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Common fragile sites in colon cancer cell lines: Role of mismatch repair, RAD51 and poly(ADP-ribose) polymerase-1

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ABSTRACT

Common fragile sites (CFS) are specific chromosomal areas prone to form gaps and breaks when cells are exposed to stresses that affect DNA synthesis, such as exposure to aphidicolin (APC), an inhibitor of DNA polymerases. The APC-induced DNA damage is repaired primarily by homologous recombination (HR), and RAD51, one of the key players in HR, participates to CFS stability. Since another DNA repair pathway, the mismatch repair (MMR), is known to control HR, we examined the influence of both the MMR and HR DNA repair pathways on the extent of chromosomal damage and distribution of CFS provoked by APC and/or by RAD51 silencing in MMR-deficient and -proficient colon cancer cell lines (i.e., HCT-15 and HCT-15 transfected with *hMSH6*, or HCT-116 and HCT-116/3+6, in which a part of a chromosome 3 containing the wild-type *hMLH1* allele was inserted). Here, we show that MMR-deficient cells are more sensitive to APC-induced chromosomal damage particularly at the CFS as compared to MMR-proficient cells, indicating an involvement of MMR in the control of CFS stability. The most expressed CFS is FRA16D in 16q23, an area containing the tumour suppressor gene *WWOX* often mutated in colon cancer. We also show that silencing of RAD51 provokes a higher number of breaks in MMR-proficient cells with respect to their MMR-deficient counterparts, likely as a consequence of the combined inhibitory effects of RAD51 silencing on HR and MMR-mediated suppression of HR. The RAD51 silencing causes a broader distribution of breaks at CFS than that observed with APC. Treatment with APC of RAD51-silenced cells further increases DNA breaks in MMR-proficient cells. The RNAi-mediated silencing of PARP-1 does not cause chromosomal breaks or affect the expression/distribution of CFS induced by APC. Our results indicate that MMR modulates colon cancer sensitivity to chromosomal breaks and CFS induced by APC and RAD51 silencing.

1. Introduction

Common fragile sites (CFS) are specific chromosomal areas that are prone to form gaps and breaks when cells are exposed to various stresses that perturb DNA replication [1]. In particular, low doses of aphidicolin (APC), an inhibitor of DNA polymerases α, β and γ, are well known to induce formation of gaps and breaks at the CFS [2]. CFS extend over hundreds of kilobases and commonly contain long stretches of AT rich sequences which favour formation of the secondary structures that delay or block the progression of replication forks [3]. CFS contribute to genomic instability because they behave as hotspots for recombination, favouring the evolution of species or tumour formation. In this regard, it should be noted that CFS also harbour a number of tumour suppressor genes that are disrupted in tumour cells, such as *FHIT* (fragile histidine triad) at FRA3B in 3p14.2 or *WWOX* at FRA16D in 16q23.3 [4,5]. The involvement of these genes in tumour transformation and the expression of specific CFS vary according to the cell type [6–8]. Under conditions of replication stress, such as that provoked by APC, when the replication fork encounters the fragile site regions, it can stall and slow down the duplicative process or collapse to generate DNA double stand breaks (DSB) [9,10]. The stability of CFS is regulated by many factors, the principal among which is the Ataxia-telangiectasia and Rad3 Related (ATR)-dependent DNA damage checkpoint pathway, because the cells lacking ATR have a dramatic increase in the occurrence of CFS [3,11]. In response to stalled or collapsed replication

Abbreviations: CFS, common fragile sites; APC, aphidicolin; DSBs, double strand breaks; HR, homologous recombination; NHEJ, non-homologous end joining; MMR, mismatch repair system; PARP-1, poly(ADP-ribose) polymerase-1; TMZ, temozolomide; siRNA, short interfering RNA; γH2AX, phosphorylated H2AX.

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forks, the ATR kinase acts as DNA damage sensor, which along with downstream effector molecules (e.g. p53, BRCA1, ChK1), inhibits cellular entry into mitosis favouring DNA repair. Hence, CFS stability may also be controlled by different genes belonging to DNA repair systems and cell cycle checkpoints activated by DSB. Indeed, it has recently been demonstrated that down-regulation of RAD51 and of DNA–protein kinase, the key components of the two major DSB repair pathways, namely the homologous recombination (HR) and of non-homologous end joining (NHEJ), respectively, leads to an increase in CFS expression under replication stress [12]. In addition, DSBs generated by APC have been shown to be repaired by HR [12].

The mismatch repair system (MMR) is involved in the control of genomic integrity correcting errors of DNA polymerases that escape the detection by their proofreading activity. The MMR is also implicated in DNA damage responses such as cell cycle checkpoints activation and apoptosis induction after treatment with genotoxic agents. In particular, MMR is required for the cytotoxic activity of a variety of anticancer agents, including methylating compounds, 6-thioguanine and cisplatin [13]. It has been recently demonstrated that MMR inhibits HR likely by aborting strand exchanges between homeologous (i.e., not completely homologous) sequences [14–18]. Furthermore, the complex hMSH2/hMSH6 has been shown to bind to Holliday junctions and to play a direct role in HR [19]. Besides being mutated in the hereditary nonpolyposis colorectal cancer of patients affected by Lynch syndrome, MMR is also mutated in about 15% of sporadic colorectal cancers [20].

In the present study, we investigated the influence of MMR and RAD51 on the expression and distribution of CFS upon APC treatment using near-diploid human colon cancer cell lines with intact or defective MMR components. Moreover, since poly(ADP-ribose) polymerase (PARP)-1 is involved in NHEJ-mediated repair of DSBs and ensures regulation of replication fork progression by HR on damaged DNA [21–23], we analyzed also the sensitivity to APC of colon cancer cell lines stably silenced for PARP-1 expression.

