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Report

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KEY WORDS

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ABSTRACT

A common polymorphism at codon 72 in p53 gene leads to an arginine to proline aminoacidic substitution which affects in an age-dependent manner the susceptibility of cells to undergo apoptosis after oxidative stress. Here we report that dermal fibroblasts from Proline allele carriers (Pro+) display a higher expression of p21 WAF1 gene, in both basal conditions and after treatment with doxorubicin or camptothecin. This phenomenon is accompanied by a lower susceptibility of Pro+ cells to undergo apoptosis, a lower capability to over cross G_1-S transition and an increased propensity to express markers of cell senescence, with respect to fibroblasts from Arginine homozygotes (Pro-). All these phenomena are particularly evident in cells from centenarians. We conclude that the functional difference between the two p53 codon 72 alleles exerts a broad impact on the capability of cell from aged people to respond to stressors such as cytotoxic drugs.

ABBREVIATIONS

DFs, dermal fibroblasts; Pro-, p53 arginine homozygote; Pro+, p53 proline carrier; FCS, foetal calf serum; <2c cells, cells with hypodiploid DNA content; PI, propidium Iodide; BrdU, bromo deoxy uridine; DMEM, Dulbecco's modified Eagle's medium; cpt, camptothecin; doxo, doxorubicin; SEM, standard error of the mean; TBE, Tris-boric acid-EDTA buffer; TRF, terminal restriction fragment; SDS-PAGE, sodium dodecyl sulphate poly-acrylammide gel electrophoresis

INTRODUCTION

p53 gene harbours a common sequence variation which yields an Arginine (Arg) to Proline (Pro) aminoacidic substitution at codon 72.[1,2] This aminoacid substitution lies in the Proline-Rich domain, which is known to affect a number of p53 activities including the interaction with a variety of proteins. Accordingly, the two isoforms display different biochemical properties, and they differentially interact with oncogenic viral proteins of HPV, as well as with the cell transcriptional machinery and other member of p53 family such as p73.[3-5] A variety of studies have recently shown that p53Arg allele is endowed with a greater capacity to bind to MDM2, to be targeted at the mitochondria, where it interacts with Bcl-2/Bcl-xL and triggers the cleavage of caspase 3.[6-8] As far as the transcriptional difference between the alleles, contrasting data have been obtained.[6,7,9] In particular, the p53Pro allele was found to be capable of eliciting p21WAF1 gene transcription at a higher extent in some in vitro models, but not in others.[3,9] We recently found that in cells from aged subjects p53 codon 72 alleles are able to modulate stress-induced apoptosis, this phenomenon being particularly evident when cells from centenarians are considered. Such an in vitro finding is paralleled by data obtained in vivo in patients suffering by myocardial acute ischaemia, a pathological event where oxidative stress-induced cell death takes place.[8]

In this paper we tested the relevance of p53 codon 72 polymorphism for the response of dermal fibroblasts (DFs) from young people and centenarian subjects to apoptotic agents such as doxorubicin and camptothecin, two drugs widely used in cancer therapy acting on proliferating cells. Accordingly, we evaluated the capacity of these drugs to induce apoptosis or cell senescence, and we investigated the role of p21WAF1 in this process, as this gene is transcriptionally regulated by p53 and, beside its role as a wide inhibitor of cyclin/CDK complexes regulating transition between different stages of cell cycle, it is also a senescence inducing factor and it has a well known antiapoptotic activity.[10,11]

MATERIALS AND METHODS

Cells from healthy subjects with known p53 codon 72 genotype and drug treatments. Dermal fibroblasts (DFs) were obtained from subjects previously assessed for the p53 Codon 72 genotype.[12] Namely, 8 healthy young people (aged 30 ± 3 years), and 6 healthy centenarians, categorised "A" for their healthy status, as previously described.[13,14] All the subjects were devoid of any clinical, or biochemical abnormalities at the moment of the skin biopsy. p53 codon 72 proline/proline genotype is quite rare in the Italian population (about 8–10%), and the proline allele is likely to exert a dominant effect on the arginine one.[12,15,16] Accordingly, two groups of subjects were considered for this study: Pro+ (proline/proline and proline/arginine genotypes) and Pro- (arginine/arginine genotype) subjects.

DFs long term cultures were established from young people were four Pro+ (two proline/proline and two arginine/proline) and four Pro-; those established from centenarians were three Pro+ (two proline/proline and one proline/arginine) and three Pro- subjects. DFs were cultured in DMEM supplemented with 10% FCS. DFs from the 6th to the 14th passage were used for all the experiments. DFs cultures were allowed to reach subconfluence prior of being exposed to the chemiotherapic treatment. As for experiments of apoptosis detection, DFs were allowed to synchronise as described,[8] then reseeded at low density (60,000/cm^2) in FCS-supplemented medium in presence or absence of 5 µM camptothecin or 500 ng/ml doxorubicin. Cells were collected after 48 and 72 hours of treatment. Note that cells were allowed to attach to the plastic substrate before drug exposure. Spontaneous and drug-induced β-galactosidase activity was assayed by using in situ β-galactosidase kit (Stratagene, La Jolla, CA, USA). A minimum of 100 cells were counted for each sample. The percentage of positive cells was calculated as follows: number of positive cells/total number of cells*100.

Cytofluorimetric analysis. All the cytofluorimetric analyses were performed using a FACScalibur cytometer (BD, San José, CA, USA) equipped with an Argon ion laser tuned at 488 nm. In all analyses, a minimum of 10,000 cells per sample were acquired in list mode and analysed with Cell Quest software (BD).

Cell death assay. The DNA content in DFs was assessed using Propidium Iodide (PI) staining. The appearance of a hypodiploid peak of PI fluorescence was took as an index of apoptosis. Briefly, cells were detached with cell scraper and resuspended in hypotonic solution containing 0.1% sodium citrate, 0.1% Triton X-100 and 50 µg/ml PI, and kept for at least 1 hour at 4°C. Therefore, cells underwent FACS analysis, and those with low PI fluorescence were considered apoptotic.

