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CCR5 Expression Influences the Progression of Human Breast Cancer in a p53-dependent Manner

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Abstract

Chemokines are implicated in tumor pathogenesis, although it is unclear whether they affect human cancer progression positively or negatively. We found that activation of the chemokine receptor CCR5 regulates p53 transcriptional activity in breast cancer cells through pertussis toxin–, JAK2-, and p38 mitogen–activated protein kinase–dependent mechanisms. CCR5 blockade significantly enhanced proliferation of xenografts from tumor cells bearing wild-type p53, but did not affect proliferation of tumor xenografts bearing a p53 mutation. In parallel, data obtained in a primary breast cancer clinical series showed that disease-free survival was shorter in individuals bearing the CCR5Δ32 allele than in CCR5 wild-type patients, but only for those whose tumors expressed wild-type p53. These findings suggest that CCR5 activity influences human breast cancer progression in a p53-dependent manner.

Key words: chemokine receptor • breast cancer • p53 • CCR5 polymorphism • p38

Introduction

Many cancers express an extensive network of chemokines and chemokine receptors (1, 2). Studies in animal models suggest that chemokines may act on tumor cells and/or tumor-infiltrating leukocytes (3–6). Tumor-produced chemokines are thought to have distinct roles in the biology of primary and metastatic disease, including (a) directing leukocyte infiltration into the tumor, (b) regulating the antitumor immune response, (c) controlling tumor angiogenesis, (d) functioning as autocrine or paracrine growth and survival factors, and (e) controlling tumor cell movement.

Current evidence does not establish whether these chemokine biological activities in the tumor microenvironment contribute to cancer growth and spread, or to host antitumor response and cancer regression. Studies using the CC chemokine CCL5 (RANTES) as a model reported that elevated CCL5 levels in the tumor environment contribute to improving the immune response against breast carcinomas (7), but also correlate with poor prognosis in breast cancers (8, 9). CCL5 also has a role in the chemotaxis and metastasis of breast cancer cell lines (10–12). Selected cell lines with an enhanced chemotactic response to CCL5 nevertheless showed decreased growth potential in nude mice, suggesting an inverse correlation between CCL5-induced chemotactic and proliferation signals (12). Compatible with this, basic fibroblast growth factor–induced proliferation of endothelial cells was inhibited by distinct chemokines, including CCL5, via a noncompetitive mechanism (13). These results indicate that the role of chemokines in tumor progression is complex and poorly understood.

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Abbreviations used in this paper: BrdU, bromodeoxyuridine; DFS, disease-free survival; MAPK, mitogen-activated protein kinase; siRNA, small interfering RNA.

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Here we studied the role of CCR5 in breast tumor progression by analyzing proliferation of xenografts derived from tumor cells expressing wild-type or mutated p53 in combination with wild-type or mutated CCR5. We also took advantage of the fact that ~1% of the Caucasian population is homozygous for the CCR5Δ32 polymorphism, which renders a nonfunctional CCR5 receptor, to study the effect of this mutation in a clinical series of breast cancer patients. We describe a new mechanism by which CCR5 influences human breast cancer progression, depending on the status of the p53 tumor suppressor.

Materials and Methods

Cell Lines and Stimulation. MCF-7 and MDA-MB-231 cells (American Type Culture Collection) were transduced with retroviral pLZ-KDELΔ32-IRES-gfp or pLZ-IRES-gfp (mock) supernatants, and green fluorescent protein–expressing cells were selected by fluorescence-activated cell sorting (11). The percentage of $CCR5^{+}$ cells observed after subtracting the control value was multiplied by average fluorescence intensity to calculate surface CCR5 expression in mock and KDELΔ32-expressing cells (14). MCF-7-$p53^{175H}$ cells were obtained by transfection with pWZL-Hygro-$p53^{175H}$ (provided by M. Serrano, Centro Nacional de Biotechnologia, Madrid, Spain; reference 15), followed by hygromycin selection. p53 silencing by small interfering RNA (siRNA) was performed by transfecting MCF-7 cells with a pool of p53-specific siRNA (SMARTpool kit; Dharmacon) at 100 nM using Oligofectamine (GIBCO BRL). Transfection efficiency was 75 ± 5.1% as estimated using a fluorescent-labeled siRNA. As a control, MCF-7 cells were transfected with nonspecific pooled siRNA duplexes (Dharmacon). Finally, MCF-7 cells were transfected with Flag-tagged dominant negative mutants for MAP kinase kinases 3 and 6 (dnMKK3 and dnMKK6; provided by R.J. Davis, Howard Hughes Medical Institute, University of Massachusetts, Worcester, MA).

Serum-depleted mock, KDELΔ32-, $p53^{175H}$-, siRNA-, dnMKK3-, dnMKK6-, or dnMKK3 plus dnMKK6–expressing MCF-7 cells were stimulated with 100 nM CCL5 (PeproTech), γ irradiated (30 Gy) or UV irradiated with UVC (254 nm, 30 J/m^2), and then incubated at 37°C for the times indicated. Equal protein amounts from cell lysates prepared with RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40) were analyzed by Western blot with anti-p53 (Oncogene Research Products), anti-p21 and anti-Mdm2 (Santa Cruz Biotechnology, Inc.), anti-p38 and anti–phospho-p38 mitogen-activated protein kinase (MAPK; no. 9211; New England BioLabs, Inc.), and anti-Flag (Sigma-Aldrich) antibodies. Protein loading was confirmed with anti-tubulin antibody (Sigma-Aldrich). For some experiments, cells were pretreated for 14 h with 100 μM tyrphostin AG 490, 10 μM LY 294002, 10 μM PD 98059, 10 μM SB 203580 (all from Calbiochem), 0.75 μg/ml pertussis toxin (Sigma-Aldrich), or DMSO before CCL5 stimulation. Densitometric analysis of Western blots was performed with NIH Image software.

