patients with positive HCC cytoplasmic expression of APE1/Ref-1 remained significant (2.2; 95% CI 1.1–4.3, $P = 0.021$), whereas the HR of poorly differentiated HCC (G3-G4 grading) was no longer significant (1.6; 95% CI 0.8–3.0, $P = 0.171$), indicating that the cytoplasmic localization of APE1/Ref-1 is a predictor of survival independent from grading.

DISCUSSION

HCC is one of the most common neoplasms worldwide (1), and it almost invariably develops as the natural outcome of a chronic hepatitis or cirrhosis (5,8,11,35). An early diagnosis is of the utmost importance to improve prognosis and therapeutic efficacy (36). A deeper understanding of the molecular events associated with the transformation from normal into neoplastic liver cells is necessary.

The increased cellular turnover associated with chronic liver injury and HCC occurs in a milieu of chronic inflammation (37) and cellular oxidative stress conditions (38). Some viral proteins such as NS5A of HCV and HBx may increase the intracellular concentration of ROS and the cytoplasmic Ca^{2+} content, which stimulates the activation of NF-κB and STAT3 (15,16,39) (Figure 5).

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 (40) and NF-κB (41), and DNA repair functions (Figure 5). APE1/Ref-1 has been demonstrated to play a prognostic role in a variety of human tumors (21,22,24,25,34). We observed that in the forty-seven HCC cases, APE1/Ref-1 expression was significantly higher in HCC tissue than in the surrounding cirrhosis (for cytoplasmic or nuclear localization), and this was confirmed by Western blot and Q-PCR analysis, which showed increased APE1/Ref-1 protein and mRNA in HCC.

Several studies demonstrated that different oxidative agents promote an increase of APE1/Ref-1 mRNA and protein expression, and that the protein up-regulation is always associated with an increase in both transcriptional and AP endonuclease activity (42,43). 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 (19). 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 histological differentiation grading. APE1/Ref-1 cytoplasmic expression in HCC tissue was significantly higher in poorly differentiated than in well-differentiated tumors. No difference was found in nuclear expression between the two groups. It is worth mentioning that in normal liver, APE1/Ref-1 was found to be localized only in the nuclei of hepatocytes and endothelial and biliary ductal cells suggesting that 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 (44), breast cancer (25,45), thyroid carcinomas (24), and epithelial ovarian cancers (22). In each of these tumors, cytoplasmic expression of APE1/Ref-1 was demonstrated for tumor cells whereas nuclear reactivity was found mostly in the normal cells. Increased cytoplasmic expression of APE1/Ref-1 was also found in malignant melanoma (34). Collectively, our data point to the conclusion that, as in other types of cancer, HCC is associated with a significant expression of

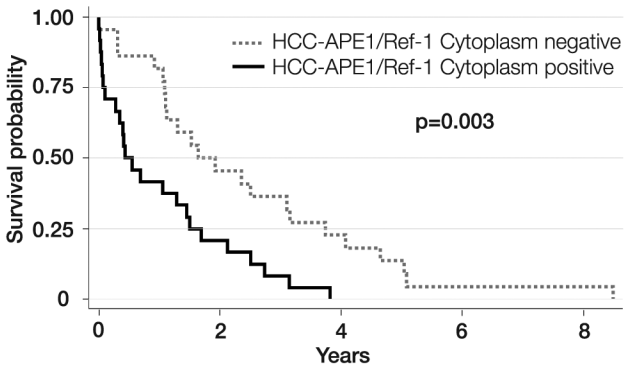

Figure 4. Kaplan-Meyer survival curve according to the cytoplasmic localization of APE1/Ref-1 in HCC tissue. Dashed line indicates cases negative for cytoplasmic presence of APE1/Ref-1, solid line indicates cases where APE1/Ref-1 was found.

Table 3. Univariable Cox regression analysis.

	HR	95% CI	*P*
HCC, APE1/Ref-1 cytoplasm positive (20%)	2.5	1.3–4.8	0.004
Age	1.0	0.9–1.1	0.496
Male sex	1.1	0.5–2.1	0.864
Child-Pugh score	0.9	0.4–1.9	0.735
G3/G4	2.0	1.1–3.7	0.026

APE1/Ref-1 in the cytoplasm of tumor cells.

This peculiar distribution was also shown to correlate with the aggressiveness and prognosis of the neoplasia. In breast cancer, APE1/Ref-1 nuclear localization was always associated with a better prognosis, low angiogenesis, and negative lymph node status, whereas cytoplasmic expression was predictive of a poor prognosis (25,45). From our data, this seems to be true also for HCC, because patients with cytoplasmic localization of APE1/Ref-1 showed a median survival time after resection significantly shorter than patients with negative cytoplasmic staining. These data underline the strong association of the cytoplasmic localization of APE1/Ref-1 with HCC and reinforce the hypothesis that APE1/Ref-1 cytoplasmic accumulation in HCC tissue could be related to a poor prognosis. This is further supported by the finding that the hazard rate of subjects with APE1/Ref-1 cytoplasmic localization was 2.5-fold higher than those negative for the protein. This difference holds when the grading is included in the multivariable analysis, again pointing to the prognos-