2. Materials and methods

2.1. Cell lines

The MMR-deficient colon cancer HCT-15 cell line (ATCC-LGC Promochem, Milan, Italy) contains mutations in both alleles of the *hMSH6* gene [24]. A MMR-proficient HCT-15 clone, obtained by stable transfection with a vector carrying the wild type *hMSH6* cDNA, was a gift of Dr. J. Jiricny (Institute of Molecular Cancer Research, University of Zürich, Switzerland) [25]. The MMR-deficient colon cancer HCT-116 cell line has a hemizygous nonsense mutation in the *hMLH1* gene located on chromosome 3, whereas the MMR-proficient HCT-116/3-6 cell line was created by microcell chromosome transfer of a single normal human chromosome 3 into HCT-116 cells [26,27]. HCT-116 and HCT-116/3-6 cell lines were kindly provided by Dr. G. Marra (Institute of Molecular Cancer Research, University of Zürich, Switzerland). The MMR-deficient LS174T colon carcinoma cell line, which contains mutations in both alleles of the *hMSH6* gene, was purchased from ATCC-LGC (Teddington, UK) [28]. Analysis of the karyotype in all cell lines confirmed data previously reported showing that the majority of the cells are circa diploid: HCT15 46, XY; HCT 116 45, X; LS174T 45, X [29,30].

The human B lymphoblastoid cell line TK-6 and its subline MT-1 [31] were provided by W.G. Thilly (Massachusetts Institute of Technology, Cambridge, MA, USA). TK-6 cell line is MMR-proficient, whereas the MT-1 line is MMR-deficient, harbouring different missense mutations in both alleles of the *hMSH6* locus [32].

Cell lines were cultured in DMEM (Sigma–Aldrich, Milan, Italy), supplemented with 10% foetal calf serum, 2 mM L-glutamine and antibiotics. MMR-proficient transfected HCT-15 and HCT116/3-6 cells were cultured in the presence of 800 μg/ml geneticin (Sigma–Aldrich).

2.2. Analysis of CFS

For the analysis of induction and distribution of CFS cells were exposed for 24 h to APC [0.05–0.3 μM; stock solution 10 mM in dimethyl sulfoxide (DMSO); Sigma–Aldrich]. During the last 2 h of incubation, colchicine (0.8 μg/ml; Sigma–Aldrich) was added to the cultures. The final concentration of DMSO in drug-treated cultures was kept below 0.5% (v/v) and did not contribute to clastogenicity (i.e., the ability to induce chromosomal aberrations) (data not shown).

Chromosome preparations were obtained by slight modifications of standard techniques [33]. Chromosomal damage was evaluated by counting chromatid and chromosomal gaps, breaks and rearrangements (each one counted as 2 chromatid or chromosomal breaks) on 100 metaphases per each experimental point. Mitotic index, i.e. the ratio between the number of cells in mitosis and the total number of cells, was evaluated examining 1000 nuclei in the same slides used for cytogenetic analysis.

Statistical analysis of chromosomal damage was performed using the normal standardized deviate, calculated according to the formula: $(m1 - m2)/\sqrt{(m1 + m2)^2}$, where $m1$ and $m2$ are the mean values, expressed as absolute numbers, of chromosomal aberrations in 100 metaphases [34]. This statistical analysis is based on the assumption that the number of chromosomal breaks follows the Poisson distribution and not the normal distribution.

In order to localize CFS at specific chromosome bands, G-banded chromosomes were obtained by a standard mild trypsin digestion, considering a human karyotype of 319 bands. Different numbers of mitoses were examined in the various cultures in order to obtain the localization of 200–400 CFS from 3 or more different cell cultures for each experimental point.

Breakpoints were considered as fragile sites according to the statistical method proposed by Mariani [35]. This method is based on the expected random distribution, assumed to be equal to a Poisson distribution, where the expected number of events per band is used as mean. The Chi square analysis was used to compare the expression of FRA3B and FRA16D in the different clones taking into account the number of chromosomes showing specific CFS and the number of intact chromosomes 3 and 16.

2.3. Transient inhibition of RAD51 by short interfering RNA (siRNA)

HCT-15 cells were seeded in 24-well plates and transfected with siRNA SMART pool targeting human RAD51 gene (100 nM, Dharmacon, Lafayette, CA, USA) using DharmaFECT 1 reagents (Dharmacon) according to the manufacturer's instructions. For each experiment, control cells were exposed to transfection reagents in the absence of the oligonucleotides. To verify the efficiency of siRNA inhibition, cells were analyzed by real-time PCR or by immunofluorescence analysis 48 h after transfection. For real-time PCR, total RNA was extracted from approximately 3×10^5 cultured cells, using the Qiagen Rneasy mini kit (Qiagen, Milan, Italy). cDNA synthesis was performed using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA) and 200 ng of total RNA; 1/10 of the total reaction was, then, subjected to PCR amplification on an ABI-7500 SDS instrument (Applied Biosystem, Foster City, CA, USA) using the Platinum SYBR Green qPCR SuperMix UDG with ROX (Invitrogen). The primer sequences for RAD51 amplification were as follows: AGC GTT CAA CAC AGA CCA CCA G (+) and ATC AGC GAG TCG CAG AAG CAT C (−). The data have been elaborated as $2^{-\Delta\Delta Ct}$ values, using ABI SDS software. Immunofluorescence staining, using a monoclonal antibody directed against RAD51 (3C10, Santa Cruz Biotechnology, CA, USA), was previously described [14].

Treatment with APC was performed 24 h after RAD51 silencing and cytogenetic analysis was assayed as described above.

2.4. Stable silencing of PARP-1

Stably PARP-1 silenced HCT-15 [14] and HCT-116 cells were obtained by transfection of the pBS-U6-SiP912 vector [36], using the CalPhos Mammalian transfection Kit (Clontech, Palo Alto, CA, USA). The pBabe vector (provided by Dr. Robert Weinberg, Whitehead Institute for Biomedical Research, Cambridge, MA, USA), carrying the puromycin resistance gene, was co-transfected with pBS-U6-SiP912 vector (1:3). Antibiotic resistant clones were isolated by ring cloning and maintained in the presence of 5 μg/ml puromycin.

2.5. Immunoblot analysis

Nuclear extracts were prepared using fractionation kits from Medical & Biological Laboratories (MBL, Watertown, MA, USA). Western blot analysis was performed using monoclonal antibodies directed against PARP-1 (clone C2-10, BD Biosciences, Milan, Italy; 1/2000), hMSH6 (clone 44, BD Biosciences; 1/500), hMLH1 (clone G168-15, BD Biosciences; 1/500) and lamin A/C (clone 14, BD Biosciences; 1/1000). Signals were quantified using a Kodak densitometer (Rochester, NY, USA).