Breast Tumor Xenograft Models and Immunohistochemistry. Eight female BALB/c-SCID mice were injected subcutaneously in both flanks with 3×10^6 mock or KDELΔ32-expressing MDA-MB-231 or 5×10^6 MCF-7 cells. In the case of MCF-7, mice were pretreated with 1 μg/ml 17α-ethynyl estradiol (Sigma-Aldrich) in drinking water for 1 wk before cell injection and during the experimental period. 4 wk after cell injection, mice received two intraperitoneal injections of bromodeoxyuridine (BrdU; 10 mg/ml, 50 μl/mouse) 6 h apart, and primary tumors were removed 4 h after the last injection. Some tumors were fixed in 70% ethanol, paraffin embedded, and analyzed for BrdU incorporation using anti-BrdU antibody (Becton Dickinson) or active p38 MAPK using anti–phospho-p38 antibody (no. 9216; New England BioLabs, Inc.). Other tumors were snap frozen in Tissue Freezing Medium (Sakura Finetek) and cryosections were used for in situ apoptosis determination by the TUNEL method (MEBSTAIN Apoptosis kit II; Immunotech) or p21 detection. Finally, some tumors were mechanically disrupted. Cell lysates were prepared with RIPA buffer and used both to measure human CCL5 (Cytoscreen; Biosource International) and for p53, p21, and Mdm2 analysis in Western blot. CCL5 levels, BrdU incorporation, and TUNEL data were compared using the two-tailed Mann-Whitney test.

Δ32 Genotyping of the Breast Cancer Cohort. The breast cancer cohort comprised primary (nonmetastatic) breast cancer patients diagnosed at the Hospital Universitario 12 de Octubre, Madrid, Spain, from January 1992 to December 1995. Stage IV patients at diagnosis were excluded from this series. DNA was isolated from biopsies of 547 patients and the CCR5Δ32 allele determined with an automated method based on real-time PCR in a Lightcycler system (16). Patients included in this study were disease free after surgery and showed no initial metastases or metastases within 3 mo after surgery.

Chemotherapy was administered to premenopausal patients with tumors larger than 1 cm, postmenopausal patients with negative estrogen receptor expression, and patients showing affected lymph nodes. Patients with conservative surgery and those showing four or more axillary lymph nodes affected were treated with radiotherapy. Patients whose tumors expressed hormone receptors were treated with tamoxifen for 5 yr. Clinical follow-up was performed according to institutional protocols by physical examination every 3 mo in the first 2 yr after surgery, every 6 mo in the next 3 yr, and every year after the sixth year. Mammography was performed yearly and other diagnostic tests were performed when relapse was suspected. Disease-free survival (DFS) was defined as the time (in months) from diagnosis of disease, usually coinciding with surgery, to first relapse or to the last clinical revision for patients with no recurrence. A total of 133 out of 541 patients relapsed (24.5%).

Allelic frequencies between patient groups where compared using standard χ^2 tests with Yates correction, the Mantel-Haenszel test, or Fisher's exact test where necessary, using Statcalc software (EpiInfo 5.1). For statistical analysis of genotypes, Δ32/+ and Δ32/Δ32 individuals were grouped to avoid values of <7. DFS was estimated using the Kaplan-Meier method and compared by the log-rank test.

Online Supplemental Material. Fig. S1 shows DFS curves in breast cancer patients grouped according to CCR5 status. Fig. S1 is available at http://www.jem.org/cgi/content/full/jem.20030580/DC1.

Results

CCR5 Signaling Increases p53 Transcriptional Activity. CCR5-mediated signals may regulate transcription of several p53 target genes in certain cell types (17). To study the association between CCR5 and p53 in cancer cells, we analyzed CCR5-dependent signaling in the MCF-7 breast carcinoma line, which expresses CCR5 and wild-type p53.

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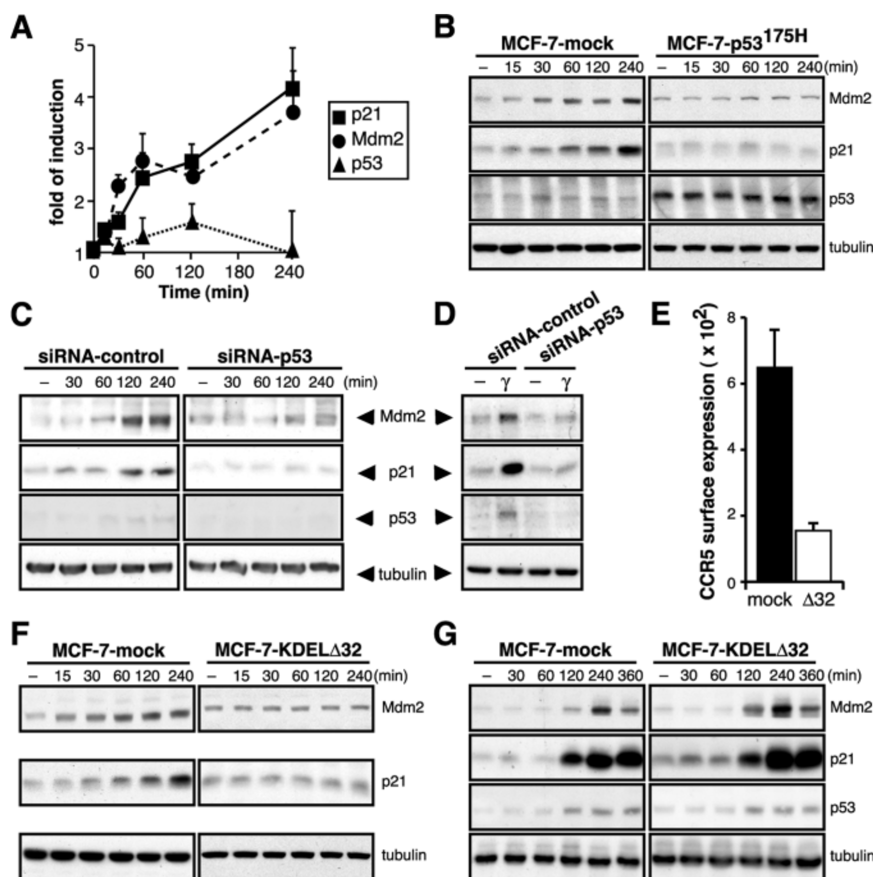