Cell cycle analysis. Cell cycle was evaluated by classical biparametric analysis of DNA content and BrdU incorporation. Briefly, DFs were pulsed with 20 mM BrdU for 30 minutes, then permeabilised with PBS + 0,5% Tween 20, and stained with anti-BrdU monoclonal antibodies (BD) and counterstained with PI solution according to standard procedures. Cells positive for BrdU staining were considered to be in S phase.

p21^{WAF1} detection. Cells were collected and fixed for 10 minutes in paraformaldehyde, permeabilised with 0.05% Triton-X100 and incubated
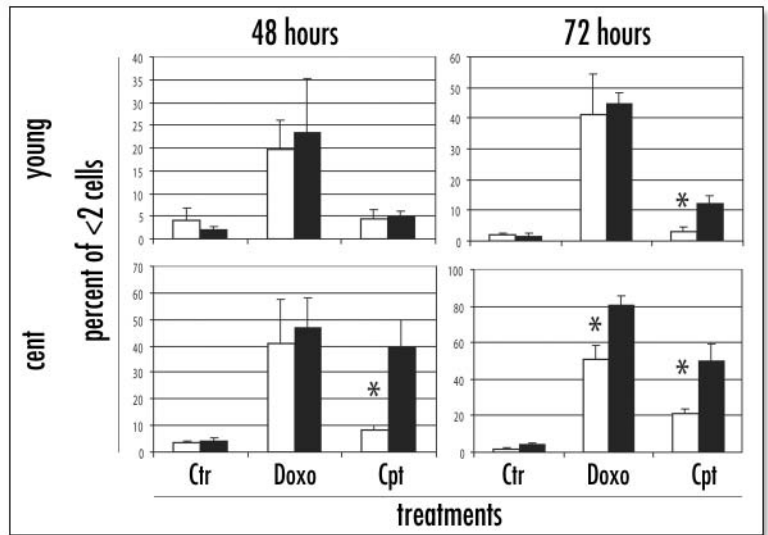

Figure 1. Flow cytometric analysis of drug-induced apoptosis on DF cultures. DF cultures from eight young people (four Pro+ vs four Pro-, upper panels), and from six centenarians (three Pro+ vs three Pro-, lower panels) were studied. Data refer to the percentage of DFs with hypodiploid DNA content (<2c cells) and are expressed as percentage ± SEM. *p < 0.05; by Student' t test. Left panels: 48 hours of treatment; right panels: 72 hours of treatment. Ctr: control untreated cells; Doxo: doxorubicin 500 ng/ml; Cpt: camptothecin 5 µM. White bars: Pro+ subjects, black bars: Pro- subjects.

Figure 2. Analysis of cell cycle. (A) Representative example of flow cytometric analysis of BrdU incorporation and PI staining in DFs from centenarians (Pro+ on the left and Pro- on the right) after 48 hours from replating. Numbers represent the percentage of BrdU positive cells. (B) Statistical analysis of S phase in DFs eight from young subjects (four Pro- vs four Pro+, upper panel), and from six centenarians (three Pro- vs three Pro+, lower panel) as assessed by the above flow cytometric technique, assayed after 48 hours in presence or absence of treatment. Data refer to the percentage of cells positive for BrdU, and are expressed as mean ± SEM. *p < 0.05, Pro+ vs Pro-, by Student' t test. Ctr, control untreated cells; Doxo, doxorubicin 500 ng/ml; Cpt, camptothecin 5 µM. White bars: Pro+ subjects, black bars: Pro- subjects.
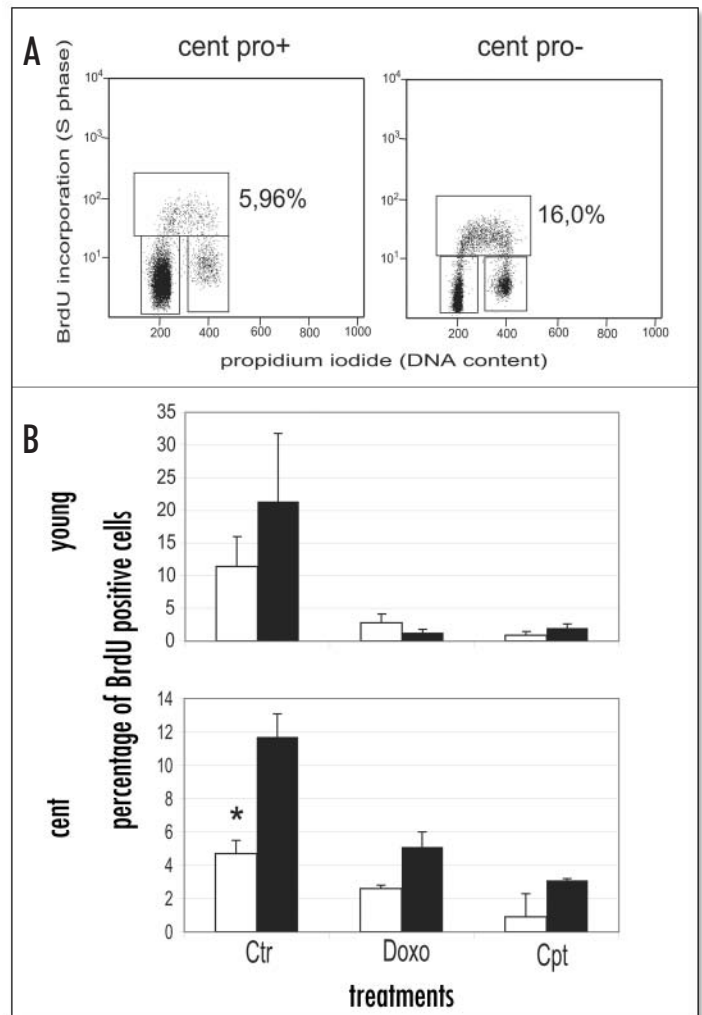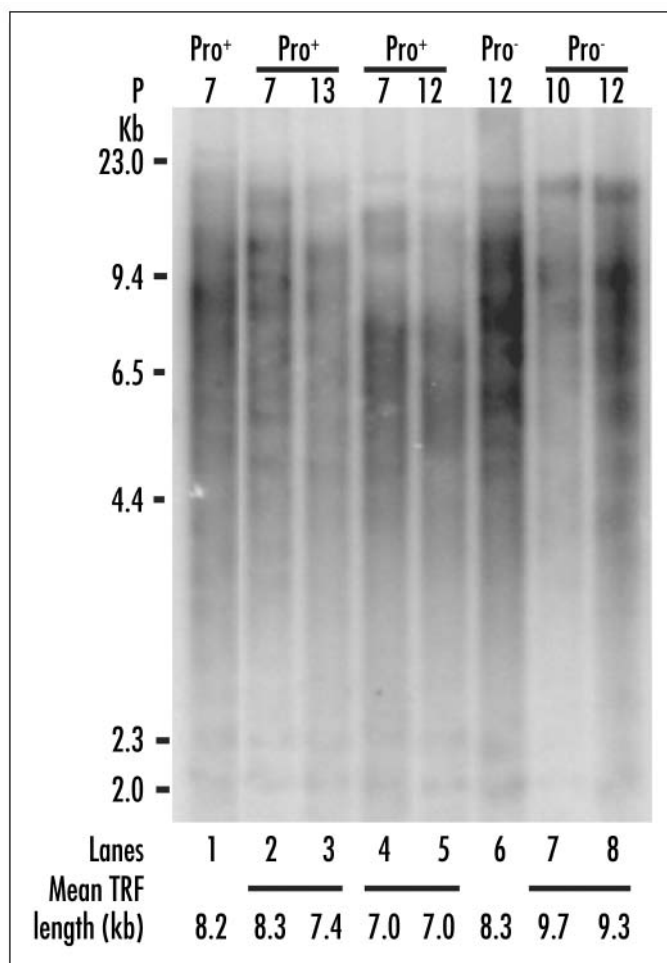
Figure 3. Telomere length assessment in DFs from centenarians. Analysis of the terminal restriction fragments (TRF) distribution in DNA samples prepared from DFs cultures of centenarians. Lanes 1–5: DNA samples obtained from three Pro+ centenarians; lanes 6–8: DNA samples obtained from two Pro- centenarians. The size (kb) and the position of the DNA molecular weight markers are indicated on the left. Culture passage (P) and genotype of cultures are reported above the lanes. Below each lane, the mean length (in Kb) of the TRF is reported.

