Cytoplasmic p21$^{WAF1/CIP1}$ correlates with Akt activation and poor response to tamoxifen in breast cancer

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Abstract. P21$^{WAF1/Cip1}$ (p21) translocates to the cytoplasm inducing cell cycle progression and survival upon Akt/PKB activation. We studied whether heregulin ß1 (HRGß1), that activates the PI3K/Akt and MAPK pathways, also misallocates p21. We also explored whether HRGß1 interferes with the effects of tamoxifen. The clinical material studied helped us to clarify whether p21 was associated with phosphorylated Akt, recurrence-free survival and response to tamoxifen. MCF-7 cells treated with HRGß1 -/+ E2 were analyzed by flow cytometry to observe how the different compounds affected tamoxifen-induced cell cycle arrest and apoptosis. Total cell lysate and nuclear and cytoplasmic fractions were used to detect p21, phospho-Akt and other proteins by Western blotting. Immunofluorescence was used to visualize p21$^+$ cells upon HRGß1 and E2 stimulation. The localization of p21 in breast cancer was studied by immunohistochemistry in frozen tumor sections from 280 patients. In MCF-7 we found that HRGß1 counteracted the inhibition of p21 expression by tamoxifen and caused p21 cytoplasmic accumulation. HRGß1 partially counteracted the cytostatic effect of tamoxifen but abrogated its cytotoxic effect. The clinical material revealed that nuclear p21 (P=0.022) and cytoplasmic p21 (P=0.00001) were associated with phospho-Akt. Based on p21 cell location, we identified 3 subgroups of ER$^+$ patients: the p21N$^+$/C$^-$ group for whom tamoxifen was needed otherwise the survival was poor (P=0.0082), the p21N$^+$/C$^+$ or p21N$^-$/C$^-$ group, that responded to tamoxifen (P=0.034), and the p21C$^+$/N$^-$ group, that might not benefit from this treatment (P=0.7). In conclusion, HRGß1 inhibits tamoxifen-induced apoptosis, contributes to p21 cytoplasmic expression while the cellular localization of p21 interacts with the benefit from tamoxifen treatment.

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Introduction

Tamoxifen inhibits estrogen receptor (ER)-mediated gene transcription leading to both G_0/G_1 arrest (1,2) and apoptosis (3-5). The efficacy of tamoxifen in the treatment of ER$^+$ breast cancer patients is well known. The drug has few side effects and has demonstrated to increase both disease free and overall survival (6-8). However, many of the initial responders among ER$^+$ patients acquire resistance to tamoxifen while still expressing the ER. Therefore, it is important to study the factors that interact with the ER or with tamoxifen to modulate cell cycle progression and apoptosis.

In MCF-7 breast cancer cells, it has been shown that tamoxifen causes cell cycle arrest through up-regulation of p21$^{WAF1/Cip1}$ (p21) and p27^{Kip1} (p27). When these cells were depleted of p21 or p27 by anti-sense technology, the effect of tamoxifen was lost, demonstrating that both, p21 and p27 are critical mediators of the therapeutic effect of this drug (9).

The cyclin dependent-kinase (Cdk) inhibitor p21 conforms a family together with p27 and p57^{Kip2}. P21 was isolated as a Cdk2 associated protein that inhibited cell cycle progression by binding and inactivation of both the Cdk2-cyclin E complex (10,11) and proliferating cell nuclear antigen (PCNA) (12). Since these processes occur in the nucleus, the inhibitory activity of p21 has been restricted to this cellular compartment.

However, recent findings indicate that p21 can also act outside the nucleus inducing cell cycle progression and cell survival (13-17). According to a new model, cell cycle progression remains dependent on free Cdk2-cyclin E complex while p21 stabilizes cdk4/6-cyclin D1 complex (14,18). On the other hand, cytoplasmic p21 has been found to bind the pro-caspase 3 and the apoptosis signal-regulating kinase 1 (ASK1), which inhibit both caspase 3 activity and the stress activated kinase cascade respectively (13,19,20).

The mechanisms that regulate the cytoplasmic localization of p21 remain under study but it has been shown that the nuclear localization signal (NLS) (aa140-165) of p21 can be altered either by truncation (21) or by phosphorylation at consensus sites Thr145 and Ser146. Activation of the PKB/Akt pathway by the erbB2 receptor is one cause of p21 phosphorylation and cytoplasmic localization (22). This finding is interesting since both Akt/PKB activation and

erbB2 expression have been associated with poor response to tamoxifen (23-28).

Based on these results, we aimed to study the connection between the growth factor heregulin ß1 (HRGß1), which activates the PI3K/Akt pathway through the erbB family, and the shift in the subcellular location of p21 from the nucleus to the cytoplasm. We also aimed to see whether HRGß1 could interfere with the effects of tamoxifen *in vitro*. Moreover, we extended our investigation to clinical material from 280 post-menopausal breast cancer patients allowing us to analyze the expression and localization of p21 in breast tumors, its association with other clinicopathological factors, phosphorylated Akt (p-Akt), erbB2 and ER as well as its association with recurrence-free survival and response to tamoxifen.

Materials and methods

Reagents. Seventeen-ß-estradiol (E2), 4-hydroxytamoxifen (Tam) and the mouse monoclonal anti-ß actin antibody were purchased from Sigma (St. Louis, MO, USA). Recombinant human HRGß1 (EGF domain) was from R&D Systems (Minneapolis, MN, USA). Rabbit anti-phospho-Akt (Ser473) was purchased from New England Biolabs (Beverly, MA, USA). Mouse anti-p21 WAF1 OP64 and sc817 were from Oncogene (San Diego, CA, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, USA) respectively. M30 CytoDeath antibody was from Roche Molecular Biochemicals (Mannheim, Germany), the IgG2b and the secondary antibody R-phycoerythrin (RPE) conjugate were purchased from Dako (Glostrup, Denmark). Charcoal/dextran striped foetal bovine serum (CS-FBS) was obtained from Hyclone (Hyclone Road, Logan, UT, USA). Slow Fade light antifade kit, the cell nuclei dye To-Pro-3 and secondary antibodies goat anti-mouse-Alexa 488 and goat anti-rabbit-Alexa 594 came from Molecular Probes (Eugene, OR, USA). Cell culture medium and supplements were obtained from Invitrogen (Invitrogen, UK).

Cell culture. The breast cancer cell line MCF-7 was obtained from Dr H. Arnqvist (Department of Cell Biology, University of Linköping, Sweden). MCF-7 cells were cultured in RPMI-1640 supplemented with 10% heat-inactivated foetal bovine serum (FBS), 2 mM L-Glutamine and a mixture of penicillin-streptomycin (complete medium). Prior to every experiment, the cells were allowed to attach in complete medium for 24 h. To avoid interference with growth factors we used phenol red-free medium and the serum was replaced by 5% CS-FBS or totally removed (starvation medium). Before the medium change cells were washed with pre-warmed sterile PBS.