Figure 1. CCR5 regulates p53 transcriptional activity. (A) Time course induction of p53 (dotted line, ▲), p21^{WAF1} (solid line, ■), and Mdm2 (dashed line, ●) in CCL5-stimulated MCF-7 cells. Western blots from four independent experiments were quantified by densitometry and the values were normalized using the tubulin loading control. Data points are plotted relative to mean values obtained before chemokine addition ($n = 5$). (B) CCL5-induced p53 targets in MCF-7 cells expressing the p53^{175H} mutant. One representative experiment of three is shown. (C and D) MCF-7 cells transfected with control or p53-specific siRNA oligonucleotides were stimulated with CCL5 (C) and incubated at 37°C for the times indicated or γ irradiated (30 Gy; D) and incubated for 3 h at 37°C. Cell lysates were analyzed by Western blot. One representative experiment of three is shown. (E) CCR5 was detected in live mock- or KDELΔ32-expressing MCF-7 cells by FACS®. Surface CCR5 expression in mock- and KDELΔ32-expressing cells was estimated by multiplying average fluorescence intensity by the percentage of CCR5$^+$ cells ($n = 3$). (F and G) Mock- and KDELΔ32-expressing cells were stimulated with CCL5 (F) or γ irradiated (30 Gy; G) and incubated at 37°C for the times indicated. Cell lysates were analyzed by Western blot. One representative experiment of three is shown.

Stimulation of MCF-7 cells with CCL5, a ligand for CCR5, increased levels of well-established p53-regulated genes, such as p21$^{WAF1/CIP1}$ and Mdm2 (Fig. 1 A). p53 protein levels were essentially unaltered after CCL5 stimulation, although a slight increase was observed 60 min after stimulation. To determine whether CCL5-mediated p21^{WAF1} and Mdm2 up-regulation is dependent on p53 transcriptional activity, MCF-7 cells stably expressing a dominant negative p53^{175H} mutant (15) were stimulated with CCL5. CCL5 did not increase p21^{WAF1} or Mdm2 protein levels in MCF-7-p53^{175H} cells (Fig. 1 B). Concurrently, transfection of MCF-7 cells with p53-specific siRNA prevented CCL5-induced p21^{WAF1} and Mdm2 up-regulation (Fig. 1 C). As a control, p53-specific siRNA also inhibited γ irradiation–induced up-regulation of p53 activity (Fig. 1 D). These results suggest that CCL5-induced transcriptional activation of p53 target genes, such as p21^{WAF1} and Mdm2, requires functional p53 protein.

To analyze whether CCL5-induced p53 activation requires CCR5-mediated signaling at the cell surface, we specifically blocked CCR5 in MCF-7 cells with a CCR5 dominant negative mutant. We previously showed that overexpression of a CCR5 deletion mutant (KDELΔ32), which is phenotypically similar to the natural CCR5Δ32 (Δ32) mutant, abrogates CCR5 function in MCF-7 cells (11). This effect is probably due to a trans inhibitory effect of the KDELΔ32 mutant on CCR5 transport to the cell surface (18). KDELΔ32 expression reduced CCR5 levels by ~70% (Fig. 1 E). CCL5 did not induce p21^{WAF1} or Mdm2 in KDELΔ32-expressing MCF-7 cells (Fig. 1 F), indicating that CCR5 is the specific receptor by which CCL5 induces these genes. p21^{WAF1} and Mdm2 up-regulation were similar, however, in mock- and KDELΔ32-expressing MCF-7 cells after γ irradiation, suggesting that KDELΔ32 expression did not affect the p53 pathway in these cells (Fig. 1 G).

p53 Transcriptional Activity Requires CCR5-mediated p38 Activation. To study the mechanism by which CCR5 activates p53, we used chemical inhibitors to block CCR5-mediated signaling. Binding of chemokines to their receptors leads to activation of heterotrimeric G proteins and JAK family tyrosine kinases. These in turn initiate multiple signaling cascades, including phosphatidylinositol-3 kinase as well as p38 and p42/p44 MAPK pathways (19). Incubation of MCF-7 cells with the inhibitors LY 294002 and PD 98059, which block phosphatidylinositol-3 kinase and p42/p44 MAPK pathways, has only a marginal effect on CCR5 induction of p21^{WAF1} and Mdm2 (Fig. 2 A). In contrast, CCR5-mediated induction of p21^{WAF1} and Mdm2 was inhibited by treatment of MCF-7 cells with pertussis toxin, AG 490, and SB 203580 (Fig. 2 A), which impede G_i, JAK-2, and p38 MAPK activation, respectively. In addition, CCL5 induced p38 phosphorylation in mock-transfected MCF-7 cells, but not in KDELΔ32-expressing cells (Fig. 2 B), indicating that CCR5 mediates p38 MAPK acti-