with specific monoclonal antibody against p21^{WAF1} (Santa Cruz Biotechnologies, CA, USA), following standard procedure for intracellular antigens staining.[17] Briefly, cells were first incubated with complement-deprived human serum, in order to saturate Fc receptors, then incubated with primary antibody for 1 hour at 4°C. Cells were washed twice in cold PBS and incubated with FITC-conjugated secondary antibody for 1 hour at 4°C, washed in cold PBS and analysed. For each sample a negative control (i.e., sample stained with an irrelevant antibody) was performed. To quantify the amount of antigen present into cells, we subtracted the linearized median value of the negative sample (spontaneous autofluorescence) to the linearized median value of positive samples, as described.[18]

Analysis of SaOs-2 cells after transfection for simultaneous detection of EGFP fluorescence and DNA content. Cells were collected after 24 hours of transfection with 40 ng of pCMS-EGFP plasmid as described below. and fixed according to Lamm et al. to preserve EGFP fluorescence.[19] Briefly, cells were treated with 2% paraformaldehyde in NaCl 100 mM, Sucrose 300 mM, MgCl₂ 3 mM, EGTA 1 mM and PIPES 10 mM, pH 6.8 for 30 min., then washed twice in PBS and post-fixed overnight with ice-cold ethanol 70% at -20°C. Afterwards cells were stained as described for cell death assay. and simuoltaneously analysed for EGFP and PI fluorescences.

RT-PCR and competitive quantitative RT-PCR. DFs from p53 codon 72 arginine and proline homozygous subjects were lysed in TriPure Isolation Reagent (Roche MB, Basel, Switzerland) and total RNA was ethanol-precipitated according to standard procedures. The amount and the purity of the extracted RNA was measured by spectrophotometry ($OD_{260\ nm}$, $OD_{260/280\ nm}$), its integrity was assessed by electrophoresis on agarose gel. cDNA was synthesised in a 20 µl reaction mixture containing 0.1 µg Oligo(dT)$^{12-18}$, 0.5 mM dNTP, 10mM DTT, 1X First strand buffer, 40 units Recombinant Ribonuclease Inhibitor, 200 units M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA), carried out at 37°C for 50 min., followed by 15 min. at 70°C to inactivate the enzyme.

Full length sequence of p53 was amplified from cDNA by Pfx Taq Polymerase (Invitrogen), using the following primers:

p53Fw: 5'-CCATGGAGGAGCCGCAGTCAGATCC-3',
p53Rv: 5'-GAAGTGGAGA ATGTCAGTCTGAGTCAGGCC-3'.

A PCR fragment of 248 bp from p21^{WAF1} cDNA was obtained by means of two specific primers:

p21-F 5'-CTCTAAGGTTGGGCAGGGTG-3';
p21-R 5'-GAAGAAGGGTAGCTGGGGCT -3'

selected using the dedicated software GENE TOOL (available at web site www.biotools.com). To create a p21^{WAF1} Competitor (p21COMP), a third specific primer (p21-COMP 5'-GAAGAAGGGTAGCTGGGGCTAGG-GAAAAGGCTCAACACTG-3'), sharing the 5' sequence with p21^{WAF1}-D and including a downstream 20 bp sequence was employed in combination with p21^{WAF1}-U. The amplified fragment of 222 bp was directly cloned in to the TOPO TA™ vector (Invitrogen), and quantitated by Spectrophotometric analysis. Competitive PCR reaction was carried out by adding scalar amounts of p21^{COMP} (on a Log$_{10}$ scale first, and Log$_{2}$ later) to a fixed quantity of cDNA. After amplification, PCR products were resolved by gel electrophoresis, stained with Ethidium Bromide and quantified by densitometric analisys by means of a Biorad GelDoc 2000 software.

Transient transfection and luciferase assay. p53-null SaOs-2 cells were cultured in DMEM 10% FCS. SaOs-2 cells were seeded in 6 well plates at a concentration of 2 x 10⁵ per plate, and after 24 hours were transiently transfected with 40 ng of pCMS-EGFP, either empty, or carrying the p53 arginine (pCMS-p53Arg) or proline (pCMS-p53Pro) allele, using the Effectene system, according to the manufacturer instructions (Qiagen, Valencia, CA, USA). After 24 hours, cells were collected to be evaluated for luciferase assay. To perform luciferase assay, cells transfected with the plasmids above were co transfected with p21Luc (100 ng) and pTK Renilla (10 ng) reporter plasmid (gently provided by Carol Prives, Columbia University, New York, USA). Cells were lysed after 48 hours and assayed by means of Promega Dual luciferase assay, according to manufacturer instructions (Promega).