Total cell lysate and subcellular fractionation. To obtain the total cell lysate $3x10^6$ cells were plated in 10 ml complete medium in 100 mm Ø Petri dishes. After 24 h the cells were incubated with starvation medium + 5% CS-FBS with the different compounds (1 nM E2, 1 or 4 μM Tam and 50 ng/ml HRGß1). After 24, 48 and 72 h, the cells were lysed in RIPA buffer with protease inhibitors. Aliquots were stored at -70˚C until use. Subcellular fractions were prepared from $10x10^6$ cells treated with E2 and HRGß1. After 1, 4, 8 and 24 h the cells were scraped in ice-cold PBS and centrifuged at 293 x g for 5 min. The lysis was performed similar to the Cell-Lytic nuclear extraction protocol from Sigma. The cell pellet (≈100 μl) was incubated for 15 min with 500 μl 1X hypotonic buffer, pH 7.9 (HEPES 10 mM, $MgCl_2$ 1.5 mM, KCl 10 mM and protease inhibitor cocktail). The cells were observed under a microscope to detect cell swelling. The swollen cells were incubated with 0.6% of NP-40 (final concentration) and vortexed vigorously for 10 sec. The cell lysates were centrifuged at 11000 x g for 30 sec at 4˚C and the supernatant (cytoplasmic fraction) saved in a fresh tube and stored at -70˚C until use.

The cell pellet was washed 3 times with 500 μl hypotonic buffer and then diluted in extraction buffer (HEPES 20 mM, $MgCl_2$ 1.5 mM, NaCl 0.42 M, EDTA 0.2 mM, 25% glycerol, 0.1 M DTT and protease inhibitors). The tubes were then agitated in a vortex mixer for 30 min, centrifuged for 5 min at 20000 x g. The supernatant (nuclear fraction) was snap-frozen in aliquots and saved at -70˚C. Prior to electrophoresis, the protein concentration was estimated using the BCA protein assay from Pierce (Rockford, IL, USA).

Western blotting. The lysates were diluted in 3X SDS sample buffer (Tris-HCl 187.5 mM, pH 6.8, 6% SDS, 30% glycerol, 150 mM DTT, 0.03% bromophenol blue), and the same amount of protein/lane was loaded in a 4-20% gradient pre-cast gel (Criterion, Bio-Rad). For Western blot analysis, the proteins were transferred to PVDF membranes that were further incubated overnight with antibodies against the different proteins. To control the equal loading of the samples, the membranes were coated with anti-Akt (phospho-Akt), anti-ß actin (cytoplasmic fraction) and anti-PCNA (nuclear fraction) antibodies. Binding of the antibodies to the membranes was detected using a commercial ECL-Plus kit.

Immunofluorescence staining. Cells were grown in 8 wells chamber slides in 500 μl complete medium at a density of $4x10^4$ cells/well. After 48 h the cells were starved overnight and then incubated with HRGß1 -/+ E2 for 1, 4, 8 or 24 h. Fixation was performed in 4% paraformaldehyde for 5 min and the slides stored at -20˚C until all the incubation periods were completed. All the washing steps were performed in BSS-HEPES buffer/0.1% saponin to permeabilize the cell membrane. Non-specific antibody binding sites were blocked with 10% normal goat serum. Primary antibodies (mouse anti-p21 sc817 or mouse IgG negative control) at 2 μg/ml were incubated for 1 h at room temperature. Secondary antibodies conjugated with Alexa were used at 5 μg/ml and incubated for 30 min at room temperature. The fluorescent cells were visualised in a Nikon Eclipse E600/Nikon D-Eclipse C1 confocal fluorescence microscope using the EZ-C1 1.70.134 Software (Nikon Instech Co., Kawasaki, Japan). Cell nuclei were counterstained using To-Pro-3 reagent and the color reprogrammed by the same software to be blue.

Flow cytometric determination of S phase fraction and apoptosis. Cells growing at a density of $3x10^5$ cells/well in 6-well plates were incubated -/+ E2, HRGß1 and Tam. After 24, 48 and 72 h the medium from each well was saved, the cells were detached from the wells by trypsinization and pooled together with their respective medium. To measure the fraction

of cells in S phase (SPF) we treated the cells according to Vindelöv *et al* (29) and analyzed the samples by flow cytometry using a Becton Dickinson FACSCalibur (Becton-Dickinson, San José, CA, USA). SPF was calculated with the program ModFit LT for Mac software (Verity Software House Inc., Topsham, ME, USA). Fragments from cytokeratin 18 were used as an indicator of apoptosis. The cells were fixed in -20˚C cold methanol for 30 min and incubated for 1 h with the primary antibodies M30 or mouse IgG2b (1:250). The secondary antibody (RPE-conjugate used at 1:20) was incubated for 30 min and the samples were analyzed by flow cytometry. The CellQuestPro software was used to calculate the percentage of $M30^+$ cells: a marker was drawn in the control and the M30 histograms respectively. By subtracting the % of cells in the marked area of the control from the % of cells in the same area of the M30 sample we obtained the percentage of $M30^+$ cells.

Patients. In 1976, the Stockholm Breast Cancer Group initiated a trial to compare postoperative radiotherapy with adjuvant chemotherapy (30). The trial included premenopausal and postmenopausal patients with a unilateral, operable breast cancer. Using a 2x2 factorial study design, the postmenopausal patients were also randomized to tamoxifen treatment or no endocrine treatment. There was thus a total of four treatment groups: adjuvant chemotherapy, adjuvant chemotherapy plus tamoxifen, radiotherapy, and radiotherapy plus tamoxifen. Tamoxifen was given postoperatively at a dose of 40 mg daily for 2 or 5 years. Surgery consisted of modified radical mastectomy. The patients were required to have either histologically verified lymph node metastases or a tumor diameter, measured on the surgical specimen, exceeding 30 mm. Patient accrual started in November 1976 and ended in April 1990. The current study included a subset consisting of 280 postmenopausal patients for whom frozen tumor tissue was still available after hormone receptor assays had been performed in routine practice. This subset showed no bias in comparison with all the 679 postmenopausal patients in the trial in terms of tumor characteristics and treatment.

Radiotherapy was given with a high-voltage technique. The dose was 46 Gy with 2 Gy per fraction 5 days a week for a total treatment time of about 4.5 weeks. The target volume included the chest wall, the axilla, the supraclavicular fossa and the internal mammary nodes. For most of the patients randomized to chemotherapy, the treatment consisted of 12 courses of CMF according to the original Milan protocol (100 mg/m^2 cyclophosphamide orally at days 1-14, 40 mg/m^2 methotrexate intravenously on days 1 and 8, and 600 mg/m^2 5-fluorouracil intravenously on days 1 and 8). During the first 18 months of the trial, however, cyclophosphamide was replaced by 10-15 mg chlorambucil orally on days 1-8, and up to 18 months was allowed for the 12 courses to avoid dose reductions. The patients were followed for a median period of 11 years. A distant recurrence was registered in 128 patients.

Immunohistochemistry. Sections from frozen tumor tissue were fixed in cold acetone for 10 min, pretreated with 3% H_2O_2-methanol solution for 5 min to block endogenous peroxidase activity, and then incubated in 10% rabbit serum to prevent unspecific binding of the secondary antibody. The primary antibody against human p21 (5 μg/ml) was incubated at 4˚C overnight. Next day, the sections were incubated with a rabbit anti-mouse biotin conjugate (20 μg/ml) for 1 h followed by streptavidin-horseradish peroxidase (HRP) for 30 min. The immunoreactive sites were visualized with a solution of 3.3'-diaminobenzidine (DAB)-H_2O_2. The nuclei were counterstained with haematoxylin. All the washing steps were done in PBS-0.5% BSA. The slides were visualized in a Leica DM LS (Leica Microsystems; Wetzlar, Germany) microscope and the pictures taken with an Olympus camera model DP 11-P (Olympus optical Co. Ltd., Japan).