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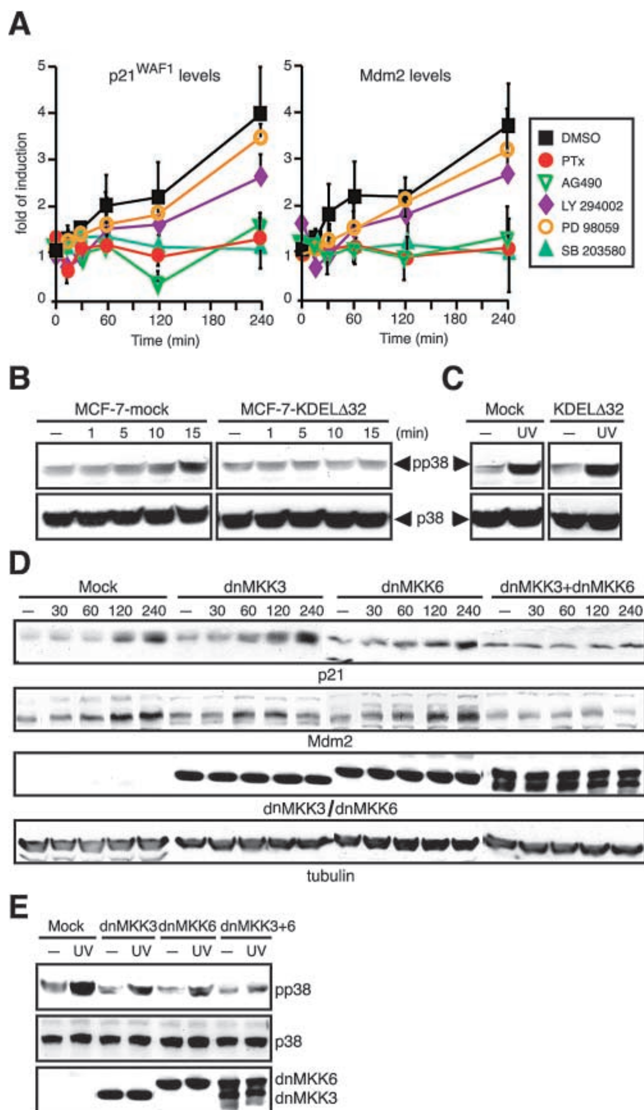

Figure 2. CCR5 regulates p53 activity through in a G_i-, JAK2-, and p38 MAPK–dependent manner. (A) Measurement of p21^{WAF1} and Mdm2 induction analyzed by Western blot after CCL5 stimulation of MCF-7 cells pretreated with the indicated chemical inhibitors. Data points represent the mean ± SD of densitometric values obtained in two independent experiments for each inhibitor. Data are plotted relative to those obtained in DMSO-treated cells before CCL5 addition. (B and C) Mock- and KDELΔ32-expressing cells were stimulated with CCL5 (B) or UV irradiated (30 J/m^2, C), and then incubated at 37°C for the times indicated (for CCL5) or for 30 min (for UV irradiation). Cell lysates were analyzed by Western blot with anti–phospho-p38 (pp38) or anti-p38–specific antibodies. One representative experiment of three is shown. (D and E) Mock-, dnMKK3-, and dnMKK6-expressing cells and cells coexpressing dnMKK3 and dnMKK6 were stimulated with CCL5 (D) or UV irradiated (30 J/m^2, E), and then incubated at 37°C as above. CCL5-induced p21^{WAF1} mdm2 up-regulation was visualized by Western blot (D). In the case of UV irradiation (E), cell lysates were analyzed by Western blot with anti–phospho-p38 (pp38) or anti-p38–specific antibodies. In all cases, dnMKK3 and dnMKK6 expression was detected using an anti-Flag antibody. One representative experiment of two is shown.

vation. p38 phosphorylation was nonetheless similar in mock and KDELΔ32 cells after UV irradiation, indicating that KDELΔ32 expression does not affect p38 activation per se (Fig. 2 C). Finally, we used dominant negative mutants for MKK3 and MKK6 to block p38 MAPK activation (20). Overexpression of dnMKK3 or dnMKK6 mutants produced only modest inhibition of the CCL5-induced increase in p21^{WAF1} and Mdm2 levels. Maximum inhibition was observed when dnMKK3 and dnMKK6 were coexpressed (Fig. 2 D). Likewise, UV-induced p38 phosphorylation was maximally inhibited in cells coexpressing dnMKK3 and dnMKK6 (Fig. 2 E). The results suggest that specific CCR5 activation at the cell surface enhances p53 transcriptional activity in MCF-7 breast cancer cells by a G_i-, JAK-, and p38-dependent mechanism(s).