Protein extraction and immunoprecipitation. To analyse protein functions DFs are lysed in CoIp buffer (10 mMTris-HCl pH 7.6, 140 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40) and 10X Protease Inhibitor Cocktail (Sigma). Nonidet-P40-solubilized cell extracts (200 µg protein per immuno-precipation) prepared as above were immunoprecipitated by incubation with 8.0 µl Anti-Caspase-3/CPP32 monoclonal antibody (Transduction Laboratories) overnight at 4°C. The resulting immune complexes were precipitated by incubation with 20 µl of protein A/G PLUS-Agarose microbeads (Santa Cruz Biotechnology) for 2 hours at 4°C. The pellets were washed three times with CoIp buffer and resuspended in Leammli Loading 1X buffer and heated at 95°C for 5 minutes to detach the microbeads from the proteins. After centrifugation, the immunoprecipitates (supernatant) were separated by SDS-PAGE on 10% gel (polyacrilamide) and transferred to nitrocellulose membrane (Trans-Blot™, BIORAD). Filters were then probed for p21^{WAF1} to evaluate the presence of such a protein in the immunoprecipitates.

Chromatin immuno precipitation. To analyse the different affinity of the two p53 isoforms to p21^{WAF1} promoter, a Chromatin Immuno Precipitation

assay has been performed on SaOs-2 cells transfected as above described with pcDNA.3.1 plasmids (Invitrogen) containing either p53 Arg or p53Pro. The main consensus sequence for p53 binding on p21^{WAF1} promoter region has been studied as the target of p53 binding using specific primers (see below). DNA-protein cross linking was obtained by adding to the culture medium 40% formaldehyde to reach a final concentration of 1% and incubating for 15 minutes at room temperature and moderate shaking. The reaction was blocked by glycin 125 mM. Cells were washed and collected using ice-cold PBS with 0.1X Protease Inhibitor Cocktail (Sigma) and cell scraper. Cells were then washed twice with PBS + Protease Inhibitor Cocktail and centrifuged to eliminate supernatant. The cell pellett was then resuspended in 300 μl of RIPA buffer+Protease Inhibitor Cocktail and PMSF and the lysis went on for 1 hour. Sonication was performed as follows: two cycles of 30 seconds each, at 30% of the maximum power of a MSE Sonicator. Resulting material was centrifuged 15 minutes at 13,000 rpm, 4°C and supernatant was recovered. Ten microliters of this supernatant was conserved (input material) as a control for equimolar loading. The supernatant was subjected to immunoprecipitation by overnight incubation at 4°C with a preformed anti-p53 antibody-A/G PLUS-Agarose microbeads complex p53 protein, using a mix of 10 μl of anti p53 from clone AB-1 (Oncogene) and 10 μl of anti p53 from clone CBL 429 (Cymbus Biotech). The obtained material was washed five times with RIPA buffer (twice), LiCl$_2$ detergent buffer (0,5% deoxycholic acid, 1 mM EDTA, 0,5% Nonidet P-40, 10 mM Tris-HCl, pH 8), TBS 1X buffer (150 mM NaCl, 20

Figure 4. p21 ^{WAF1} mRNA and protein levels in DFs of different p53 codon 72 genotype. (A) Competitive quantitative RT-PCR analysis of p21 ^{WAF1} mRNA in DFs. The amount of PCR product was constant and coamplified with decreasing amount of competitor (p21^{WAF1COMP}). The "#" indicates the concentration of p21^{COMP} equalling that of p21 ^{WAF1} mRNA. (B) Western blot analysis of p21^{WAF1} immunoreactivity in DFs in absence of stimulation. One subject for each group is showed. (C) Flow cytometry semi-quantitative analysis of p21 ^{WAF1} immunoreactivity of DFs in absence of stimulation. Data are presented as the median channel of p21^{WAF1}-related fluorescence emitted by DFs marked with anti p21^{WAF1} monoclonal antibody and FITC-conjugated secondary antibody (fluorescence arbitrary units). Data are expressed as mean ± SEM of four Pro$^-$ and four Pro$^+$ DFs from young subjects and of three Pro$^-$ and three Pro$^+$ DFs from centenarians. *p < 0.05, by Anova test. White bars: Pro$^+$ subjects, black bars: Pro$^-$ subjects.

mM Tris-HCl, pH 7.6) and TE 1X buffer (pH 7.6) respectively. The pellet was finally resuspended in TE buffer 1X pH 7.6 + Proteinase K 2% and SDS 0.25% and incubated at 65°C for four hours to break cross links, then DNA fragments were extracted according to standard protocols using phenol-cloroform-isoamylic alcohol solution. The obtained DNA and the input material were then subjected to PCR using primers specific for the sequence upstream to the main p53 binding site on p21^{WAF1} promoter according to MatInspector software (Genomatix Softwares, Germany):

F: 5'-TGGCATAGAAGAGGCTGGTGGC-3' (22 bp)
R: 5'-AAGGCAAGGGAGCAGGCTGTTAA-3' (23 bp).

The PCR products were subjected to LE 1% agarose gel migration in 0,5X TBE buffer stained with 0,5 μg/ml Etidium Bromide. Bands were then analysed with Gel Doc 2000 (BIO-RAD) and quantified with respect to those related to the input material using Quantity One software.

Western blot assay. To obtain whole cell lysates for Western blot analysis, DFs and SaOs2 cells were lysed in CoIP Buffer (0,5% Nonidet P-40, 10 mM Tris-HCl, pH 7.6, 140 mM NaCl, 5 mM EDTA, pH 8.0), added of protease inhibitor cocktail (Sigma) and NaVO$_4$. For Western blot analysis the used antibodies were: anti p21^{WAF1} polyclonal (C-19, Santa Cruz), anti Caspase 3 (Transduction Laboratories), anti actin polyclonal (C-11, Santa Cruz), anti β-tubulin monoclonal (D-10, Santa Cruz). All primary antibodies were probed by a secondary Horse Radish Peroxidase (HRP) conjugated antibody (Bio Rad Laboratories, CA). Chemiluminescent assay was used for detection (Santa Cruz).