Evaluation and grading. Two independent observers performed the evaluation of the slides. The slide was analyzed if the section had enough tumor cells and the staining of the tumor cells was clearly distinctive from the background. From 280 tissue sections, 262 fulfilled these criteria. Positive sections were classified in two groups: those containing 1-25% and those with more than 25% of stained cells. Otherwise the section was classified as negative (<1%). Nuclear and cytoplasmic staining was scored independently.

Statistical anayses. The experiments of S phase fraction and apoptosis were done in triplicate and the statistical significance between the two groups of treatment was assessed by Student's t-test for independent samples. The relationship between the different variables in the clinical material was assessed by the χ^2 test or Spearman's rank correlation (when more than two groups were compared). The rate of distant recurrence in relation to tamoxifen treatment was estimated and tested by use of Cox's proportional hazards model. A test for the potential interaction between p21 localization and the benefit from treatment was performed by a Cox model including the covariates p21, treatment, and the interaction variable treatment x p21. The product-limit method was used for estimation of cumulative probabilities of distant recurrence-free survival. The criterion for statistical significance was $P<0.05$. All the procedures are included in the statistical package 'Statistica' (Statsoft, Inc. 1999 Statistica for Windows).

Results

Effect of HRGß1, Tam and E2 on Akt activation and p21 expression. HRGß1 activates Akt in MCF-7 cells. In Fig. 1A we can see that this activation lasted for 72 h and was not further improved in presence of E2 or affected by Tam. The levels of Akt protein remained constant. On the other hand, at 24 h we detected a certain decrease in the levels of p21 upon treatment with Tam and E2 (Fig. 1B) which was more clearly observed at 72 h. HRGß1 alone was able to stimulate p21 protein expression and this effect lasted for 72 h. Indicating also a possible stabilization of p21 upon HRGß1 treatment. Presence of Tam or E2 did not further change p21 levels upon HRGß1 treatment.

Subcellular localization of p21 upon treatment with HRGß1. We performed subcellular fractionation in order to localize p21 upon treatment with HRGß1 (Fig. 2). Cytoplasmic and nuclear fractions from cells treated with HRGß1 and E2 were analyzed by Western blotting after 1, 4, 8 and 24 h. In

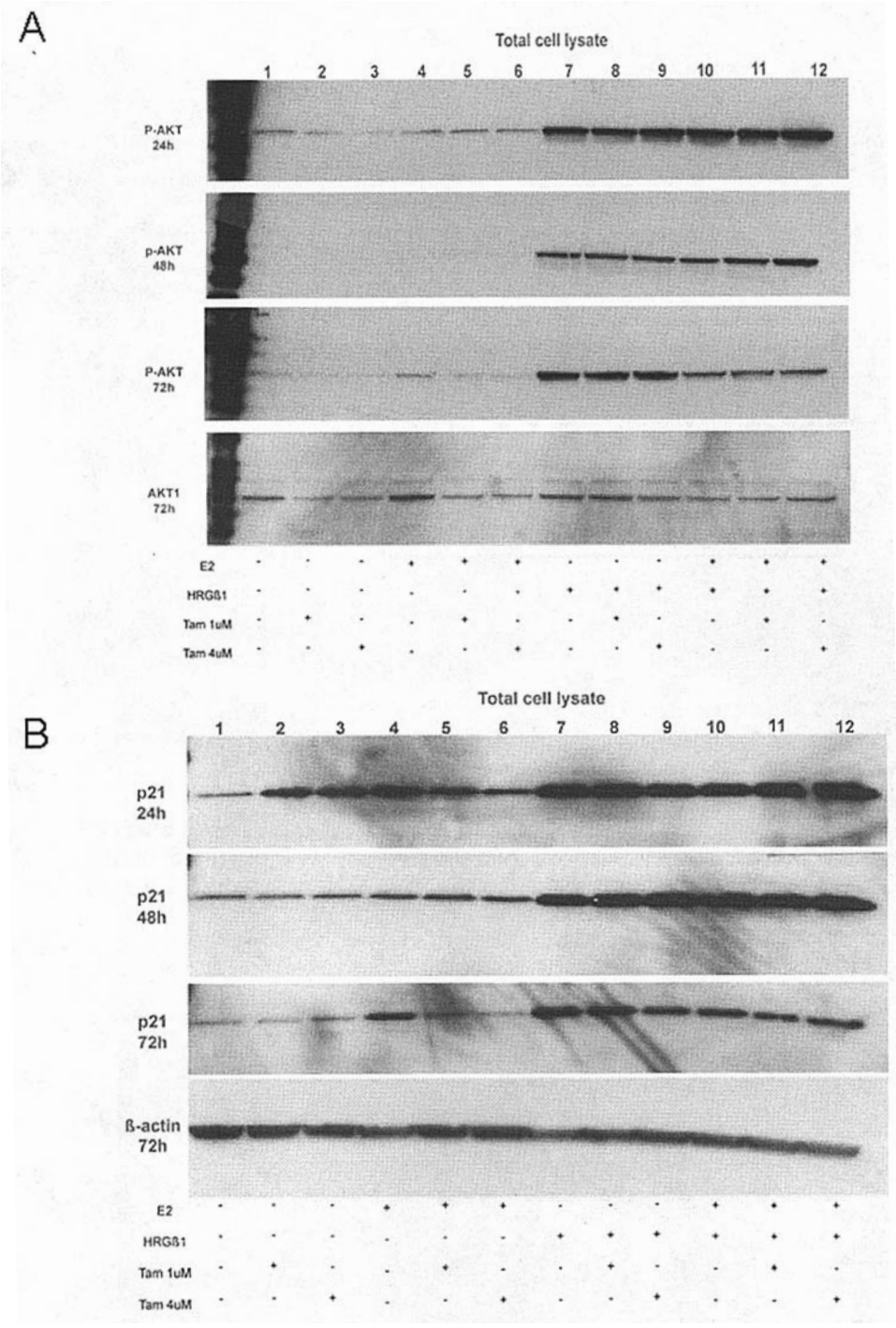

Figure 1. Expression of phospho-Akt, Akt (A) and p21 (B) in the total cell lysate of MCF-7. The cells were incubated for 24, 48 and 72 h in 5% CS-FBS without or with 1 or 4 μM Tam (lanes 1-3). Some cells were additionally treated with 1 nM E2 (lanes 4-6); 50 ng/ml HRGß1 (lanes 7-9) or HRGß1 + E2 (lanes 10-12). Staining against ß-actin helped to verify that equal amounts of proteins were loaded/lane. This figure represents one of two experiments.

absence of HRGß1 (only E2) we could observe that p21 was present in the nucleus and to some extend in the cytoplasm of MCF-7 cells. After 1-h treatment with HRGß1 the protein level increased in both fractions. However, at 24 h the levels of p21 appeared to be higher in the cytoplasmic fraction in comparison with the nuclear. The presence of p21 in the different cell compartments was visualized by immunofluorescence (Fig. 3). The cells were treated in the same way as before. After 1 h, in absence of HRGß (only E2), p21 was visualized in the nucleus but after addition of HRGß1 p21 also appeared in the cytoplasm. After 8 h most of the p21 is in the cytoplasm and at 24 h the protein still remained in this cell compartment. Upon treatment with HRGß1 in absence of E2 we could also observe p21 in the cytoplasm.