2.6. Immunofluorescence microscopy of γ-H2AX

Cells were grown on poly L-lysine coated glass coverslips and treated with APC. After 24 h, slides were washed twice with PBS and fixed in 4% (w/v) paraformaldehyde in PBS for 20 min. Cells were permeabilized with 0.1% Triton X-100 in PBS for 2 min, incubated in 2.5% goat serum–PBS for 20 min and with rabbit anti-γ-H2AX polyclonal antibody for 1 h (Abcam, Cambridge, UK; 1/100 in 2.5% goat serum–PBS). After washing in PBS, cells were incubated with goat anti-rabbit-Alexa 488 secondary antibody (Molecular Probes, Eugene, OR, USA; 1/2000) for 1 h. Slides were counterstained by vectashield antifade solution containing 4,6-diamidino-2-phenylindole (DAPI, Vector laboratories, Burlingame, USA) and examined with a fluorescent microscope (Nikon Eclipse, E600, Yokohama, Japan). Images were analyzed with the Arkon FISH program (Nikon). For quantitative analysis, foci were

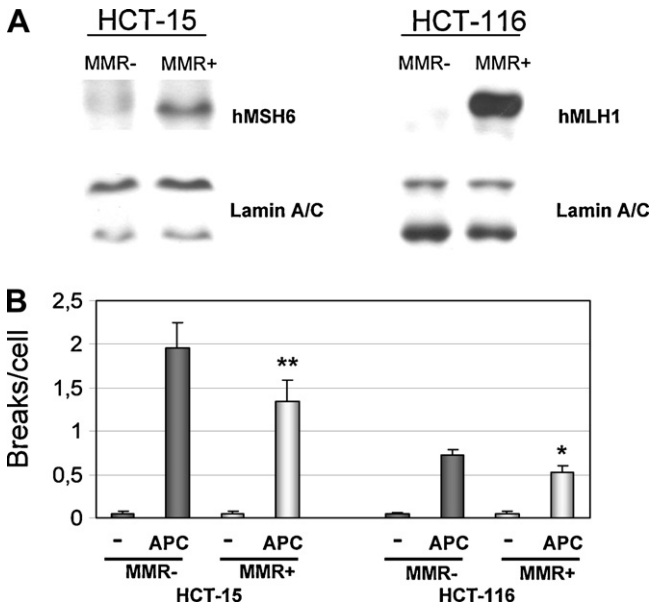

Fig. 1. MMR-deficient colon cancer cells are more prone to formation of chromatid breaks induced by APC than MMR-proficient counterparts. (A) Immunoblot analysis of MMR-deficient (MMR-) HCT-15 cells, MMR-proficient (MMR+) HCT-15 cells transfected with *hMSH6*, MMR-deficient HCT-116 cells and MMR-proficient HCT116/3–6 cells in which the *hMLH1* gene has been replaced. Nuclear extracts (10 μg) were electrophoresed and analyzed for the expression of hMSH6 (HCT-15), hMLH1 (HCT-116) or lamin A/C. (B) Cells were incubated for 24 h in culture medium or culture medium containing APC (0.3 μM in HCT-15 cells, 0.1 μM in HCT-116 cells) and then processed for cytogenetic analysis. Each value represents the mean (+SE) of three independent experiments. Statistical analysis, according to normal standardized deviate, was as follows: in HCT-15 cells, differences between APC treated MMR− and MMR+ cells, $P < 0.001$ (**); in HCT-116 cells, differences between MMR− and MMR+ cells, $P < 0.05$ (*).

counted by eye during the microscopic and imaging process by using a 100× objective. Cells with at least 5 foci were considered positive for γ-H2AX expression [37].

3. Results

3.1. MMR-deficient colon cancer cells are more prone to formation of APC-induced chromosomal breaks than MMR-proficient cells

In order to investigate the implication of MMR pathway in influencing the chromosomal damage induced by APC, we initially analyzed the chromatid breaks in MMR-deficient and -proficient colon cancer cells. To this end, we used the MMR-deficient colon cancer HCT-15 and HCT-116 cell lines lacking hMSH6 and hMLH1, respectively, and their MMR-proficient counterparts in which MMR has been rescued by introduction of the corresponding wild-type gene (Fig. 1A). Restoring of MMR function in HCT-15 cells was verified by the analysis of chromosomal damage induced by temozolomide (TMZ, Sigma–Aldrich), a well-known methylating agent of clinical interest. In fact, it is known that MMR function is required for the cytotoxic and clastogenic activity of methylating compounds [38]. Our results indicate that hMSH6 transfection in HCT-15 cells restored MMR function, since these cells were more sensitive to TMZ (400 μM) with respect to the parental MMR-deficient cells, having 0.42 ± 0.06 and 0.18 ± 0.01 breaks per cell, respectively ($P < 0.05$) (data not shown). The higher sensitivity to the chromosomal damage induced by methylating agents in MMR-proficient HCT116/3–6 cells with respect to MMR-deficient HCT-116 cells was previously reported [34].

We observed that APC-induced chromatid and chromosomal breaks occurred in a dose-dependent manner in colon cancer cells after exposure to graded concentrations of APC (0.1–0.3 μM for HCT-15 cells and 0.05–0.1 μM for HCT-116 and LS174T cells) (data not shown). Interestingly, MMR-deficient cells showed a higher number of APC-induced chromatid and chromosomal breaks with respect to their MMR-proficient counterparts (Fig. 1B). It is noteworthy that here and in subsequent studies, we treated the HCT-15 cells with 0.3 μM APC whereas HCT-116-derived cells with 0.1 μM APC, because for the latter, the doses higher than 0.1 μM APC caused too many chromosomal breaks that often hampered their localization at specific chromosome bands.

Similar results were obtained in another set of MMR-deficient and -proficient cell lines of lymphoblastoid origin: MT-1 and TK-6 (breaks per cell after treatment with 0.1 μM APC were 0.69 ± 0.08 and 0.49 ± 0.05, respectively, $P < 0.05$) (data not shown).