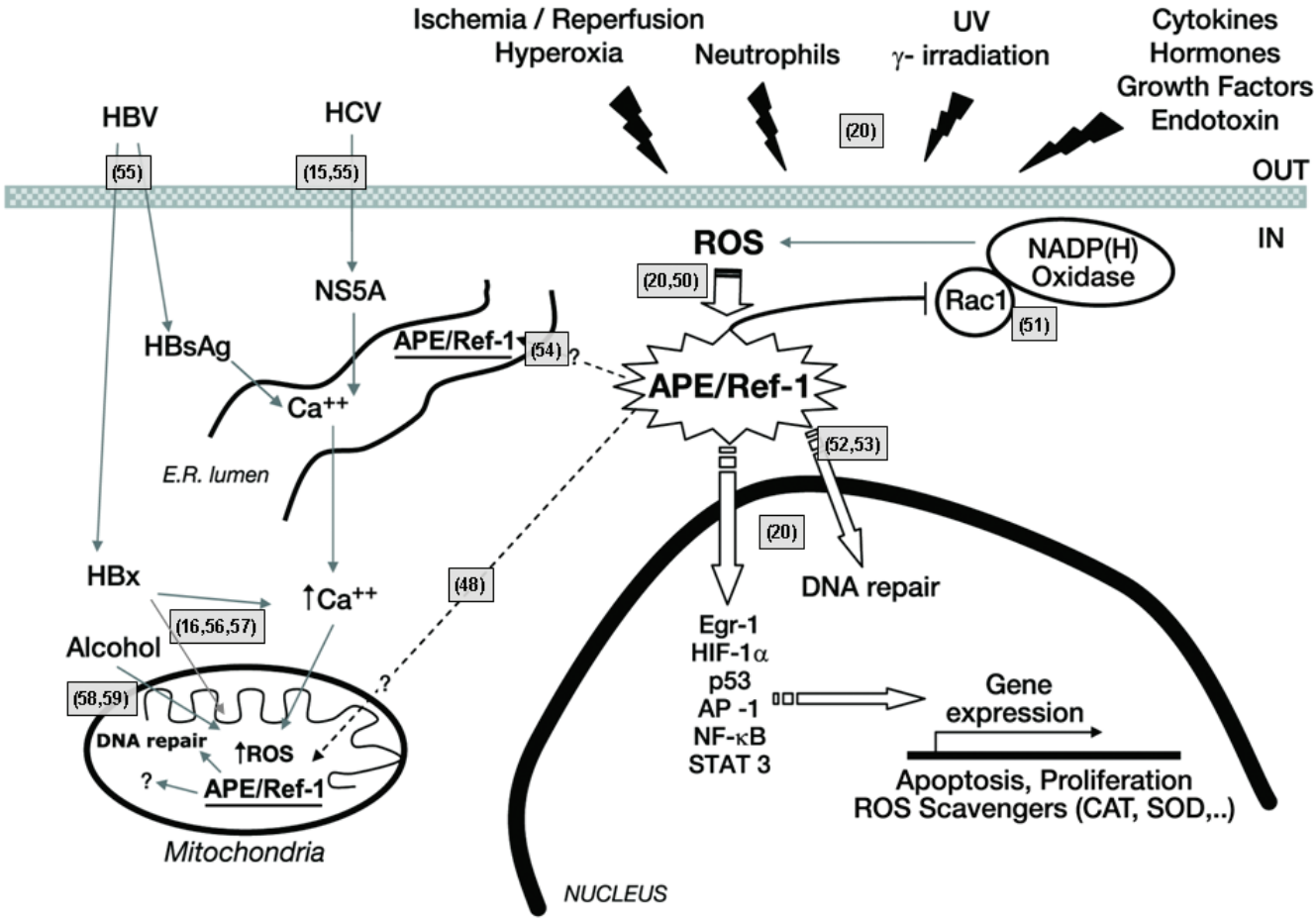

Figure 5. Schematic representation of APE1/Ref-1 stimulation and function. HBV, hepatitis B virus; HCV, hepatitis C virus; HBsAg, hepatitis B virus antigen S; HBx, hepatitis B virus X protein; NS5A, hepatitis C virus nonstructural 5A protein; Rac1, EGR-1, early growth response 1; HIF-1α, hypoxia-inducible factor 1; p53, p53 tumor suppressor protein; AP-1, transcription factor AP1; STAT3, signal transducer and activator of transcription 3.

tic role of the cytoplasmic localization of the protein.

The intracellular expression and the localization of APE1/Ref-1 are strictly regulated processes. Stimuli that promote APE1/Ref-1 expression are also able to control its intracellular trafficking (20). As shown in Figure 5, several different intracellular and extracellular stimuli activate APE1/Ref-1 (20). Each of these stimuli, particularly ROS production, has been described to act in the maintenance of chronic liver injury (46). The finding that HCC showed more elevated cytoplasmic levels of APE1/Ref-1 than SLC suggests that neoplastic transformation of liver cells is associated with a possible role of the protein in the propagation of cellular damage. During cellular response to oxidative stress, neosynthesized APE1/Ref-1 is rapidly translocated into the nuclear compartment by means of its N-terminal bipartite nuclear localization sequence that also guarantees its nuclear maintenance (47). However, whereas its nuclear roles as transcriptional coactivator and in the BER pathways of DNA damage are well established, very little is known about APE1/Ref-1 extranuclear functions. Within the cytoplasmic compartment, APE1/Ref-1 has been associated with mitochondria (48,49) and with the endoplasmic reticulum, although its function in these districts is completely unknown. A cleavage at Lys 31, giving rise to a truncated form of the protein with higher electrophoretic mobility, regulates the mitochondrial localization of the protein by removing its nuclear localization signaling sequence (48). Therefore, the loss of the N-terminal part may be responsible for APE1/Ref-1 cytoplasmic retention by inhibiting its nuclear accumulation. Additional data are needed to provide an explanation on the molecular events at the basis of these observations.

In conclusion, APE1/Ref-1 is a multifunctional protein playing a central role in cellular response to oxidative stress, involving both control of gene expression and maintenance of genome stability. HCC is characterized by an increased expression of APE1/Ref-1 compared with surrounding liver cirrhosis, with cytoplasmic localization of the protein. This higher cytoplasmic accumulation is associated with a lower degree of differentiation and a significantly shorter survival time. 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.

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