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Relationship between p53 and p27 expression following HER2 signaling

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Summary HER2, frequently associated with low p27 expression in breast tumors, when activated has been found to upmodulate p53 in tumor cells. The aim of this work was to investigate the role of p53 in the connection between HER2 and p27. Fifty-two breast tumor specimens, characterized for p53 mutations, were analyzed immunohistochemically (IHC) for HER2, p53 and p27 expression. p27, inversely associated with HER2, was found in 29% of tumors with IHC-negative mutated p53 versus 93% of tumors with accumulation of p53 protein and 59% with wild-type p53 ($p = 0.001$), indicating a direct association between p53 and p27 expression. HER2-overexpressing cell lines carrying wild-type or null p53 protein, and treated with heregulin $\beta 1$ (HRG), were analyzed for expression and subcellular localization of p53 and p27. In HER2-overexpressing cells stimulated with HRG, p27 protein expression increased in parallel with p53 with no corresponding increase in p27 transcript. No p27 increase was observed in p53-null cells. Transfection with wild-type p53 restored p27 upmodulation in HRG-stimulated cells, indicating a crucial role of p53 in determining p27 upmodulation following HER2 activation. Together, our data demonstrate the crucial role of p53 in determining p27 upmodulation following HER2 activation. This could have implications in the response to Transtuzumab therapy.
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Introduction

HER2 is a member of the epidermal growth factor receptor (EGFR)-related family of tyrosine kinase receptors comprised of HER1, -2, -3 and -4.[1] HER2 overexpression is a frequent event in several human cancers and has been correlated with poor prognosis.[2,3] However, several lines of evidence demonstrate that, under certain conditions, activation of these receptors leads directly to the apoptotic death of the cell.[4,5] HER2 receptor activation has been found to upmodulate expression of p53,[6,7] a major determinant of growth, differentiation and induction of apoptosis, i.e., wild-type p53 leads to apoptosis/differentiation, whereas mutated p53 leads to proliferation.[7,8] It has also been shown that heregulin β1 (HRG) stimulation of HER2-overexpressing cells leads to enhanced c-Myc protein synthesis through activation of the PI3K/Akt/mTOR pathway,[9] which, in turn, might act as a transcriptional factor for p53 and lead to its upregulation. The association of HER2 overexpression with proliferation in cell lines bearing a mutated p53 gene is consistent with the frequent overexpression of HER2 in breast tumors with p53 alterations.[10] However, HER2 overexpression in p53-wild-type tumors suggests the presence of other alterations related to the apoptotic/cell cycle pathway in tumor cells that allow the shift from apoptosis to proliferation.[7,11] One possible candidate is an altered p27^{kip1} (p27) gene, a member of the cyclin-dependent kinase inhibitor (CDKI) family and encoding a putative tumor suppressor. Many human tumors exhibit a variable loss of p27 protein and such loss has been described as an independent prognostic factor in various human cancers, including breast, colon, and prostate adenocarcinomas.[12] On the other hand, loss of heterozygosity of the 12p13 locus encompassing the p27 gene as well as p27 gene biallelic losses or mutations are very rarely found in breast carcinomas.[13] Thus, the decrease in p27 levels in some human tumors has been related to increased and deregulated degradation. In fact, Akt-mediated phosphorylation of p27 reportedly impairs its nuclear import and leads to cytoplasmic p27 accumulation, functionally inactivating the growth inhibitory properties of p27 and favoring the proliferation of breast cancer cells.[14–16]

Decreased immunoreactivity of the p27 protein in patients with early-stage breast carcinoma correlates with HER2 overexpression and with benefit from a single course of perioperative chemotherapy in patients with lymph node negative-status.[17] It has been demonstrated that induction of p27 protein is one of the key mechanisms of action of HER2-targeting antibodies.[18,19] It is therefore of interest to elucidate the pathways downstream HER2 involved in the regulation of p27.

In this study, we examined the relationship among HER2, p53 and p27; immunohistochemical (IHC) analysis of human breast tumor tissue revealed a direct association between p53 and p27 expression. In vitro studies confirmed the importance of this correlation, given that following HER2 activation, p53 expression upmodulates in parallel with p27 induction in cellular models expressing wild-type p53.

Materials and methods

Patients

Breast carcinoma specimens were obtained from patients surgically treated at National Cancer Institute of Milan in 2002. Primary tumor diameter, axillary nodal status, histological grade, and hormone receptors status were obtained from histological reports. Patients characteristics are: median age 59 (range 38–87); size >2 cm, 79%; grade III, 45%; node positive, 62%; hormone receptors positives, 73%.

Immunohistochemistry (IHC)

IHC staining of paraffin-embedded tissue was carried out using the following monoclonal antibodies: anti-HER2 reagent included in the HercepTest™ (Dako), with HER2 overexpression interpreted according to the supplier's instructions, it was considered as positive, samples scored 2+ and 3+; anti-p53 DO7 (1:500 dilution; Visionbiosystems, Newcastle-upon Tyne, UK), with staining of $\geqslant$10% nuclei considered positive; and anti-human p27 clone SX53G8 (1:100 dilution, Dako), considering low and high immunoreactivity, respectively, as nuclear staining in $<$50% and $\geqslant$50% of tumor cells. The association analysis of p27 with HER2 or p53 was based only on the downmodulation of p27 nuclear staining since cytoplasmic localization of p27 was observed in 58% of cases with low expression in nuclei and in 60% of cases with high nuclear expression.