Analysis of phosphorylation of histone H2AX (γ-H2AX) serves as an indicator of DSBs repair, and we observed that in HCT-15 and HCT-116 cells treated with APC, a higher number of cells were positive for γ-H2AX foci in MMR-deficient cells with respect to the MMR-proficient counterparts (Fig. 2).

3.2. MMR does not affect the distribution of CFS induced by APC

We initially analyzed CFS in the MMR-deficient HCT-15, LS174T and HCT-116 cells treated with APC. For each cell line examined, at least 200 breaks were localized on G-banded chromosomes. The percentages of statistically significant CFS induced by APC, for at least one of the three colon cancer cell lines, are indicated in Fig. 3. In these cells, most breaks (70–80%) were coincident with the most frequently expressed CFS; FRA16D in 16q23 was always the most frequently expressed, differently from human lymphocytes in which FRA3B in 3p14 was, instead, the most expressed [39]. It should be noted that since chromosomal rearrangements at 3p14 has not been reported in any of these cell lines, this excludes any possible underestimation of CFS at this site [29,30]. Fig. 4 shows partial G-banded metaphases showing CFS at 3p14 and 16q23 in HCT-15 cells treated with APC.

We then compared the distribution of the chromosome bands containing CFS, showing gaps or breaks, after treatment with APC in MMR-deficient and -proficient cell lines. Table 1 reports the distribution of the significantly expressed CFS (indicated in bold), according to the statistical evaluation by Mariani's test. The numbers of significantly expressed CFS in MMR-deficient and -proficient HCT15 cells treated with APC were 11 and 8, respectively and represented 80–83% of the total evaluated breaks (Table 1, APC columns for each MMR-phenotype). Although presently 84 areas in human karyotype have been defined as CFS, the low number of significantly expressed CFS detected in colon cancer cell lines is comparable to that reported in other cell models [2,39,40]. The distribution of statistically significant CFS induced by APC in MMR-deficient and -proficient cells was comparable; in fact, G band analysis revealed that 7 CFS out of 8 (in MMR-proficient) or out of 11 (in MMR-deficient) were located in the same chromosome regions in both cell lines (Table 1). Similar results were obtained when MMR-deficient and -proficient HCT-116 cells were analyzed (data not shown).

3.3. Sensitivity to APC of RAD51 silenced cells with intact or defective MMR

Since down-regulation of the HR component RAD51 in a breast cancer cell line has been shown to increase CSF expression [11], we investigated whether siRNA-mediated transient silencing of RAD51 might differentially affect CFS expression and distribution depending on the functional status of MMR. The real time PCR of RAD51 gene transcript, at 48 h after transfection, indicated more than 90% suppression in the expression of RAD51 as compared to mock-transfected control, thus demonstrating a strong

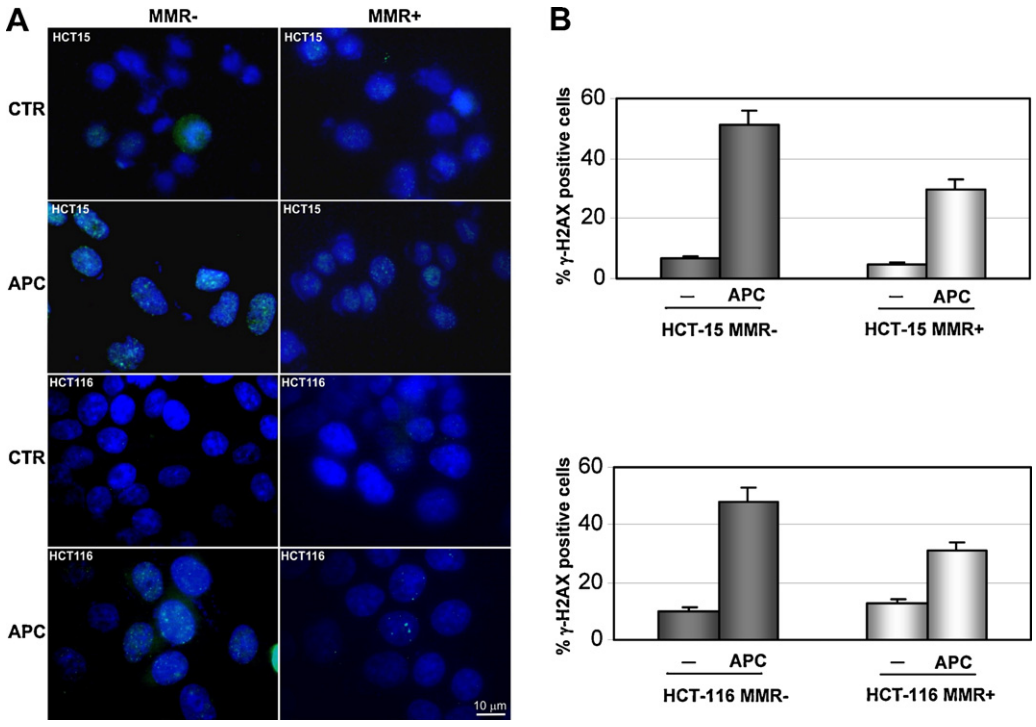

Fig. 2. Effect of APC on formation of γ-H2AX foci. MMR− or MMR+ HCT-15 and HCT-116 cells were incubated for 24 h with 0.3 μM or 0.1 μM APC, respectively. (A) Immunofluorescence analysis of γ-H2AX foci (green) in untreated (CTR) or APC treated cells. Nuclei were stained with DAPI (blue). (B) The mean percentage of cells (+SE) with ≥5 γ-H2AX foci of three independent experiments is presented (50 cells counted for each experiment). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

knock-down effect of transient siRNA (Fig. 5A). Immunofluorescence staining also confirmed a significant down-regulation of RAD51 positive cells (data not shown). Even though the abrogation of RAD51 expression has been associated with inhibition of cell growth, this effect is not an early event, since it occurs after an arrest at G2/M phase when mitoses can be still analyzed [10,41]. Therefore, we performed the cytogenetic analysis at a suitable time point after transfection (i.e., 48 h), when the mitotic indexes of the

Table 1
CFS distribution in siRAD51 and MMR-deficient and -proficient HCT-15 colon cancer cells.