Effect of Tam and HRGß1 on SPF, and apoptosis. The percentage of cells in S-phase was significantly lower in the samples treated with Tam (1 and 4 μM) than in the untreated samples. In presence of E2 in the medium we obtained the same result (Fig. 4). The addition of HRGß1 stimulated cell

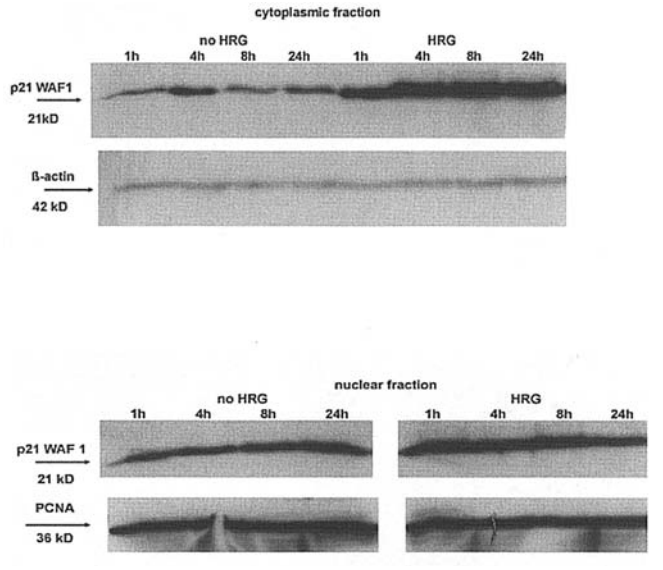

Figure 2. Expression of p21 in the nuclear and cytoplasmic fraction of MCF-7. MCF-7 cells were treated with E2 (1 nM) or with E2 + HRGß1 (50 ng/ml). After 1, 4, 8 and 24 h the cytoplasmic and nuclear fractions were isolated from the cell lysates. The levels of p21 in both fractions were assessed with the mouse anti-p21 (DCS60). To verify that equal amounts of proteins were analyzed the levels of ß-actin (cytoplasmic fraction) or PCNA (nuclear fraction) were also detected. This experiment was repeated 3 times.

growth and decreased the effect of 1 μM Tam on SPF (from 48 h in the absence of E2, Fig. 4B and C and at 72 h in the presence of E2, Fig. 4F). At the higher concentration of 4 μM Tam, the drug still had an effect independently of HRGß1 in the medium though the SPF is lower in the absence of the growth factor. On the other hand, we could not detect apoptosis at 24 h. However, in the absence of E2 the percentage of $M30^+$ (apoptotic) cells increased at 48 and 72 h upon Tam treatment (Fig. 5A-C). The addition of HRGß1 completely inhibited Tam-induced apoptosis at 48 and 72 h. The same trend was observed in the presence of E2, though not statistically significant (Fig. 5D-F).

Expression of p21 in tumors. Association with clinical variables. Two representative tumors showing nuclear p21 (N^+) and cytoplasmic p21 (C^+) staining are shown in Fig. 6A and B respectively. Nuclear p21 was found in 38 tumors (14.5%) while cytoplasmic p21 was positive in 70 tumors (26.7%). There existed no significant association between p21 and lymph node infiltration, tumor size or ER expression. Cytoplasmic p21 was more often visualized in $erbB2^+$ tumors but this association did not reach statistical significance. On the other hand, there was a strong association between pAKT and cytoplasmic p21 (P=0.00001). A weaker association, although statistically significant, was seen between pAkt and nuclear p21 (P=0.022) (Table I). Dividing p21 positive cases into subgroups (1-25% and >25%) did not provide further information.

Subcellular localization of p21 in relation with distant recurrence-free survival. Considering the four possible p21 phenotypes (N^-/C^-; N^+/C^-; N^-/C^+ and N^+/C^+), the worst distant recurrence-free survival was registered in the p21 N^+/C^- group (P=0.034) (Fig. 7). The 179 patients whose tumors were ER^+

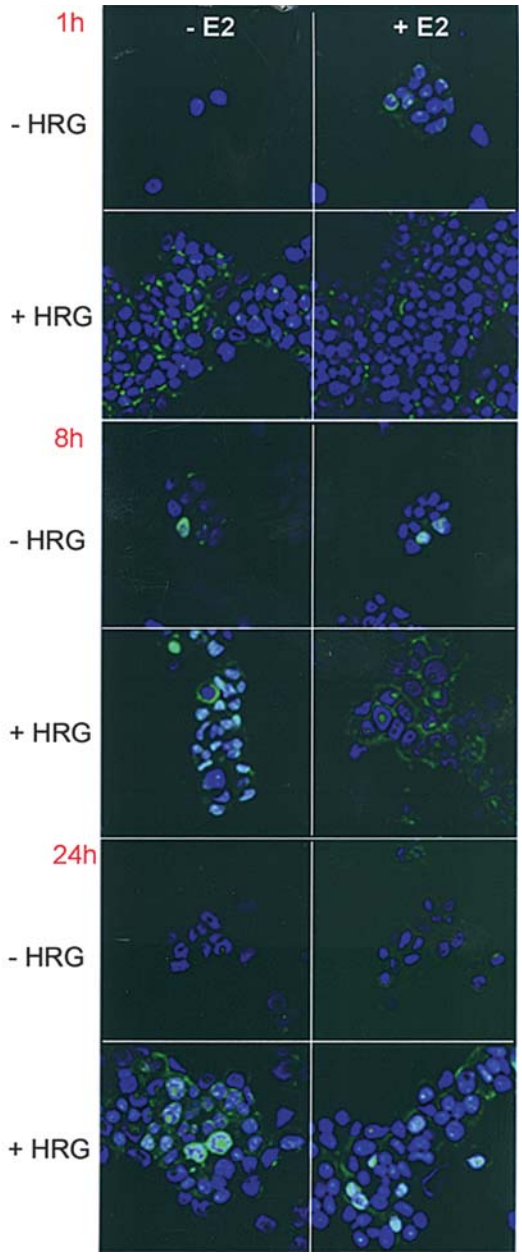

Figure 3. Immunofluorescence representing the cellular distribution of p21 in the cytoplasm and nucleus of MCF-7 cells. The cells were incubated without or with HRGß1 (50 ng/ml) for 1, 8 or 24 h. The left panel represents the cells incubated without E2 while the right panel represents the cells incubated with 1 nM E2. The expression of p21 was detected with the anti-mouse sc817 antibody at 2 μg/ml. Cell nuclei are shown in blue while p21 is shown in green. The slides were visualized with a magnification of x600.

were analyzed in order to see the effect of tamoxifen in relation to p21 location. Fig. 8 shows the distant recurrence-free survival for p21 N^+/C^- (A); p21 N^-/C^- or N^+/C^+ (B) and p21 N^-/C^+ (C). Patients with tumors classified as p21 N^+/C^- benefited significantly from tamoxifen (P=0.0082), as did the patients that had either the p21 N^-/C^- or N^+/C^+ phenotypes (P=0.034). In contrast the p21 N^-/C^+ group appeared not to respond to tamoxifen (P=0.7). The relative risk of distant recurrence for the different groups, comparing tamoxifen vs no tamoxifen, is shown in Table II. The interaction between the effect of tamoxifen and p21 localization was statistically significant (P=0.00095).