Fluorescence microscopy. DFs were cultured on plastic chamber slides Lab Tek II (Nalge Nunc, Naperville, IL, USA) and treated with or without 5 μM cpt for 48 hours, then stained with 80 nM MitoTracker Red CMXRos (Molecular Probes) 45 minutes before the end of cpt incubation, then fixed with 4% paraformaldehyde in PBS for 10 minutes, permeabilised with 0,1% Triton X-100 and stained according to standard protocols for
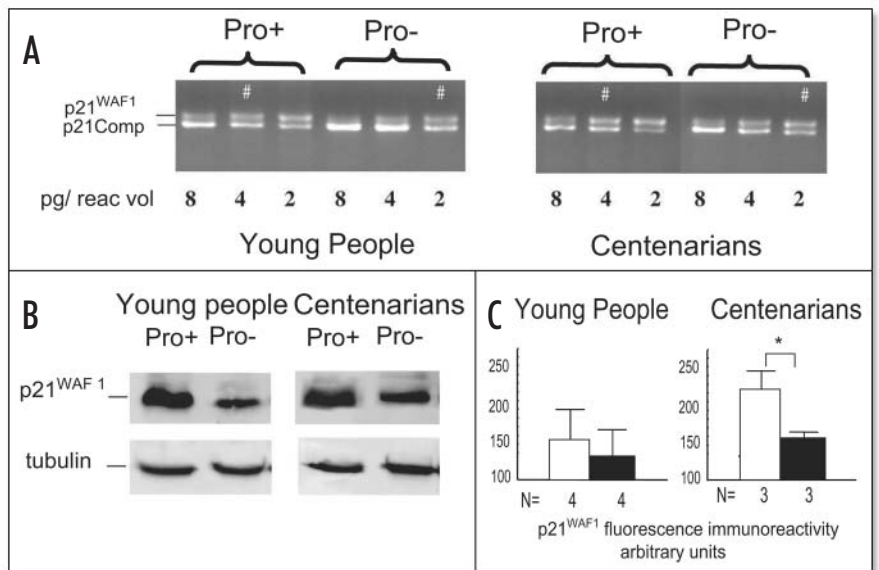
intracellular antigens with polyclonal antibody against p21^{WAF1} (clone C-19, Santa Cruz) revealed by a FITC-conjugated secondary antibody (Dako, Denmark). Exposure time and fluorescence enhancement were fixed at a value by which green and red fluorescences resulted undetectable in unstained samples. Slides were mounted with ProLong antifade (Molecular Probes) and then observed with a Axioplan fluorescent microscope (Zeiss, Germany) with 60x oil-immersion magnification. Images were collected with a Kodak MDS 290 photocamera (operated by a Kodak Microscopy Documentation System) and analysed with Thumbs Plus 5 software (Cerious Software, Charlotte, NC, USA).

Telomere length analysis. Telomere length was analyzed by determining the mean length of the terminal restriction fragments (TRFs) by Southern blotting, as described elsewhere.[20] Briefly, DNA samples were digested with the restriction enzymes *Hin*fI and *Rsa*I (Amersham), resolved on a 0.6% agarose gel, and transferred to a nylon membrane (Hybond N; Amersham). The filters were hybridized with a probe for the telomeric TTAGGG repeats, a mixture of synthetic (TTAGGG)n fragments ranging in size from 100 to 400 bp. The probe was labeled with ^{32}P using the Mega-Prime DNA labeling system (Amersham). After an overnight hybridization at 65°C, the final washing was performed in 0.2x SSC at 65°C. To detect the hybridization signal, the filters were exposed to a Phosphor-Imager (Applied Biosystems) screen for 24–48 h. The hybridization signal was smeared, reflecting the length heterogeneity of the TRFs. The signal intensity along each lane (between migration distances corresponding to molecular weights just over 23 kb and to 2 kb) was quantified by the Image-Quant software and the data were used to determine the mean length of TRFs.

Statistical analysis. ANOVA test, Chi square test and Student' *t* test were used in the statistical analysis to verify differences in quantitative variables. Data analysis was performed by SPSS 10 Package (SPSS, Chicago, IL, USA).
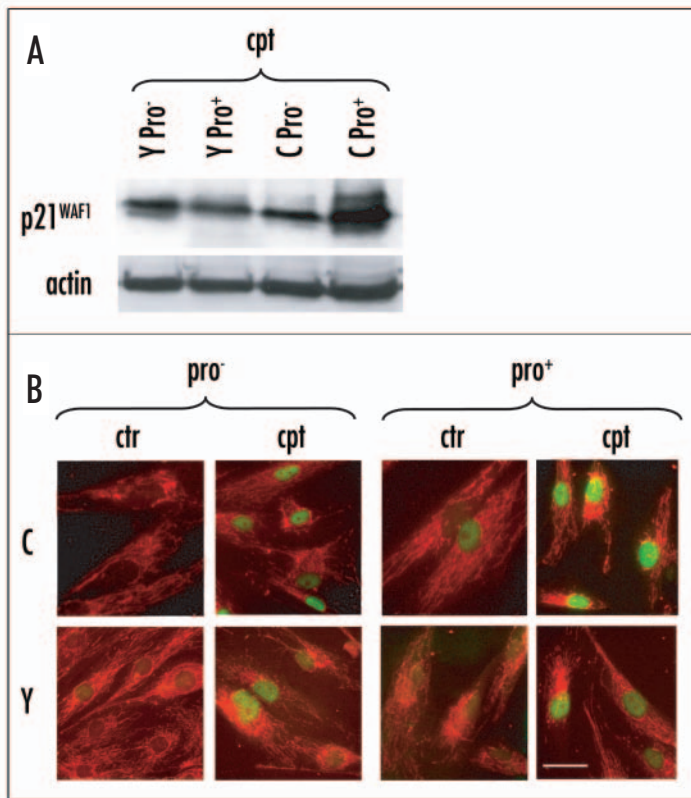
Figure 5. p21^{WAF1} induction by cpt in DFs of different p53 codon 72 genotype. (A) Western blot analysis of p21^{WAF1} immunoreactivity in DFs after treatment with cpt 5 µM for 48 hours. Y, young subject; C, centenarian; Ctr, control untreated cells; cpt, camptothecin 5 µM. One subject for each group is showed. (B) Fluorescence microscopy of DFs in presence and absence of cpt 5 µM for 48 hours. Cells were probed with MitoTracker CMX Ros Red (red fluorescence) for mitochondria and with anti p21^{WAF1} polyclonal antibody (C-19) revealed with FITC-conjugated secondary antibody (green fluorescence). Pro$^+$ DFs from centenarians have a higher intensity of p21^{WAF1}-related fluorescence. Note also that the whole p21^{WAF1}-related fluorescence is mostly nuclear. Ruler: 20 µm.