G band	MMR−			MMR+		
	APC[a] %	siRAD51[a] %	siRAD51 APC[a] %	APC[a] %	siRAD51[a] %	siRAD51 APC[a] %
1p36	1.2	**2.4**	**3.5**	1.1	**2.7**	**2.7**
1p32	0.3	**3.6**	2.1	0.3	**4.8**	**2.7**
1p22	0.5	**2.6**	0.7	0	1.4	1.1
1q21	0.3	**2.4**	**3.5**	1.4	**5.5**	0.8
1q25	1.2	**4.0**	1.8	0.8	**3.4**	1.6
1q41	0.2	**3.2**	0.0	0	**3.1**	1.1
1q44	0.3	**2.2**	2.1	0.8	1.7	**2.7**
2p24	0.7	**2.2**	1.1	1.1	1.4	1.9
2p16	1.6	**1.8**	1.1	0	2.1	1.3
2q23	0.7	0.6	1.4	**3.8**	0.7	0.0
2q33	**3.1**	**4.4**	**2.5**	1.4	**4.8**	1.1
2q35–37	0.5	1.4	1.8	0.3	0.3	**3.8**
3p24	1.4	1.4	**2.8**	0.8	**5.2**	**2.7**
3p14	**16.2**	**11.2**	**12.8**	**17.2**	**10.0**	**13.9**
3q27	**2.1**	**4.8**	**2.8**	**2.2**	1.7	**2.1**
6p22	**3.5**	**2.2**	**5.3**	**2.7**	2.1	**4**
6q26	**3.1**	**3.4**	1.1	0.3	**6.2**	**4.3**
7p14	1.4	**3.2**	0.7	0.8	1.4	1.6
7q22	**7.1**	**1.6**	**3.2**	**6.6**	**3.8**	**5.6**
7q32	**1.7**	**5.6**	**2.5**	1.6	**2.4**	**4.8**
12q24	0.5	**2.2**	0.0	0	1.0	1.9
13q32	0.9	0.8	**2.5**	0.5	**3.4**	1.1
14q24	**2.8**	**2.4**	**3.2**	**5.5**	**3.1**	**2.1**
16q23	**35.0**	**11.0**	**28.0**	**40.2**	**11**	**19.3**
Xp22	**3.8**	1.0	2.1	**5.2**	**2.4**	1.9
Xq27	**1.7**	1.4	1.4	0	1.4	1.6
G bands with significantly expressed CFS	**11**	**20**	**12**	**8**	**15**	**13**

Bold indicates the number of significantly expressed CFS according to Mariani's test.

[a] The frequency of CFS is expressed as percentage of breaks and gaps located at CFS calculated on the total number of chromosomal breaks and gaps detected for each treatment.

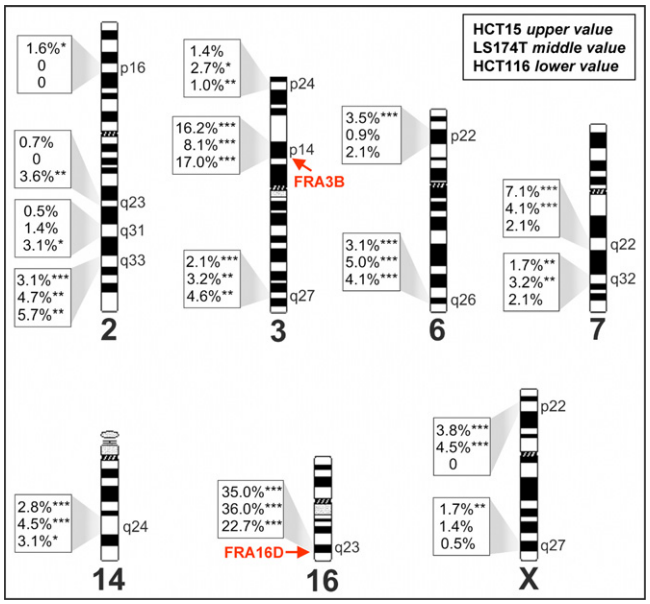

Fig. 3. Ideogram showing the most expressed CFS in MMR-deficient HCT-15, LS-174T and HCT-116 cells treated with APC. The values represent the percentages of breaks and gaps located at CFS calculated on the total number of chromosomal breaks and gaps detected for each treatment in MMR-deficient HCT-15 (upper value), LS-174T (middle value) and HCT-116 (lower value) cells. Statistical analysis, according to Mariani's test was as follows: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

cultures were only slightly decreased with respect to controls. The results indicated an increase in genomic instability in RAD51 siRNA transfected cells, as demonstrated by the spontaneous rearrangements (tri- and tetra radials, dicentrics and translocations) and chromatid breaks (Fig. 5B and C). These results suggest that siRNA oligonucleotides really abrogated RAD51 protein expression, in accordance with a previous study [14]. The RAD51 silenced MMR-proficient clone showed a higher number of breaks per cell with respect to the MMR-defective counterpart (Fig. 5C). Most breaks were localized in CFS positive bands; however RAD51 silencing provoked a more widely spread distribution of CFS with respect to APC treatment, as indicated by the higher number of significantly expressed CFS observed in RAD51 silenced cells, i.e., 20 *versus* 11 and 15 *versus* 8, respectively, in MMR-deficient and -proficient HCT-15 cells (Table 1). Once again FRA16D was the most expressed CFS in the RAD51-silenced cells, analogously to what observed after the APC treatment.

Combined treatment with APC and RAD51 silencing caused lower clastogenic effects than those provoked by APC alone in the MMR-deficient cells, whereas it caused a higher number of breaks than individual treatments in the MMR-proficient cells (Fig. 5C). The distribution of CFS in MMR-deficient cells and -proficient was similar (Table 1).