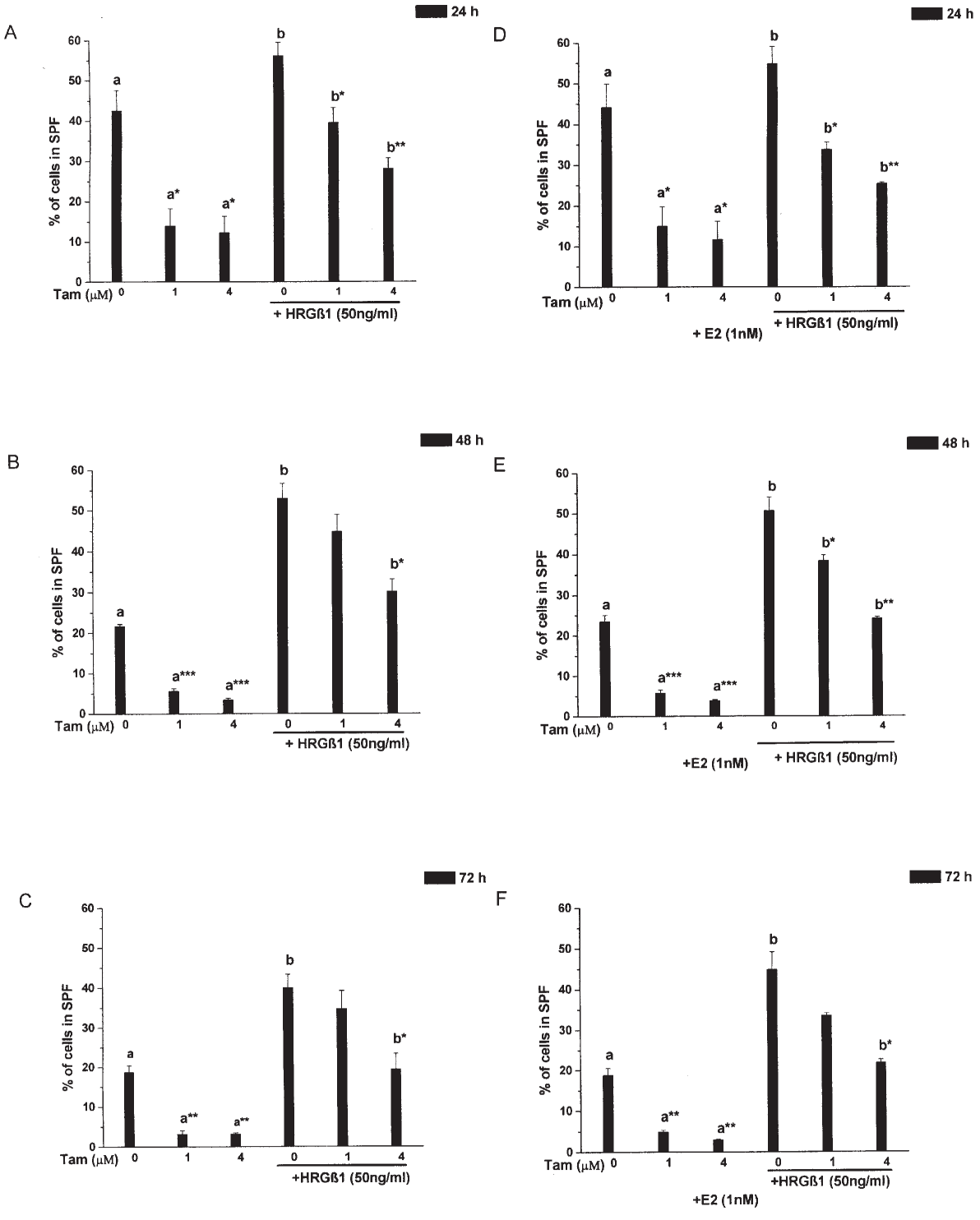

Figure 4. Percentage of cells in S phase determined by flow cytometry. The cells were treated with Tam (0, 1, 4 μM) without or with HRGß1 (50 ng/ml) (A-C) and with E2 or E2 + HRGß1 (D-F). The experiments were carried out for 24, 48 and 72 h. The percentage of cells in the S-phase was calculated using the program ModFit LT. The bars represent the mean of three independent experiments and the error bars represent SE. *P<0.05; **P≤0.005; ***P≤0.0005.

Discussion

The factors that control p21 subcellular location or the clinical significance of this protein are not completely known. Some authors have associated the nuclear localization of p21 with p53^{+} status and poor differentiation (31); others have found nuclear p21 to be inversely associated with p53 and a potential marker of good prognosis (32) yet other authors have found p21 as independent of any other established prognostic factor and irrelevant for clinical outcome (33). However, all of these studies have focused the analysis on nuclear p21 and have disregarded the cytoplasmic protein. Studies analyzing both the cytoplasmic and the nuclear p21, in a series composed of 73 patients, have found the cytoplasmic protein to be