RESULTS

p53 codon 72 polymorphism modulates the induction of apoptosis and cell senescence of DFs in response to camptothecin (cpt) and doxorubicin (doxo). p53 codon 72 alleles modulate apoptosis susceptibility.[8] However, the exposure to cytotoxic drugs is known to induce also cell cycle arrest and cell senescence.[21] On this background, DFs were exposed to cpt (5 µM) or doxo (500 ng/ml) for 48 and 72 hours. DFs were assessed for hypodiploid DNA content as a marker of apoptosis (Fig. 1), for β-galactosidase activity, a marker of cell senescence (Table 1), and for BrdU incorporation as an index of cells in S phase (Fig. 2). We found that Pro$^+$ DFs from centenarians have a lower percentage of apoptotic cells with respect to Pro$^-$ ones after 48 hours of culture (cpt treatment) and 72 hours of culture (doxo and cpt treatments), while DFs from young people display a similar feature only at 72 hours of culture upon cpt, but not doxo, treatment (Fig. 1). It is to observe that the two drugs had quite a different efficacy in inducing apoptosis. This is likely due to their different mechanisms of action, being cpt less active in slowly replicating cells as DFs since it acts on DNA replication, while doxo causes oxidative stress.

Conversely, opposite results were obtained when spontaneous and drug-induced cell senescence were evaluated. Indeed, Pro$^+$ DFs from centenarians display a higher percentage of β-galactosidase positive cells upon 48 hours of cpt treatment (Table 1) with respect to Pro$^-$ ones. A similar trend was present also in DF cultures from young people, even if at a lower extent.

Table 1   Percentage of β-galactosidase positive cells

		CTR	CPT
Y	Pro-	7.0 ± 0.6	8.3 ± 0.7
	Pro+	10.0 ± 0.7	22.0 ± 3.4
C	Pro-	6.0 ± 0.5	11.6 ± 1.1
	Pro+	11.9 ± 1.5*	78.7 ± 7.7**

Numbers represent the percentage of β-galactosidase positive cells in DFs cultures as observed after 48 hours of culture. A minimum of 100 cells was counted for each sample. One representative subject was analysed for each category. Data are expressed as mean ± standard error of the distribution. CTR, untreated cells; CPT, 5 mM camptothecin; Y, DFs from young people; C, DFs from centenarians. Chi square test, Pro$^-$ vs Pro$^+$: *$p < 0.05$, **$p < 0.01$.

Interestingly, also in absence of drug treatment, Pro$^+$ DF cultures contained a higher percentage of spontaneously senescent cells with respect to Pro$^-$ DF cultures. This difference was very slight in DF cultures from young people, nevertheless, when DFs from centenarians were analysed, the difference was statistically significant (Table 1).

As far as the analysis of cells in S phase, doxo or cpt treatment abolishes S phase almost completely (Fig. 2B), as expected. Interestingly, in absence of drug treatment, the percentage of cells in S phase was lower in Pro$^+$ than in Pro- DF cultures, this difference being statistical significant for DFs from centenariarians (Fig. 2B). Thus, it is not surprising that cpt, a topoisomerase inhibitor is more active on Pro$^-$ cultures, which proliferate more vigorously. Nevertheless, it is unclear why Pro$^-$ DFs cultures from centenarians appear to be more susceptible to cpt than the correspondent ones from young people, which have an even higher percentage of cells in S phase. This is likely a general phenomenon of cells from old people, as it is observed also when DFs are treated with doxo; moreover, a higher drug sensitiveness has been reported for cells from aged donors.[22]

Given the link between cellular senescence and telomere length, we measured the mean TRF length in Pro$^+$ and Pro$^-$ DFs from centenarians. As shown in Figure 3, telomeres were on average shorter in Pro$^+$ than in Pro$^-$ DFs, suggesting that the higher percentage of senescent cells in untreated Pro+ cultures could be due, at least in part, to the presence of shorter telomeres.

p53 codon 72 polymorphism affects p21^{WAF1} regulation. p21^{WAF1} is at the crossroad of the p53-dependent machinery which regulates cell cycle, apoptosis and cell senescence, and we reasoned that its regulation could explain the above reported results. The expression of p21^{WAF1} mRNA was evaluated by an ad hoc developed p21^{WAF1} competitive RT-PCR (Fig. 4A), and the level of p21^{WAF1} protein was evaluated by Western blot (Fig. 4B). In all the assays we found that p21^{WAF1} was upregulated in Pro$^+$ DFs in respect to Pro$^-$ DFs. In particular, using a semi-quantitative flow cytometric test we measured the p21^{WAF1}-related fluorescence in all the DFs cultures used in this study (eight from young people and six from centenarians) and found that the difference in p21^{WAF1} expression was statistically significant in DFs cultures from centenarians (Fig. 4C). It is to note that in general DFs from centenarians have more p21^{WAF1} than DFs from young people as assessed by Western blot and flow cytometry and this is in apparent contrast with RT-PCR data, where similar amount of mRNA are observed in DFs from young and centenarian people bearing the same p53 genotype (Pro$^+$ or Pro$^-$). This could be explained by a different stability of p21^{WAF1} protein in DFs from centenarians with respect to young people. As far as we know, this is at present a still unanswered question. After exposure to cpt (5 µM), Pro$^+$ DFs displayed higher levels of p21^{WAF1} protein (Fig. 5A) and immunoreactivity (Fig. 5B). To directly test the activity of exogenous p53 codon 72 alleles on p21^{WAF1} promoter-driven expression, SaOs-2 cells were cotransfected with a pCMS-EGFP plasmid carrying either a p53Arg or p53Pro allele, with a p21^{WAF1} promoter driven reporter plasmid (p21FireflyLuc), and with a control plasmid (pTKRenillaLuc). We found that, after 24 hours from transfection (Fig. 6A), the cell lysate from pCMS-p53Pro transfected cells was found to contain higher levels of Firefly luciferase activity, indicating that p53Pro allele has an increased capacity to induce p21^{WAF1} promoter, in respect to p53Arg allele. To assay the different extent at which the two