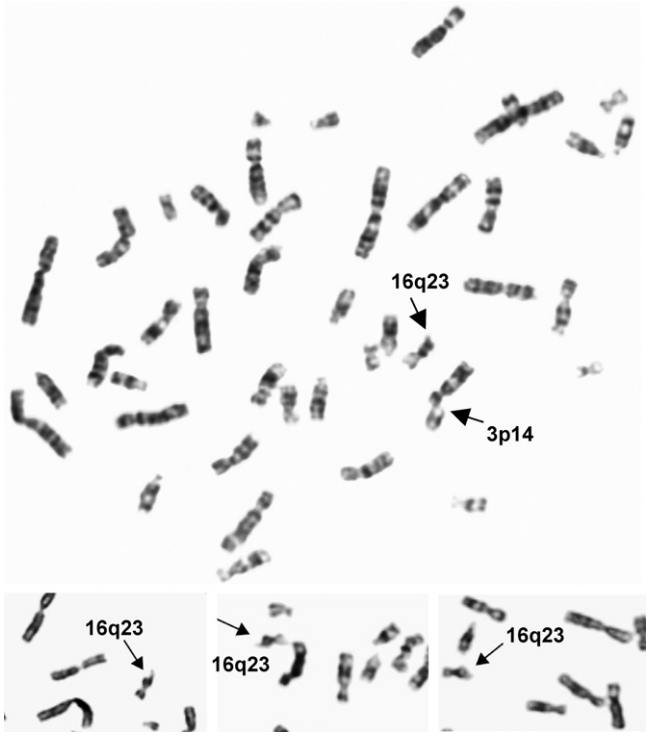

Fig. 4. Partial metaphases of HCT-15 cells treated with APC. A representative panel showing partial metaphases in the MMR-deficient HCT-15 cells which were treated with 0.3 μM APC. Arrows indicate CFS in 3p14 and 16q23.

3.4. Influence of PARP-1 depletion on sensitivity to APC

We next investigated the influence of PARP-1 depletion on chromosomal breaks and CFS expression induced by APC. To this end, clones from the MMR-deficient HCT-15 cells [14], HCT-116 and MMR-proficient HCT-116/3–6 cells were co-transfected with the pBS-U6-SiP912 vector, which targets specific PARP-1 sequences without affecting the expression of PARP-2 [36], and/or the pBabe vector expressing only the puromycin resistance gene. Drug-selected clones were then analyzed for PARP-1 expression and a clone almost completely devoid of PARP-1 protein together with a PARP-1-positive control clone for each cell line were used for further studies (Fig. 6A).

Cytogenetic analysis after treatment with APC did not show any difference in sensitivity to chromosomal breaks in PARP-1 silenced cells with respect to PARP-1 proficient cells (Fig. 6B). Similar results were obtained in PARP-1 proficient cells treated with the PARP inhibitor NU1025 at a concentration known to abrogate PARP activity (25 μM) [14] prior to exposure to APC (data not shown).

Two of the most frequently expressed CFS, i.e. FRA3B and FRA16D, were expressed at similar levels in the MMR-proficient and -deficient clones, either in the presence or in the absence of PARP-1 expression (data not shown), confirming that compromising PARP-1 activity in the cells does not alter their susceptibility to form APC-induced strand breaks at CFS.

4. Discussion

In the present study we clearly demonstrate that colon cancer cell lines treated with APC show a distribution of CFS different from the one reported for human lymphocytes. In fact, we find that the most expressed CFS in colon cancer HCT-15, HCT-116 and LS174T cells is FRA16D, which is located in 16q23, a chromosome area associated with chromosomal translocations and homozygous deletions in a variety of tumours [42], including the colon cancer HCT-116 cell line [43]. Interestingly, the *WWOX* gene, a tumour suppressor gene involved in apoptosis that is frequently mutated in tumour cell lines such as HCT-116, is also located in the 16q23 region [44]. Nevertheless, it has been shown that deletion of *WWOX* gene at 16q23 in HCT-116 did not affect the expression of FRA16D [42,45]. On the other hand, in human lymphocytes the most expressed CFS is FRA3B in 3p14, and although FRA16D is the second most expressed CFS, its frequency is much lower (9.7%) [39] than that found in the cell lines derived from colon cancers (35–40%). However, a recent report indicated that in the UML-49 normal lymphoblastoid cell line FRA16D was more expressed than FRA3B [46].