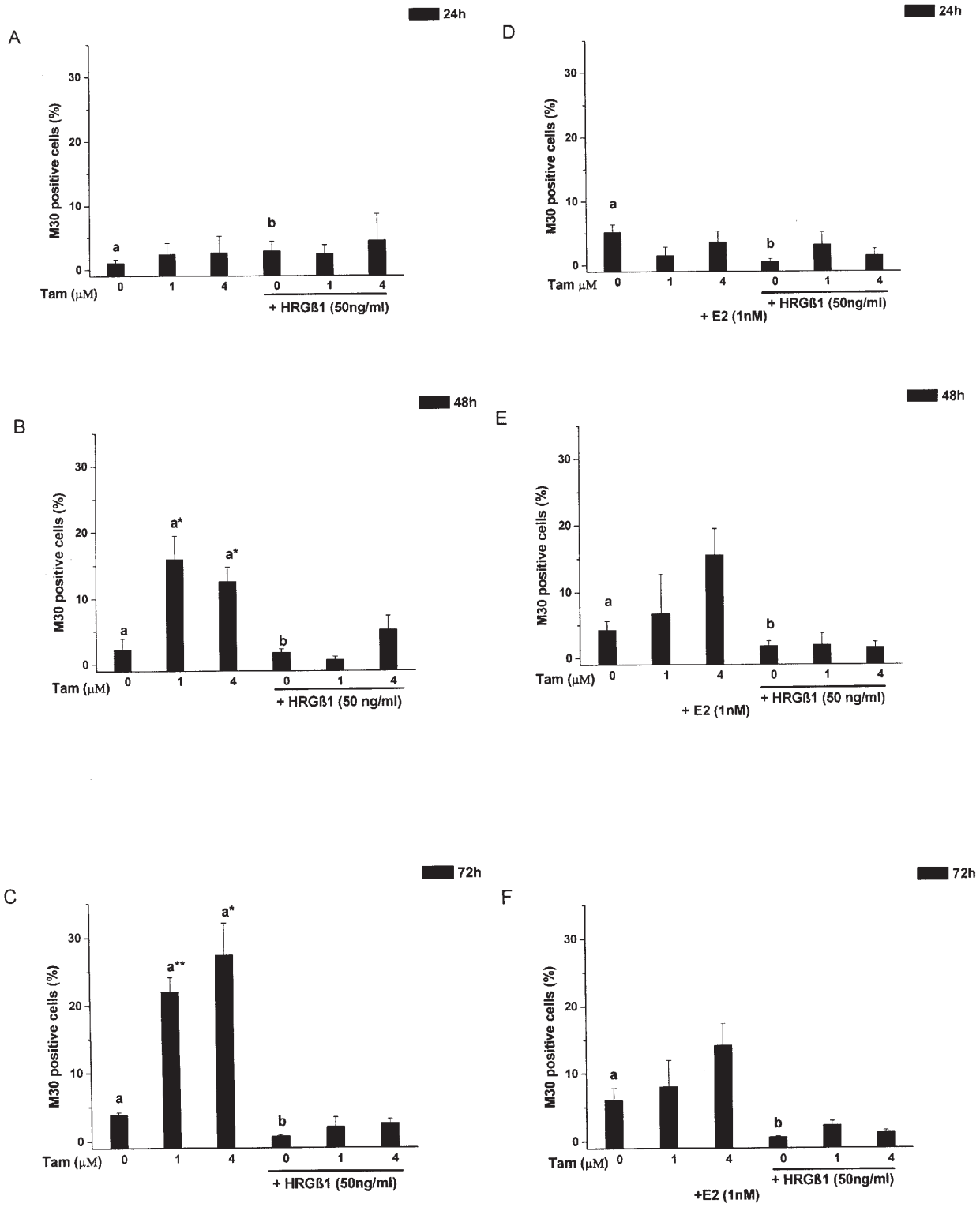

Figure 5. Apoptosis was measured by flow cytometry by detecting the fragments of cytokeratin 18 with the M30 CytoDEATH antibody. The percentage of $M30^+$ cells was calculated by the following formula: % $M30^+$ = % cells $_{M30}$ - % cells $_{IgG2b}$. The cells were treated with Tam (0, 1, 4 μM) without or with HRGß1 (50 ng/ml) (A-C) and with E2 or E2 + HRGß1 (D-F). The experiments were carried out for 24, 48 and 72 h. The bars represent the mean of three independent experiments and the error bars correspond to SE. $^*P<0.05$; $^{**}P\leq0.005$; $^{***}P\leq0.0005$.

associated with $p53^+$ status, erbB2 expression and being an independent predictor of poor recurrence-free and overall survival (34,35). Recently it has been shown that cytoplasmic localization of p21 correlates with phospho-p21, expression of erbB2 and Akt phosphorylation and all were associated with worse overall survival (36).

In this study we found that HRGß1 is able to induce p21 expression and cytoplasmic delocalization as well as to abrogate the effects of tamoxifen on p21 expression, cell proliferation and cell death. We also found that cytoplasmic p21 might have a clinical significance regarding response to tamoxifen. In the clinical material nuclear p21 and in

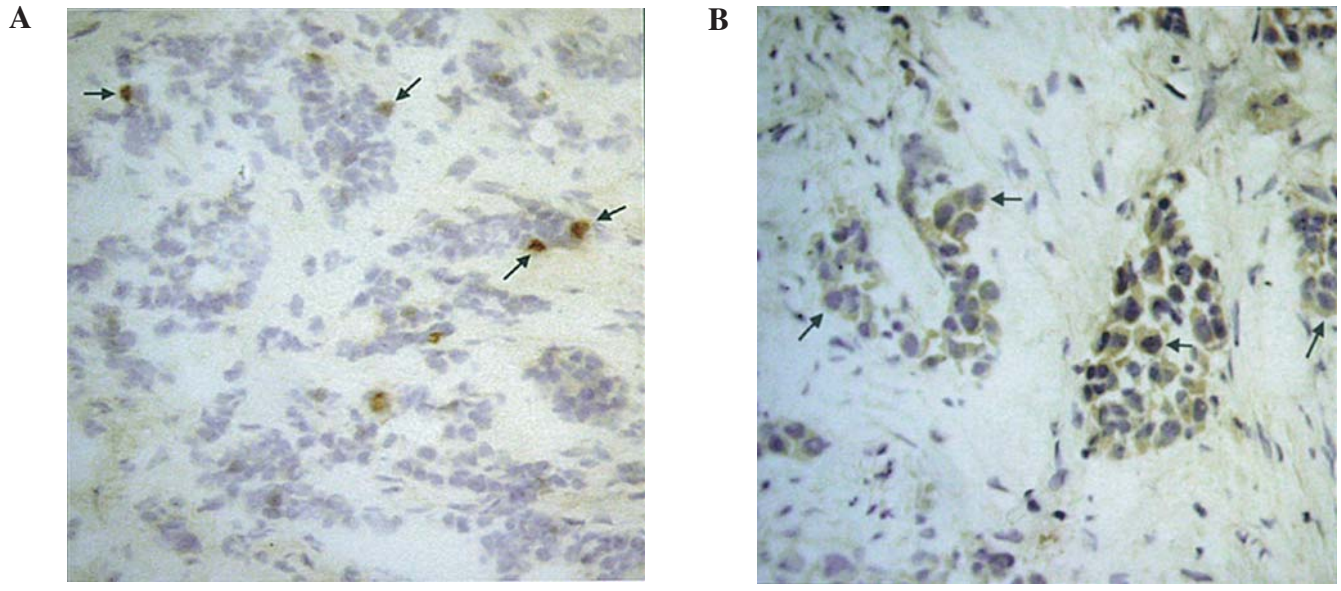

Figure 6. Immunohistochemical staining of two representative tumors showing expression of p21 in nucleus (A) and cytoplasm (B). Tumor frozen sections were stained with anti-p21 (OP64) at 5 μg/ml and visualized with an HRP-conjugate followed by DAB-H_2O_2. Nuclei were counterstained with haematoxylin. Original magnification x400. The arrows indicate the localization of p21.

Table I. Localization of p21 in nucleus and cytoplasm in relation to other variables.

	p21 N^- n (%)	p21 N^+ n (%)	p21 C^- n (%)	p21 C^+ n (%)
Nodal status/ tumor size (mm)				
N^-/>30	27 (93.1)	2 (6.9)	19 (65.5)	10 (34.5)
N^+/≤20	93 (84.6)	17 (15.4)	78 (70.9)	32 (29.1)
N+/>20	104 (84.6)	19 (15.4)	95 (77.2)	28 (22.8)
ER status				
-	71 (91.0)	7 (9.0)	57 (73.1)	21 (26.9)
+	150 (82.9)	31(17.1)[a]	133 (73.5)	48 (26.5)
erbB2 status				
-	163 (85.8)	27 (14.2)	143 (75.3)	47 (24.7)
+	56 (83.6)	11 (16.4)	46 (68.7)	21 (31.3)
Phospho-AKT				
-	165 (88.7)	21 (11.3)	151 (81.2)	35 (18.8)
+	57 (77.0)	17 (23.0)[b]	40 (54.1)	34 (45.9)[c]
Tamoxifen				
-	112 (83.6)	22 (16.4)	97 (72.4)	37 (27.6)
+	112 (87.5)	16 (12.5)	95 (74.2)	33 (25.8)

[a]P=0.089; [b]P=0.022; [c]P<0.0001. P>0.1 (not shown).

particular cytoplasmic p21 were associated with Akt activation. Moreover, cytoplasmic p21 indicated poor response to tamoxifen while patients with nuclear p21 did benefit from the same treatment.