exogenous p53 codon 72 alleles bind to the endogenous p21^{WAF1} promoter, a Chromatin Immunoprecipitation assay (ChIP) analysis was performed in SaOs-2 cells transfected with either pCMS-p53Arg or pCMS-p53Pro plasmids. Higher amounts of PCR amplified p21^{WAF1} promoter fragment were present when p53 was immunoprecipitated from pCMS-p53Pro transfected cells (Fig. 6B). Control western blot analysis on the expression of p53 after the transfection with the two plasmids is shown (Figure 6B). To check that p53 expression was not due to a different efficiency of the transfection process, SaOs-2 cells were analysed with flow cytometry after 24 hours of transfection. Similar amount of transfected cells were found, as shown in Figure 6C. Further, this analysis confirms that the two p53 isoforms have different apoptotic potential, as it results from the percentages of cells with hypodiploid DNA content (upper left quadrants), in comparison with the total percentage of EGFP-positive cells, being the Arg variant more pro-apoptotic than the Pro variant.

It has been proposed that p21^{WAF1} inhibits apoptosis by inactivating Caspase 3 at mitochondrial level.[23] For this reason, we tested the amount of Procaspase 3 bound to p21^{WAF1} as a possible mechanism of apoptosis modulation in DFs. Similar amounts of p21^{WAF1}/Procaspase 3 complex were found in DFs from young people independently from p53 codon 72 polymorphism, while in centenarians we found that higher levels of p21^{WAF1}/Procaspase 3 complex were present in Pro$^+$ DFs with respect to Pro$^-$ ones (Fig. 7). However, only scanty p21^{WAF1} mitochondrial immunolocalisation could be documented in DFs (data not shown).

DISCUSSION

In this work we report that p53 codon 72 genotype affects the response to chemotherapeutic drugs and that this phenomenon emerges with the age of the cell donors and is particularly evident in centenarians. We here demonstrate that DFs from centenarians carrying the p53Pro allele (Pro$^+$) are characterized by a lower susceptibility to undergo apoptosis after exposure to drugs such as doxorubicin and camptothecin. These data mirror what we and others have previously reported about the higher susceptibility to undergo drug-induced apoptosis of cells homozygotes for the p53Arg allele (Pro$^-$).[6,8,9] Morever, it has been reported that, upon treatment, cancer cells can undergo either apoptosis or cell senescence.[21] We report here that also for normal fibroblasts, Pro$^+$ cells from centenarians are more prone to cell senescence than Pro$^-$ ones, thus suggesting that the decision between drug-induced apoptosis and senescence in cells from aged people is under the control of p53 codon 72 alleles. These data, together with recent literature (see refs. 9, 24 and 25) strongly support the notion that genetic variability at this p53 locus is a pharmacogenetically relevant piece of information that should be obtained from patients who have to undergo anti-cancer therapy, in order to predict their response to therapy. This could be of particular importance
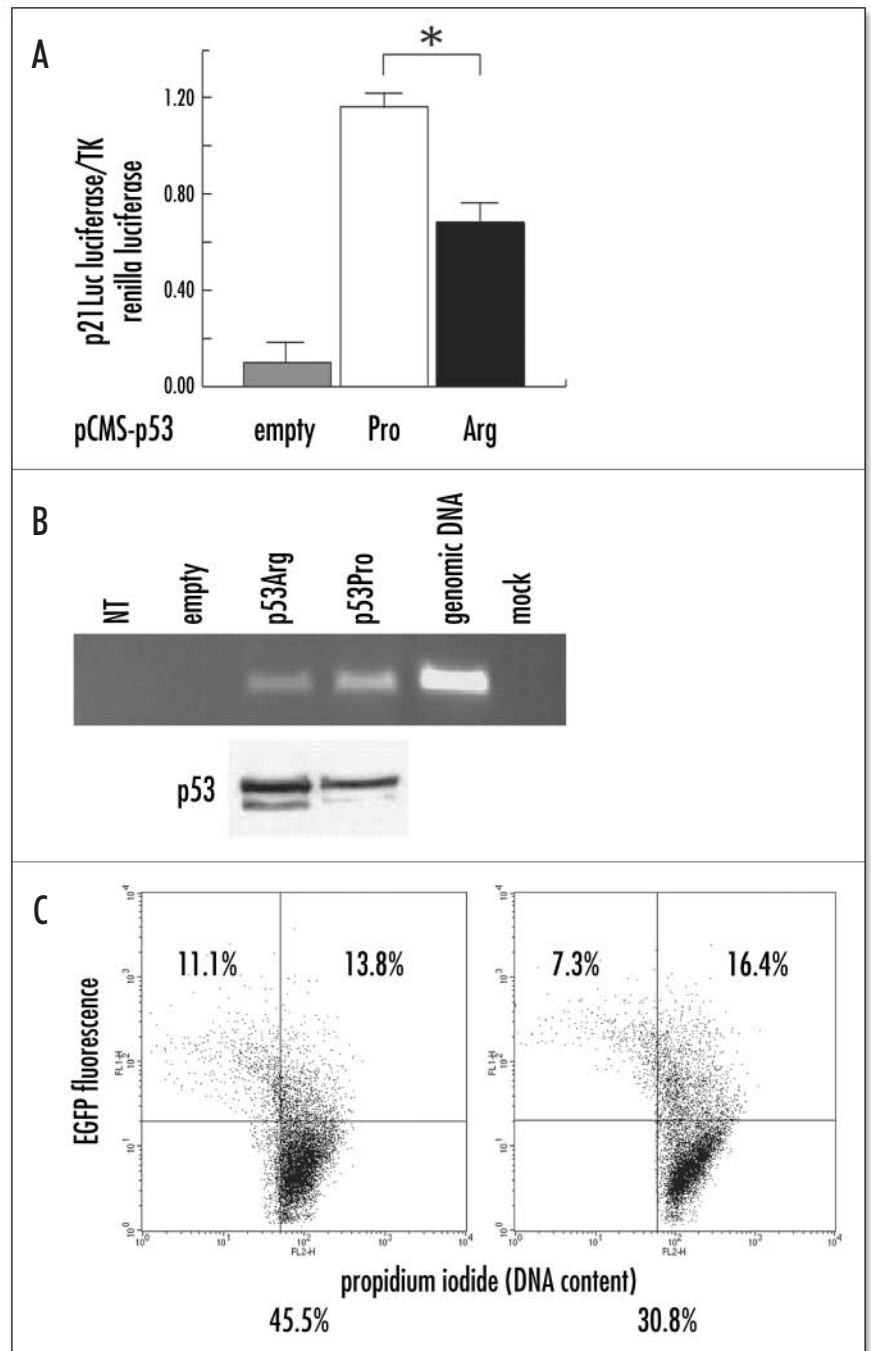