The CCR5-p53 Pathway Regulates In Vivo Proliferation of Human Breast Tumor Xenografts. To study the CCR5-p53 pathway in tumor growth, we compared in vivo proliferation of mock- and KDELΔ32-expressing MCF-7 (wild-type p53) or MDA-MB-231 (mutated p53) cells implanted into SCID mice. As for MCF-7, KDELΔ32 mutant expression in MDA-MB-231 cells reduced surface CCR5 levels by 62 ± 4.2%. We measured the amount of human CCL5 in extracts from mock and KDELΔ32 xenografts from both cell lines. CCL5 levels were comparable in mock and KDELΔ32 tumors (MCF-7-mock: 35.1 ± 7.5 pg/100 μg, n = 8; MCF-7-Δ32: 41.7 ± 10.6 pg/100 μg, n = 8, P = 0.5; MDA-MB-231-mock: 33.06 ± 5.34 pg/100 μg, n = 5; MDA-MB-231-Δ32: 50.21 ± 17 pg/100 μg, n = 7, P = 0.15, Mann-Whitney test), suggesting that all tumor types were equally exposed to CCL5.

BrdU incorporation experiments using the MCF-7 xenografts showed that the percentage of BrdU$^+$ cells was significantly higher in KDELΔ32 than in mock tumors (Fig. 3 A; $P < 0.05$, Mann-Whitney test). It should be noted, however, that subcutaneous MCF-7 xenograft implantation and growth is dependent on estradiol supplementation during the experimentation period, and that estradiol induces equal proliferation of mock and KDELΔ32-expressing cells in vitro (unpublished data). This may attenuate the differences observed in BrdU incorporation between mock- and KDELΔ32-expressing MCF-7 xenografts. Similar BrdU chase experiments using MDA-MB-231 tumors showed no BrdU incorporation differences when mock- and KDELΔ32-expressing xenografts were compared (Fig. 3 B). TUNEL assays of the xenografts showed a comparable percentage of apoptotic cells in mock and KDELΔ32 derived from MCF-7 (Fig. 3 C) or MDA-MB-231 (Fig. 3 D) tumors, indicating that CCR5 expression did not affect apoptosis.

p21^{WAF1} levels were increased in mock- compared with KDELΔ32-expressing MCF-7 xenografts (Fig. 4 A). Again, KDELΔ32 overexpression in MDA-MB-231 cells produced no differences in p21^{WAF1} levels (Fig. 4 B). In agreement, nuclear localization of p21^{WAF1} was clearly increased in mock compared with KDELΔ32-MCF-7 xenografts, but not in those derived from MDA-MB-231 cells (Fig. 4 C). Collectively, these data indicate that a reduction in functional cell surface CCR5 may increase the proliferation rate of tumor cells bearing wild-type p53, probably by diminishing levels of the CDK inhibitor p21^{WAF1}. To analyze whether p38

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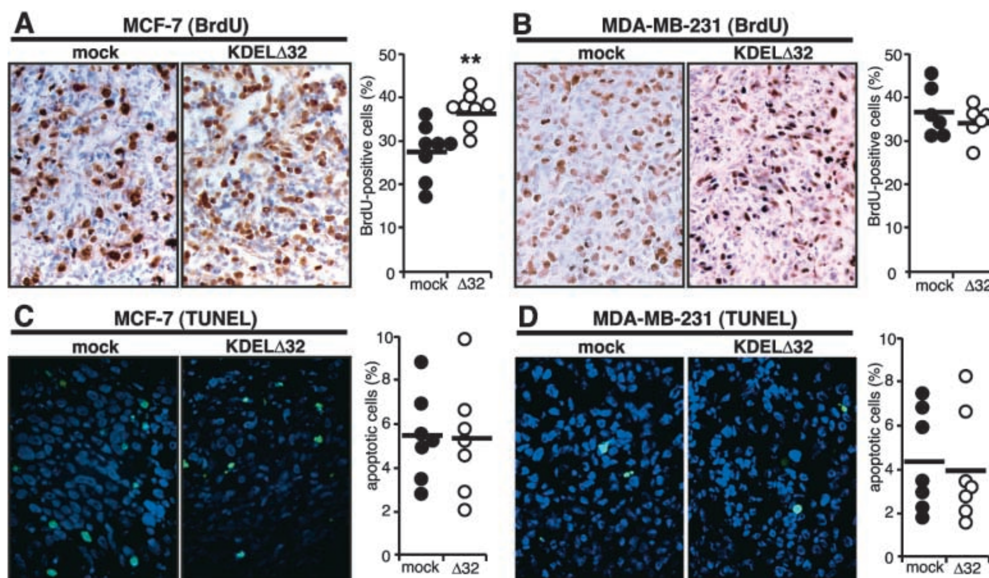

Figure 3. Cell surface levels of CCR5 specifically affect the proliferation of breast tumor xenografts with functional p53. BrdU (A and B) and TUNEL (C and D) staining of mock- and KDELΔ32-expressing MCF-7 (A and C) or MDA-MB-231 (B and D) xenografts. Counterstaining was done with hematoxylin and eosin for BrdU and nuclei were DAPI stained for TUNEL. ×40. Data points at the right represent the percentage of $BrdU^+$ or $TUNEL^+$ nuclei determined in four random fields for each tumor analyzed (two-tailed Mann-Whitney test). *, $P < 0.05$.

MAPK was involved in this pathway, we stained mock and KDELΔ32-MCF-7 xenograft sections with an antibody against the active form of p38 MAPK. Mock-derived xenografts showed greater cytoplasmic and nuclear anti–phospho-p38 staining than those derived from KDELΔ32-MCF-7 (percent nuclei stained for phospho-p38: mock 13.67 ± 3.1%, KDELΔ32 5.44 ± 1.9%; Fig. 4 D). Thus, the results suggest that the CCR5-p53 pathway is operative and regulates in vivo breast cancer cell proliferation.