It is believed that antiestrogens, including tamoxifen, decrease cyclin D1 expression thereby releasing p21 from cyclin D1-Cdk4,6 complexes. P21 is then free to associate with cyclin E-Cdk2 and to decrease its activity inhibiting

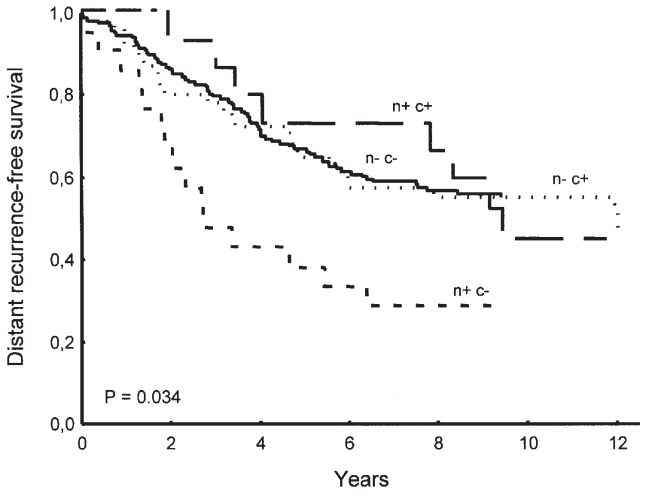

Figure 7. Kaplan-Meier curves comparing survival among the four possible p21 phenotypes. All the patients (n=262) were included in this analysis. The $p21N^+/C^-$ had the highest risk to develop distant recurrence in comparison with the other three groups (P=0.034).

Table II. The relative rate of distant recurrence, with versus without tamoxifen, for groups of ER-positive patients with different p21 localization patterns.

	Rate ratio (95% CI)	Significance	Test for interaction
p21n>p21c ($p21n^+c^-$)			
No tamoxifen	1.00		
Tamoxifen	0.06 (0.01- 0.49)	P=0.0082	
p21n = p21c ($p21n^-c^-$, $p21n^+c^+$)			
No tamoxifen	1.00		
Tamoxifen	0.55 (0.33-0.93)	P=0.034	P=0.00095
p21n<p21c ($p21n^-c^+$)			
No tamoxifen	1.00		
Tamoxifen	1.34 (0.44- 4.10)	P=0.70	

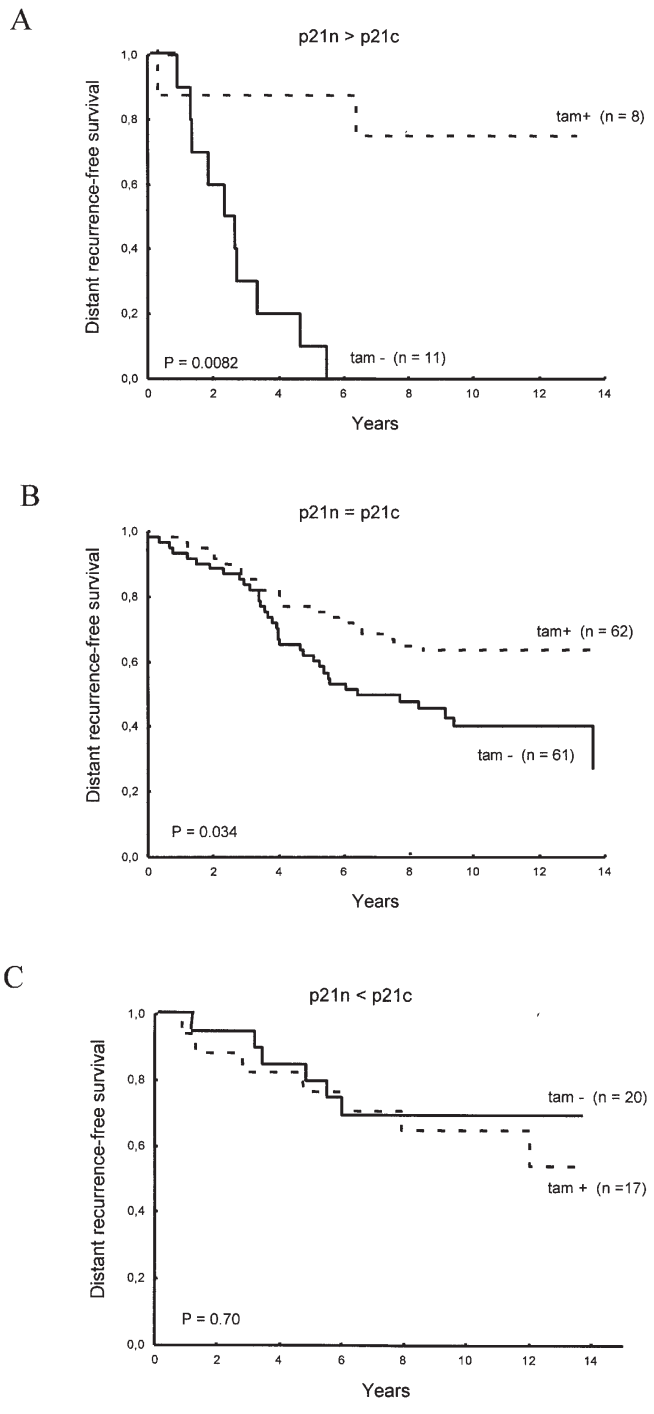

Figure 8. Distant recurrence-free survival for ER^+ patients (n=179) treated with tamoxifen or not. (A), Distant recurrence-free survival in the group p21n>p21c ($p21N^+/C^-$) (B), in the group p21n=p21c ($p21N^+/C^+$ or $p21N^-/C^-$), and (C), in the group p21n<p21c ($p21N^-/C^+$). The worst survival was registered among the $p21N^+/C^-$ that remained without endocrine treatment. The group of patients with $p21N^-/C^+$ tumors appeared not to benefit from treatment with tamoxifen.

G_1/S phase progression (37). Whether tamoxifen is able to up-regulate or down-regulate p21 protein expression is not clear. In a recent report, when the authors interrupted the mitogenic E2 signaling by treatment with tamoxifen, they observed inhibition of cell cycle progression at G_1/S phase together with an increase of p21 protein (9). Some other investigators have reported that accompanying the decrease in S phase, tamoxifen also decreased the p21 level by 50% compared to the control (37). We observed that by treating the cells with tamoxifen in the presence of E2, the levels of p21 in the total cell lysate decreased with increasing dose of the drug. This effect was observed at 24 h and it was even more pronounced at 72 h. Interestingly, the action of tamoxifen was abrogated when HRGß1 was added.

In agreement with previous studies showing that growth factors such as HRGß1 (38) and IGFI (39) are able to induce p21 expression and mediate cell proliferation, we found that HRGß1, at a dose that stimulates cell proliferation in MCF-7 cells, can induce p21 protein expression in the nucleus and the cytoplasm of these cells. By performing subcellular fractionation we could see differences in the expression of p21 in each cellular compartment. While p21 increased progressively in the cytoplasm from 1 to 24 h, the higher level of p21 in the nuclear compartment was reached at 4 h, declining after 8-h treatment, allowing us to believe that p21 could possibly accumulate in the cytoplasm under the effect of HRGß1. By immunofluorescence we could visualize the cells treated without or with HRGß1 in the absence or the presence of E2. We found p21 in both the nuclear and the cytoplasmic compartment after stimulation with HRGß1 independently of the E2 in the medium. There is an *in vitro* study that supports the role of HRG in regulating p21 expression and stabilization (40). Nevertheless these authors found that HRG-transfected cells only accumulate p21 in the nucleus. In their hands p21 nuclear accumulation abrogated the apoptotic effect of cisplatin.