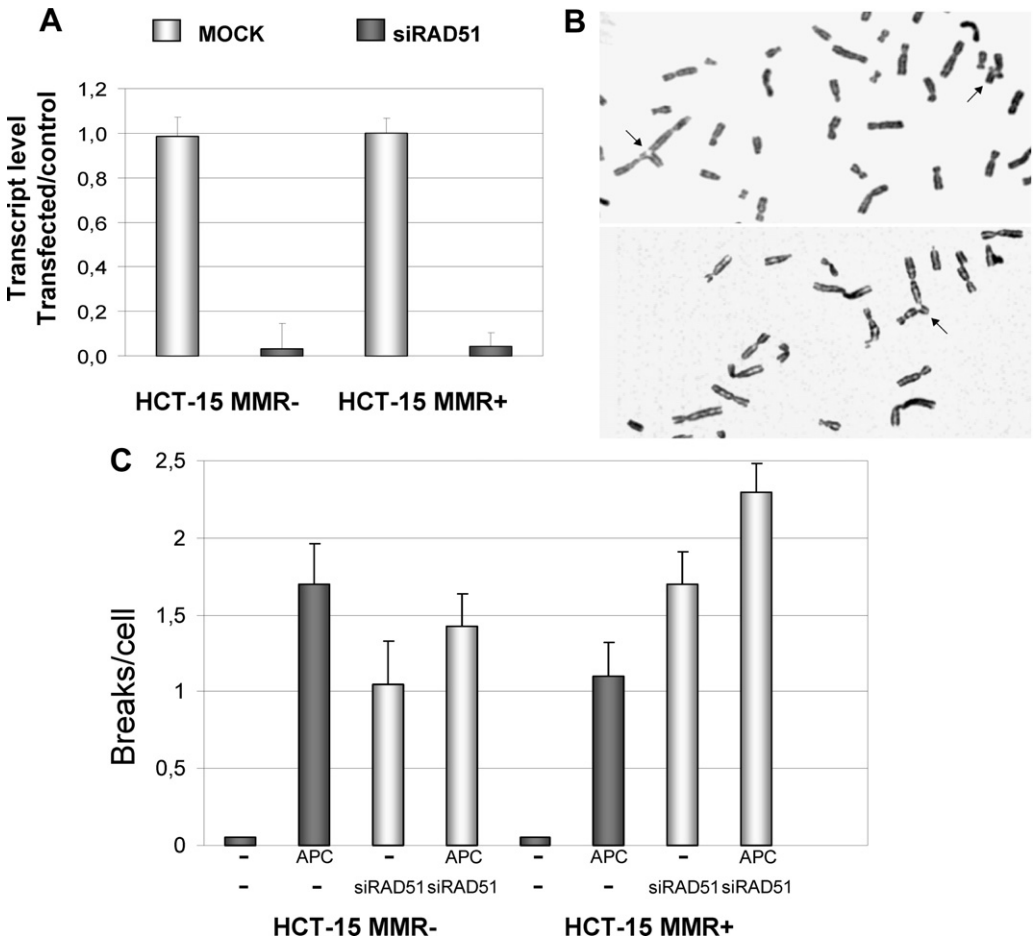

Fig. 5. Clastogenic effects of transient silencing of the HR component RAD51 in MMR− or MMR+ cells untreated or exposed to APC. (A) Analysis of RAD51 expression. Real-time PCR was performed using total RNA extracted from MMR− and MMR+ HCT-15 control cells, cells exposed to transfection reagents only (Mock) or transfected with siRNA SMARTpool targeting human RAD51 (siRAD51) 48 h after transfection. Histograms represent the ratio of specific gene transcript level between Mock and control cells (light column) or siRAD51 and control cells (dark column). Each value represents the mean (+SE) of three independent experiments. (B) Photographs of two representative partial metaphases of HCT-15 cells transiently silenced for RAD51. Arrows point chromatid rearrangements. (C) Analysis of chromatid breaks in MMR− or MMR+ HCT-15 cells with intact or transiently silenced RAD51, untreated or treated with 0.3 μM APC. Each value represents the mean (+SE) of three independent experiments. Statistical analysis, according to normal standardized deviate, was as follows: in the MMR− HCT-15 clone, differences between APC and siRAD51, $P < 0.001$; differences between either APC or siRad51 and the combined treatment, $P < 0.05$; in the MMR+ HCT-15 clone differences between all treatment groups, $P < 0.001$; differences between MMR− and MMR+ treated with APC or siRAD51, $P < 0.001$; differences between MMR− and MMR+ treated with APC+ siRAD51, $P < 0.01$.

Interestingly, MMR-deficient cells show a higher sensitivity to clastogenicity and γ-H2AX foci formation induced by APC with respect to their MMR-proficient counterparts. Our data on chromosomal damage provoked by APC reflect a higher sensitivity to the anti-proliferative effects (evaluated by clonogenic assay) of APC and other DNA polymerases inhibitors that was previously reported in MMR-deficient colon cancer cell lines, including HCT-116 derived cells [47]. Moreover, long-term primary cultures of fibroblasts derived from $MSH2^{-/-}$ mice are known to have elevated induction of gaps and breaks after exposure to APC indicating that proteins involved in MMR could be implicated in CFS stability [48]. Another study reported that treatment with APC increases the sensitivity of MMR-deficient ovarian carcinoma cell lines to the cytotoxic effects of methylating agents and cisplatin [49]. This effect was attributed to the ability of APC to inhibit the bypass of DNA lesions that may occur in the absence of a functional MMR. Of the two matched pairs of ovarian cancer cell lines differing in MMR functional status, Moreland et al. reported that only in one pair, the MMR-deficiency is associated with higher sensitivity to the anti-proliferative effects of μM concentrations of APC used as single agent [49]. It should be noted that in our study with colon cancer cells, we used much lower concentrations of APC than those tested in ovarian cancer lines, which did not profoundly affect the mitotic index of the colon cancer cell lines.

It has been demonstrated that the hMSH2/hMSH6 complex of MMR co-localizes with phosphorylated p53 and with the BLM helicase in RAD51 foci (induced by the inhibitor of DNA synthesis hydroxyurea), which might represent the sites of presumed stalled DNA replication forks [50]. In the endometrial tumour cells or colon cancer HCT-15 cells, the hMSH6-deficiency causes an increase in the number of RAD51 foci in response to replication inhibition, most likely as a result of augmented DNA damage [50]. These data suggest that a defective MMR might favour DNA breaks in the presence of an inhibitor of DNA synthesis and might explain the enhanced clastogenicity that we observed in MMR-deficient colon cancer cell lines treated with APC.

The increased sensitivity of MMR-deficient cells to chromosomal breaks induced by APC is striking in contrast to the resistance of these cells to DNA methylating agents. In the latter case, MMR is directly involved in the processing of mismatches derived from the presence of O^6-methylguanine, which inappropriately pairs with thymine during DNA replication [13]. Since MMR is directed exclusively to the newly synthesized strand and the modified base is in the template strand, the polymerase regenerates mispairs during the repair synthesis. Therefore, reiterated futile attempts of the