The CCR5-p53 Pathway Influences Human Breast Cancer Progression. Chemokine and/or chemokine receptor polymorphisms in the human population represent a unique model by which to study the contribution of specific chemokines to pathogenesis. Δ32 is a 32-bp deletion within the CCR5 coding region, which results in a frame shift that generates a nonfunctional receptor (21–23). Homozygotes for this mutation (Δ32/Δ32) do not express CCR5 on the cell surface, and receptor levels are also greatly reduced in Δ32 heterozygotes (Δ32/+). Both Δ32/Δ32 and Δ32/+ individuals appear healthy and show no apparent phenotype. Nonetheless, mutant Δ32 allele expression is associated with (a) resistance to HIV-1 infection in homozygotes and slow progression to AIDS in heterozygotes (21–23), (b) decreased severity of rheumatoid arthritis (24–26) and (c) multiple sclerosis (27), (d) long-term survival of renal transplants (28), and (e) reduced risk of myocardial infarction (29). These observations suggest a functional role for CCR5 in these pathogenic processes.

To analyze the relevance of CCR5 in human breast cancer, we determined the allelic frequency and genotypes of Δ32 polymorphism in 547 patients diagnosed of primary (nonmetastatic) breast cancer. The median follow-up period was 83 mo. Six patients were Δ32/Δ32 and 74 were Δ32/+. The allelic frequency of Δ32 in the cohort was 7.86% (86 of 1,094 chromosomes). No differences were detected in allelic frequencies between breast cancer patients and the general population ($P = 0.97$, χ^2 with Yates correction; reference 16). In addition, the genotype distribution observed in breast cancer patients was in accordance with the Hardy-Weinberg equilibrium law ($\chi^2 = 0.57$, $P = 0.75$ with two degrees of freedom). These data suggest that the Δ32 allele is not associated with susceptibility to breast cancer.

We next explored the role of the Δ32 allele in breast cancer by evaluating allelic frequency and genotypes of this marker in patients grouped according to clinical, pathological, or molecular parameters (Table I). We found no bias in Δ32 frequency when tumors were grouped according to hormone receptor (estrogen or progesterone), ErbB-2, or angiogenin expression. Significant differences in allele frequencies between groups were observed, however, when classified by tumor size. Specifically, patients with the largest tumor size (T4) had a Δ32 allelic frequency of 14.3%, a significant increase compared with the expected frequency for this mutation in the cohort ($\chi^2 = 4.07$, $P = 0.043$).

We analyzed whether the CCR5-induced p53 activation observed in breast cancer cell lines influenced breast cancer progression in humans. A larger proportion of tumors derived from Δ32 patients tended to be negative for p53 expression than those derived from CCR5 wild-type individuals (Table I). Absence of aberrant p53 expression, which usually corresponds to p53 mutation and is associated with better prognosis in breast cancer (30), is considered an indication that this tumor suppressor gene is not mutated in these cancers. We analyzed DFS in the cohort, sorted according to p53 and CCR5 status. Because of the small number of homozygous patients, Δ32/Δ32 and Δ32/+ individuals were grouped together. DFS differed significantly between Δ32 and wild-type CCR5 patients only in those individuals with tumors with wild-type p53 (i.e., $p53^-$ tumors; Fig. 5, bottom; $P = 0.021$, log-rank test). DFS was comparable between Δ32 and wild-type CCR5 patients whose tumors expressed a mutated p53 form (i.e., $p53^+$ tumors; Fig. 5, bottom; $P = 0.752$, log-rank test). When samples were grouped according to CCR5 status (Fig. S1, available at

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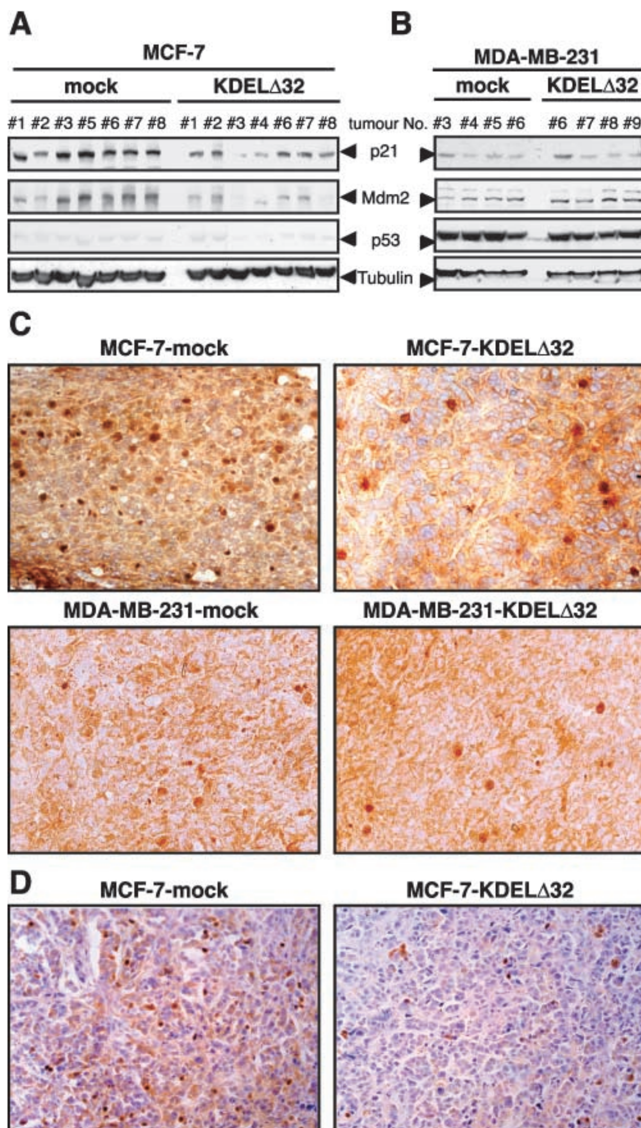