Figure 6. p53 binding capacity on p21^{WAF1} promoter. (A) Luciferase assay performed on SaOs-2 cells transiently cotransfected with pCMS-p53Arg or pCMS-p53Pro plasmids (40 ng), together with p21Luc (100 ng) and TK-Renilla reporter (10 ng) plasmids. Data are presented as the *ratio* between the fluorescence emission of p21Luc (Firefly luciferase) and TK-Renilla (Renilla luciferase), and are expressed as mean ± SEM of three independent experiments. *p = 0.01, by Anova test. (B) Chomatin Immuno Precipitation assay of SaOs-2 cells transfected with pCMS-p53Arg or pCMS-p53Pro plasmids (40 ng) and assayed for the main consensus sequence for p53 in the p21^{WAF1} promoter region. NT, cells non transfected; empty, cells transfected with empty vector. (C) Flow cytometric analysis of SaOs-2 cells after 24 hours of transfection. Cells where fixed and permeabilised then stained with Propidium Iodide to measure DNA content (abscissa). In ordinate the fluorescence intensity of EGFP is shown. Numbers in upper left quadrants represent the percentage of EGFP-positive apoptotic (<2c DNA content) cells, numbers in upper right quadrants represent the percentage of EGFP-positive non apoptotic cells. Numbers below the dot plots represent the percentage of apoptotic cells with respect to the total of EGFP-positive cells.
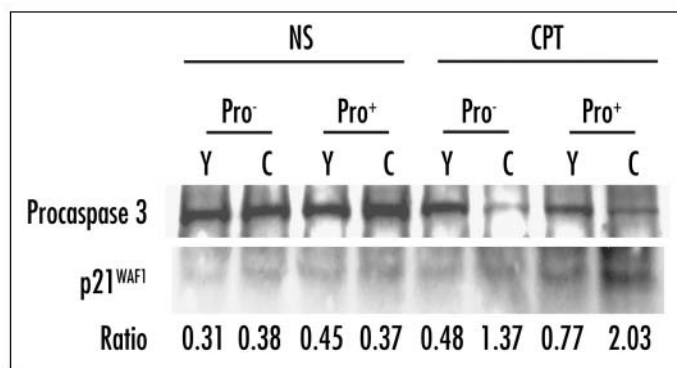
Figure 7. $p21^{WAF1}$/Procaspase 3 co-Immuno precipitation. Upper lane: detection of Procaspase 3 in protein immunoprecipitates obtained from different DF cultures using anti-Procaspase 3 and A-G PLUS microbeads. Lower lane: the same immunoprecipitates assayed with anti $p21^{WAF1}$ antibody. Bands have been quantified using Quantity One Software. Numbers represent the ratio between the intensity of corrispondent $p21^{WAF1}$ and Procaspase 3 bands. Y, young subject; C, centenarian; NS, control untreated cells; CPT, cells treated with cpt 5 μM for 24 hours.

for elderly people, which represent the most part of people affected by cancer and who are the most exposed to adverse effects of anti-cancer therapy.

Further, even in absence of the exposure to any external stress, we found that DFs from centenarians harbouring Pro⁺ or Pro⁻ p53 codon 72 genotypes have slower or faster cell cycle kinetics, respectively. This phenomenon is in agreement with recent in vitro observations.[26] This fact could provide hints to explain the difference observed in susceptibility, progression and clinical outcome among breast cancer patients carrying different p53 codon 72 genotypes, even in absence of any cytotoxic drug treatment.[27]

The functional difference between p53 codon 72 alleles has been hypothesized to be post transcriptional, being due to the greater ability of p53Arg allele to localize at the mitochondria.[6,8] Differences in the capability to induce gene transcription have been found to be less reproducible.[3,6,9] We here showed that Pro⁺ DFs have increased levels of $p21^{WAF1}$ and we obtained evidence that this phenomenon is related to a different capability to p53 codon 72 alleles to bind to $p21^{WAF1}$ gene promoter and to induce $p21^{WAF1}$ gene transcription, being this finding in agreement with a higher capability of the p53Pro allele to bind TAFII32.[3] These higher $p21^{WAF1}$ levels found in Pro⁺ DFs are associated with lower apoptotic potential, lower capability to over cross G_1-S transition and increased propensity to undergo cell senescence. $p21^{WAF1}$ protein induces not only p53-dependent cell cycle inhibition, but also p53-dependent downregulation of telomerase.[28] Thus, we are tempted to speculate that the polymorphism at the p53 codon 72, likely through differential expression of $p21^{WAF1}$, could exert an influence on the determination of telomere length in individuals, thus affecting proneness to spontaneous cell senescence. However, this possible functional link between p53 codon 72 polymorphism and telomere length remains to be elucidated in more details. As far as drug-induced senescence, it has been reported that $p21^{WAF1}$ is responsible, at least in part, for the induction of a senescence-like phenotype in HCT116 colon carcinoma cells upon doxorubicin treatment.[29] Thus, it is likely that also in the case of DFs from centenarians, the higher proneness to undergo cell senescence observed in Pro⁺ DFS is related to the higher induction of $p21^{WAF1}$ in such cells.

$p21^{WAF1}$ is an anti-apoptotic protein (reviewed in refs. by Roninson[10] and Liu[11]), being capable of directly bind Procaspase 3 at mitochondrial level, protecting it from cleavage.[23,30] In this investigation we found higher amounts of $p21^{WAF1}$ bound to Procaspase 3 in Pro⁺ DFs from centenarians. Nevertheless, we did not obtain any evidence that in DFs $p21^{WAF1}$ is localized to the mitochondria, neither before nor after stimulation with chemotherapic drugs. Thus, the mechanism throughout which $p21^{WAF1}$ and Procaspase 3 play together in the regulation of apoptosis remains to be elucidated.

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