Because the cytostatic effect of tamoxifen has been attributed to the inhibition of estrogen-dependent- proliferation we employed this drug in medium supplemented with E2. In order to study the individual effect of HRGß1 on tamoxifen actions we also used medium without E2. The experiments in this study were done using clinically achievable doses of tamoxifen and E2 (6). We assessed the cytostatic properties of tamoxifen by measuring the fraction of cells in S phase. At 24 h we found 67 and 71% reduction in SPF after treatment with 1 and 4 μM tamoxifen respectively (P=0.01 and P=0.009). The drug, as expected, had similar effect in the presence of 1 nM E2 (66%, P=0.02 and 73%, P=0.01). The addition of HRGß1 to tamoxifen treated-cells, partially abrogated the effect of tamoxifen since the % of proliferating cells decreased in comparison with the control. However, the response differed dependent on E2 in the medium. In the absence of hormone, the effect of 1 μM tamoxifen was counteracted by HRGß1 from 48 h while in the presence of E2 the same result was obtained at 72 h. The higher dose of tamoxifen (4 μM) still had an effect in the presence of HRGß1.

In addition, we aimed to explore the effect of HRGß1 on tamoxifen-induced apoptosis since this topic is not well elucidated. There are controversial reports on the ability of tamoxifen to cause apoptosis on MCF-7 cells (41,42). We found that tamoxifen at a dose of 1 and 4 μM induced apoptosis in MCF-7 as measured by the M30 cytoDeath assay. By using this method, we observed apoptosis from 48 h treatment with tamoxifen in the absence of E2. When E2 was added to the medium we did not register statistically significant increase in the percentage of apoptotic cells compared with the control though the level of apoptosis was higher in the tamoxifen-treated cells compared with the E2-treated cells. In agreement with our results, other authors have reported that micromolar concentrations of tamoxifen (up to 1 μM) caused apoptosis in MCF-7 cells and this type of cell death was inhibited by E2 (43,44). Whether the mechanism of tamoxifen-induced apoptosis is ER-dependent is also under discussion (45).

Interestingly, addition of HRGß1 counteracted this effect of tamoxifen even at its higher concentration. The mechanism behind the effects of HRGß1 could be related with its ability to activate signaling pathways such as PI3K/AKT and MAPK (27,46-48) that interfere with the effects of tamoxifen possibly by activating the ER. Taken together the results from the SPF and the apoptosis analysis allow us to speculate that HRGß1 could be the reason why some tumors that seem to respond to the cytostatic effects of tamoxifen still are able to survive its cytotoxic action relapsing later on.

The role of the induction/delocalization of p21 by HRGß1 in development of tamoxifen resistance is not documented in the literature. Other authors have reported that p21 gene expression can be induced by HRGß1 prior to S phase entry (49) and that HRG-transfected MCF-7 cells, with constitutive activation of MAPK and PI3K/AKT signaling pathways, accumulate p21 (40) but this only suggests a possible link between HRG-induced p21 expression/cytoplasmic localization and tamoxifen resistance.

We proceeded to investigate the role of p21 in clinical material. Both nuclear and cytoplasmic localization were detected in tumor cells while the stroma remained negative. The presence of the nuclear protein was seen in 14.5% of the cases while the cytoplasmic protein was present in 26.7% of the tumors. Another study reported a frequency of 21% for the nuclear p21 phenotype (34) but the results are difficult to compare with ours due to the use of different antibodies, detection systems and scoring applied to either frozen or paraffin-embedded tissue. We could not find a significant association between p21 and nodal status, tumor size or presence of ER. Cytoplasmic p21 was often registered among $erbB2^+$ tumors but this association was not significant. However, there was a strong association between cytoplasmic p21 and Akt phosphorylation and in the same material we previously reported a significant association between pAkt and $erbB2^+$ status (50).

The general idea of p21 as a cell cycle inhibitor has also changed. The nuclear protein has been found to facilitate the assembly and translocation of cyclin D-Cdk4,6 complex to the nucleus (51,52), to be associated with poor differentiation (31) and even with poor disease free-survival among patients that received CMF (53). In our study we found that, among the four p21 phenotypes, the $p21N^+/C^-$group registered the worst distant recurrence-free survival. Since the patients comprised in this study were randomized to different treatments we cannot conclude at this stage which is the therapeutic regime that fails in the presence of this p21 phenotype. We could not detect any obvious interaction between p21 and the benefit from CMF versus radiotherapy (data not shown).

To analyze the relevance of p21 regarding response to tamoxifen, we selected the ER^+ cases. Some authors had previously reported that nuclear p21 expression was significantly associated with longer breast cancer related survival in a subgroup of patients with hormone receptor positive tumors under antiestrogen treatment (31). We found that the $p21N^+/C^-$ group indeed benefited from tamoxifen but in the absence of drug these patients presented the worst outcome in comparison with the other groups. The presence of p21 in the nucleus does not always represent a low proliferative status as we have discussed before, the question is whether

there exists a specific interaction between tamoxifen and the nuclear p21 that makes this group so drug-dependent to survive. We know from studies *in vitro*, that tamoxifen is able to down-regulate cyclin D1 expression while E2 exerts the opposite effect (54). In an ER^+/tamoxifen responsive tumor, under treatment with tamoxifen, the levels of cyclin D1 should be expected to decrease making possible for p21 to join and inactivate the cyclin E-Cdk complex leading to cell cycle arrest. On the other hand, in a scenario where tamoxifen is not present, p21 would be free to join and stabilize the cyclin D1 complex while the free cyclin E-Cdk complex will drive the cell cycle progression. In agreement with this speculation, it has been reported that the elevation in cyclin D1 and D3 overcomes the inhibition of cell cycle progression by p21 in MCF-7 cells (55). Other authors have found that p21 functions as an enhancer of the ERα activity (56).

In contrast, we found that the group of patients with the $p21N^-/C^+$ phenotype did not benefit from tamoxifen although we have to consider that the number of patients was small. There is still no report explaining how cytoplasmic p21 could affect the response to tamoxifen. However, it is believed that the cytoplasmic protein can mediate cell survival through binding and inactivation of the pro-apoptotic protein ASK1 (19). The ASK1 protein acts upstream of a chain that leads to activation of stress-activated protein kinases (SAPKs), such as p38 MAPK (57). These kinases are preferentially activated by various cytotoxic stresses, which are able to trigger apoptosis and one of these is the treatment with tamoxifen (58).

The results achieved in this study indicate that HRGß1 could be a survival factor since it partially reduces the cytostatic effect of tamoxifen while its main role consists in keeping the cells alive. HRGß1 is not only able to induce p21 expression but also might contribute to p21 cytoplasmic accumulation. The role of p21 in the cytoplasm was further explored in a clinical material. The clinical findings showed a significant association between nuclear and cytoplasmic p21 and Akt activation. Furthermore, we were able to identify 3 subgroups of ER^+ patients based on the different p21 phenotypes: the ones that are likely to respond to tamoxifen ($p21N^+/C^+$ or $p21N^-/C^-$), the ones for whom the use of tamoxifen is crucial ($p21N^+/C^-$) and the ones that are not likely to benefit from this treatment ($p21C^+/N^-$). Whether HRGß1 induces p21 expression and delocalization through the PI3K/Akt signaling pathway and whether and how HRGß1-induced p21 can impair the effect of tamoxifen remain to be answered. We can suggest that p21 is an important factor in unravelling the complex problem of tamoxifen resistance.

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