Figure 4. p21^{WAF1} and phospho-p38-MAPK detection in xenografts from mock- and KDELΔ32-expressing tumor cells. (A and B) Lysates from mock- and KDELΔ32-expressing MCF-7 (A) or MDA-MB-231 (B) xenografts were analyzed sequentially with anti-p21^{WAF1}, anti-Mdm2, anti-p53, and anti-tubulin antibodies by Western blot. (C) Cryosections of xenografts derived from mock- and KDELΔ32-expressing MCF-7 and MDA-MB-231 cells, as indicated, were stained with anti-p21^{WAF1} antibody followed by a peroxidase-labeled second antibody. Counterstaining was performed with hematoxylin and eosin. (D) Paraffin sections from mock- and KDELΔ32-expressing MCF-7 xenografts were stained with an anti–phospho-p38 antibody followed by a peroxidase-labeled second antibody, and were hematoxylin and eosin counterstained. For C and D, the background staining with the second antibody was also analyzed (not depicted). ×40.

http://www.jem.org/cgi/content/full/jem.20030580/DC1), it was found that those wild-type CCR5 patients whose tumors had wild-type p53 showed a better prognosis than those with mutant p53 (P = 0.0051, log-rank test), concurring with the previously reported prognostic value of p53 (30). In contrast, there was no statistically significant difference in DFS between the p53-mutated versus the wild-type groups in Δ32 patients (P = 0.68, log-rank test).

Table I. *Clinical Characteristics of Breast Cancer Patients (n = 547)*

Clinical parameter		Allele frequency		
		CCR5wt	CCR5Δ32	P (χ^2)
Age	<50	91.35	8.65	0.59
	>50	92.46	7.53	
Menopausal status	Pre-Peri	92.72	7.28	0.73
	Post	91.96	8.04	
Histologic grade	I-II	91.64	8.36	0.86
	III	91.07	8.93	
Tumor size	T1	92.82	7.18	
	T2	92.44	7.56	
	T3	92.04	7.95	
	T4	85.71	14.29	0.04
Lymph node infiltration	0	92.41	7.58	
	1–3	93.08	6.91	
	4–9	92.96	7.04	
	>10	89.58	10.4	0.36
Stage	I	92.02	7.97	
	II	92.62	7.38	
	III	89.24	10.76	0.22
Estrogen receptor	Neg	91.21	8.79	0.52
	Pos	92.54	7.46	
Progesterone receptor	Neg	91.63	8.37	0.76
	Pos	92.32	7.68	
ErbB-2	Neg	92.87	7.13	0.62
	Pos	91.48	8.52	
Angiogenin	Neg	94.11	5.88	0.63
	Pos	92.21	7.79	
p53	Neg	91.11	8.89	
	Pos	94.11	5.89	0.1

Estrogen receptor$^+$ defined as ≥10 fmol/mg, progesterone receptor$^+$ as ≥20 fmol/mg (Abbott), ErbB-2$^+$ as ≥15 HNU/μg (Oncogene Science), angiogenin$^+$ as ≥5 ng/mg (R&D Systems), and p53$^+$ defined by Western blot with pAb 181 (Oncogene Science) according to established standards.

Discussion

Here we present genetic evidence for the role of the CCR5 chemokine receptor in human breast cancer progression. First, we show that stimulation of a human breast adenocarcinoma cell line with the chemokine CCL5 results in activation of the tumor suppressor p53 through a mechanism that depends on specific cell surface CCR5 expression and G_i, JAK2, and p38-MAPK activation. Second, using a mouse xenograft model, we show that the link

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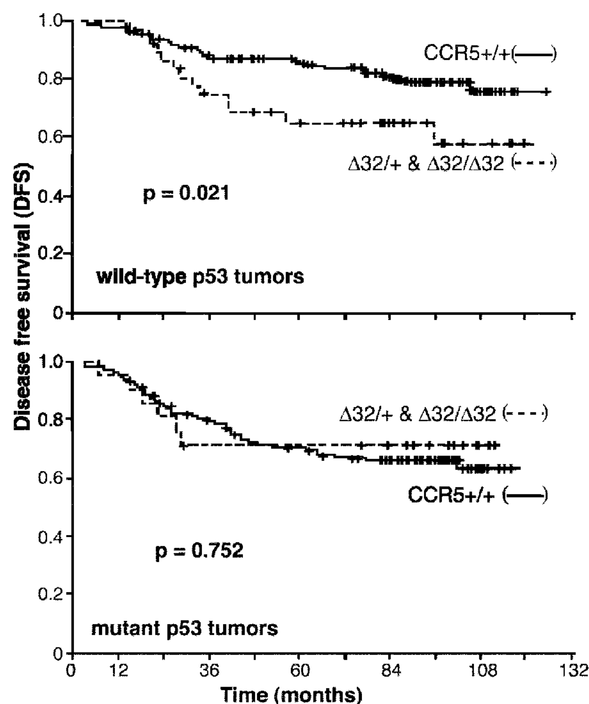