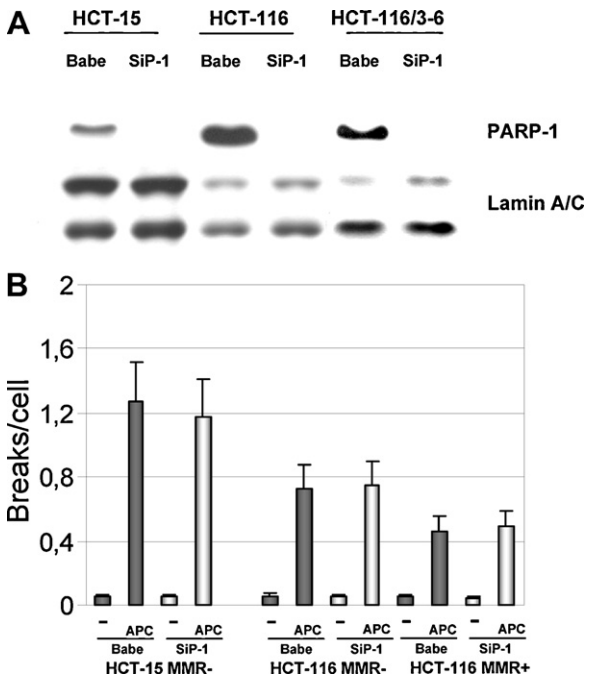

Fig. 6. Influence of PARP-1 depletion on chromosomal damage induced by APC. (A) Analysis of PARP-1 protein in control (Babe) or PARP-1 (SiP-1) silenced HCT-15, HCT-116 and HCT-116/3–6 cells. For immunoblot analysis nuclear extracts from a control clone (Babe) or from a clone transfected with pBS-U6-SiP912 vector (SiP-1) were electrophoresed and analyzed for the expression of PARP-1 or lamin A/C. (B) Analysis of chromatid breaks in cells with intact or stably silenced PARP-1. Babe and SiP-1 HCT-15, HCT-116 and HCT-116/3–6 cells were treated with APC (0.3 μM in HCT-15 cells, 0.1 μM in HCT-116 cells) for 24 h and then processed for cytogenetic analysis. Each value represents the mean (+SE) of three independent experiments. Statistical analysis, according to normal standardized deviate demonstrated no significant differences between Babe and SiP-1 HCT-15 or HCT-116 or HCT-116/3–6 cells treated with APC.

MMR system to correct the mispairs occur until the replication fork arrests, eventually leading to chromosomal aberrations, cell death and growth inhibition [51].

Our observation that the down-regulation of the HR component RAD51 in HCT-15 cells causes a high number of spontaneous chromosomal aberrations and breaks is in agreement with a previous report [14]. That most breaks were located at CFS suggests that HR might be required for maintaining the stability of CFS. Interestingly MMR-proficient cells are more sensitive to chromosomal breaks induced by silencing of RAD51 expression. This effect might be explained with the combined inhibitory effects on HR deriving from RAD51 silencing and from the suppression of HR by MMR. In accordance with our data is the observation that MMR can suppress recombination between divergent sequences containing multiple mismatches (i.e., homeologous recombination) as reported by several groups, first in bacteria and yeasts, but later also in mammalian cells [14,18,52,53]. In fact, MSH2 and MSH6-deficient cells show a hyper-recombinational phenotype [16]. Moreover, HR intermediates possess heteroduplex DNA and may contain one or few mispairs that are substrates of the MMR machinery [52]. Indeed, the MSH2–MSH6 complex was found to bind to Holliday junctions playing a direct role in HR [19]. It should be noted that RAD51 silencing causes a more widespread distribution of CFS with respect to treatment with APC, suggesting partially different mechanisms underlying generations of breaks at CFS by inhibition of HR or by replicative stress, although in both cases, FRA16D is the most expressed CFS.

Combining, APC treatment with RAD51 silencing leads to a higher number of chromosomal breaks in MMR-proficient cells with respect to APC or RAD51 silencing alone. This effect was not observed in MMR-deficient cells, in which APC action prevailed. These data are in agreement with the increased CFS expression after replicative stress that was reported in RAD51-depleted MCF-7 breast cancer cells [11], which also happen to be MMR-proficient [54].

Stable silencing of PARP-1 in MMR-deficient colon cancer cells does not increase chromosomal breaks or affect distribution of CFS caused by APC treatment. Similar results were obtained after treatment of tumour cells with the PARP inhibitor NU1025 (data not shown). Our results are in agreement with the previously reported data in the murine model, showing that regions of chromosomes known to be CFS in the mouse genome, are not more prone to DNA rearrangements in the absence of both PARP-1 and the Werner syndrome protein [55]. PARP-1, the principal member of a large family of PARPs, catalyzes the synthesis of ADP-ribose polymers using NAD^+ as a substrate [21]. Poly(ADP-ribosyl)ation of proteins represents one of the earliest responses to DNA damage, modifying chromatin structure in proximity of DNA damage; thus favouring the recruitment of DNA repair machineries, such as the base excision repair that is devoted to the correction of abasic sites and single strand breaks. PARP-1 has also been involved in regulation of the repair of DSBs by NHEJ [22]. Moreover, PARP-1 is required for slowing replication fork progression when replication fork collapse as a result of treatment with DSBs inducing agents [23]. The slowing of replication fork progression depends on the recruitment of HR pathway to the damage sites favoured by the ability of PARP-1 to suppress the inhibitory effect of NHEJ on HR [56]. Interestingly, very recent data showed that pharmacological inhibition of PARP activity or silencing of PARP-1 expression down regulates the expression of RAD51 and BRCA1 through the induction of E2F4/p130 transcription factors decreasing the efficacy of the repair mediated by HR [57]. In our model of colon cancer cell lines stably silenced for PARP-1, we do not observe a RAD51 down-regulation (data not shown) sufficient to increase spontaneous or APC-induced chromosomal breaks as, instead, observed after silencing of RAD51 by siRNA which provokes >90% decrease of RAD51 transcript.

5. Conclusions

We report that the colon cancer derived cell lines have a specific CFS distribution and the most expressed site is FRA16D, which is also the site for *WWOX* gene that happens to be frequently rearranged in colon cancers. These data are in agreement with previous findings showing that specific fragile sites are involved in chromosomal alterations present in cancer cells. Moreover, MMR-deficient cells show a higher sensitivity to the clastogenic effects of APC, as compared to their MMR-proficient counterparts. Deficiency of RAD51 causes a high level of chromosomal damage, even in the absence of other clastogenic factors, and breaks are mainly localized at CFS, even though with a broader distribution with respect to APC. MMR-proficient cells are more sensitive to chromosomal damage caused by RAD51 inhibition with or without APC as compared to their MMR-deficient counterparts. The absence of PARP-1 does not play a significant role in cellular responses to APC in our models.

Overall, the data indicate that deficit of MMR components (i.e., MLH1, MSH6) increases chromosome breaks induced by APC; on the other hand, silencing of RAD51 causes chromosome breaks at a higher extent in MMR-proficient cells.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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