Figure 5. The Δ32 polymorphism affects human breast cancer progression. Kaplan-Meier DFS curves for the 547 breast cancer patients evaluated according to p53 expression and the Δ32 polymorphism, as indicated. CCR5 wild-type (+/+) patients, solid line; Δ32/+ and Δ32/Δ32 patients, dashed line.

between CCR5 and p53 is operative in regulating in vivo proliferation of tumor cells. Abrogation of cell surface CCR5 expression enhanced proliferation of tumor cells bearing wild-type p53, but not of tumor cell lines expressing a mutated p53 form. Finally, based on the CCR5Δ32 polymorphism in humans, which renders a nonfunctional CCR5 receptor, we found that CCR5 status influenced disease progression in a genotyped cohort of 547 patients diagnosed with primary (nonmetastatic) breast cancer. We found that DFS was shorter in CCR5Δ32 than in CCR5 wild-type patients with wild-type p53 tumors. Conversely, DFS was comparable between CCR5Δ32 and CCR5 wild-type patients whose tumors had mutant p53.

Our results suggest a negative correlation between CCR5 expression and the growth of human breast tumors expressing wild-type p53. As seen in our tumor xenograft models, CCL5 production in the tumor environment does not increase apoptosis, but restricts growth of cancer cells expressing wild-type p53 in a CCR5-dependent manner. These results concur with previous data showing the ability of CCL5 to inhibit growth factor–induced cell proliferation (13), as well as CCL5-induced stabilization of p53 in neuronal and astrocytic nuclei (17). In some cases, CCL5 can also activate the apoptotic pathway in CCR5-expressing cells (31). How CCR5 transmits the signal to p53 requires future investigation, but the data presented here suggest a role for p38 MAPK in this pathway. In agreement with others (32, 33), we found that CCL5-induced p38 activation at the cell surface was CCR5 expression dependent. Moreover, the active p38 MAPK form stained more intensely in mock-derived xenografts than in those derived from cells expressing the KDELΔ32 mutant, indicating that the CCR5-p38 MAPK pathway probably operates in this in vivo model. Because p38 MAPK is a prominent p53 activator in response to stress signals and certain anti-cancer drugs (34, 35), CCR5-induced p38 phosphorylation can activate p53 transcriptional activity, leading to $p21^{WAF1}$ induction and slowing of tumor cell growth.

Concurring with this hypothesis, the data from our cohort suggest that CCR5 influences the p53 mutation rate in human tumors. The p53 tumor suppressor gene is inactivated by mutation in approximately half of all human tumors. Evidence suggests that tumor cells have developed mechanisms other than mutation to "silence" p53 function (36). We found that mutation of p53 affected 46% of tumors from CCR5 wild-type individuals, but only 35% of tumors from CCR5Δ32 patients. Moreover, no tumors from the Δ32/Δ32 patients showed mutant p53. These data strongly suggest that there is less selective pressure for p53 mutation in tumors from CCR5Δ32 individuals than from those bearing CCR5 wild-type alleles. In CCR5Δ32 patients, all pathways downstream of CCR5 are abolished (homozygous) or severely impaired (heterozygous) and CCR5-mediated p53 activation is consequently diminished. It is thus possible that p53 would be silenced in CCR5Δ32 patients under conditions in which this tumor suppressor gene is activated in CCR5 wild-type individuals.

In apparent contradiction to our results, elevated CCL5 levels are reported in patients with progressive breast cancer (8, 9), although it is noteworthy that these studies provide no information on CCR5 and p53 expression. In one of these reports, markedly elevated plasma levels of CCL5 were found in 27% of patients with progressive cancer, raising the possibility that the CCL5 mediates tumor progression in some patients but not in others (9). Indeed, in our cohort, the Δ32 polymorphism affects only breast cancer progression in patients whose tumors expressed wild-type p53.

CCR5 may also have an indirect effect on cancer progression by controlling the antitumor immune response. CCR5 participates in chemotaxis of memory and activated naive T cells and is required for T cell activation (37). Ele-

Table II. *Lymphocyte Infiltration in Breast Tumors*

Infiltration grade	+/+	Δ32/+	Δ32/Δ32
+	5	2	4
++	1	1	0
+++	1	0	0

Lymphocyte infiltration <50% (+), equal to 50% (++), or >50% (+++) per field at low magnification (×10).

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vated lymphocyte infiltration is a rare event in breast cancer, as confirmed by analysis of lymphocyte infiltration in 14 random samples from the cohort (Table II). Although additional research is needed to clarify the relevance of Δ32 polymorphism in the antitumor immune response, it is noteworthy that DFS curves in our cohort were similar between Δ32 and wild-type patients whose tumors expressed mutant p53. This suggests that the CCR5-dependent antitumor immune response does not have a major impact on this cancer progression. Alternatively, immune system impairment in Δ32 patients may only be important in those tumors with a benign prognosis (wild-type p53 tumors).

In summary, we have identified a G_i-, JAK2-, and p38-dependent pathway by which CCR5 regulates p53 transcriptional activity. This pathway appears to be silenced or severely impaired in individuals bearing the mutated CCR5 Δ32 allele. As a consequence, breast tumors with wild-type p53 grow faster and relapse sooner in Δ32 carriers than in wild-type CCR5 individuals. This connection between CCR5 and the tumor suppressor p53 represents a previously unknown mechanism controlling human tumor progression and provides a rationale for new approaches to cancer treatment using CCL5.

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