Fluorescence in situ hybridization (FISH)

All HER2 2+ cases were evaluated by FISH using the PathVysion HER-2 DNA Probe Kit (Vysis, Downers Grove, IL, USA) according to the manufacture's recommendations. Samples were stained with DAPI, coverslipped, and analyzed with a Zeiss

Axioscop 2 microscope (Carl Zeiss, Milano, Italy). Images were acquired using FISHVIEW (ver 4.0, ASI, Vista, CA, USA).

Cells and culture conditions

Ovarian carcinoma cell line IGROV1 and the HER2-transfectant cell line IGROV1/HER2 have been described.[7] Ovarian carcinoma cell line SKOV3 were purchased from ATCC. Cells were maintained at 37 °C in a humidified atmosphere (5% CO_2 in air) in RPMI-1640 medium (Sigma-Aldrich s.r.l., Milan, IT) supplemented with 10% heat-inactivated FCS (HyClone) and 2 mM L-glutamine (Sigma-Aldrich s.r.l.). IGROV1/HER2 cells were cultured as above, except that the culture medium contained G-418 (200 μg/ml).

SDS-PAGE and western blotting

Cells were treated for the indicated times with 20 ng/ml HRG (NeoMarkers, Fremont, CA) in complete medium, or left untreated (controls), harvested by scraping and lysed in SDS buffer (62.5 mM Tris-HCl, pH 7.4, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue). Lysates were cleared by centrifugation. Protein concentration was determined by the BCA protein assay (Pierce Biotechnology, Inc. Rockford, IL). For each sample, up to 50 μg of total protein extract was fractionated by SDS-PAGE as described[20] and blotted onto an Immobilon-P transfer membrane (Millipore Corp., Bedford, MA). After blocking for 1–2 h in 5% skim milk (Merck KGaA, Darmstadt, DE) in Tris-buffered saline containing 0.1% Tween-20 (TBS-T), blots were probed with the following antibodies for 1 h at room temperature: clone sc-12352-R (Santa Cruz Biotecnology, Santa Cruz, CA) at 1:5000 to detect P-HER2, DO7 (Visionbiosystems) at 1:1000 dilution to detect p53 protein; clone C19 (Santa Cruz Biotecnology, Santa Cruz, CA) at 1:200 dilution to detect p27; and clone AC40 (Sigma-Aldrich) at 1:500 dilution to detect actin. Blots were incubated with peroxidase-conjugated secondary antibodies raised against mouse or rabbit immunoglobulins and visualized using the ECL detection system (Amersham Biosciences) according to the supplier's instructions. All experiments were repeated at least two times.

Northern blotting

Total RNA was isolated as described[7] from HRG-treated cells (10 ng/ml HRG in complete medium) or untreated cells. Each sample (10 μg) was analyzed by Northern blotting using the following [^{32}P]dCTP-labeled (Amersham Biosciences, Cardiff, UK) random-primed (Roche, Mannheim, Germany) probes: full-length p53 cDNA, obtained by BamHI digestion of pC53-SN3[21]; and a 613-bp region of p27 obtained by PCR amplification from IGROV1/HER2 cDNA (forward primer: aaagatgtcaaacgtgcgagt, reverse primer: aattcgagctgtttacgtttg). After stripping, membranes were hybridized with a control [^{32}P]dCTP-GAPDH probe (full-length coding sequence). Densitometric analysis was performed by phosphorimager scanning using the ImageQuant System (Molecular Dynamics, Sunnyvale, CA). Band intensity was expressed as a proportion of the GAPDH value.

Indirect immunofluorescence and confocal microscopy

Cells were seeded on glass coverslips, allowed to attach, and treated with 10 ng/ml HRG in complete medium for 30 min, 1, 2, 4 and 6 h or left untreated. After treatment, cells were fixed with 3.7% formaldehyde in the medium, saturated with 3% BSA and permeabilized with 0.2% Triton-X100 in PBS. Cells on coverslips were then washed with PBS, incubated for 1 h at room temperature with anti-p53 (DO7, 1:100) or anti-p27 (clone C19, 1:200) primary antibody diluted in PBS 0.02% Triton-X100, and incubated for an additional 45 min with specific secondary FluoroLink Cy™2-conjugated goat anti-mouse or FluoroLink Cy™3-conjugated goat anti-rabbit antibody (Amersham Biosciences) (1:1000 in the same buffer as for primary antibody). After three washes with 0.02% Triton-X100 in PBS, coverslips were mounted on glass slides using Mowiol (Calbiochem, San Diego, CA) and examined under a confocal microscope (Microradiance 2000; Bio-Rad Laboratories Inc., Hercules, CA) equipped with Ar (488 nm) and HeNe (543 nm) lasers. All images were taken using a 60 × oil immersion lens (512 × 512 pixels), with laser intensity or electronic gain kept constant through the time course. Images of SKOV3-mock cells images were obtained using a 60 × objective and an inverted microscope connected to a NIKON DXM camera. All confocal images were processed using the Image PC software package and analyzed using Lasersharp 2000 software. Control cells were exposed to relevant secondary antibodies alone and showed no significant degree of labeling. For co-localization experiments, cells were incubated with anti p53 antibody for 1 h, the anti-p53 was drawn away and anti-p27 was added. After three washes, cells were incubated for 45 min with

secondary antibodies (FluoroLink Cy™2-conjugated goat anti-mouse and FluoroLink Cy™3-conjugated goat anti-rabbit antibody, Amersham Biosciences) (1:1000 in the same buffer as for primary antibody) added simultaneously, and processed as described above.

Florescence intensity has been quantified using Image-Pro Plus 5.0 program (Media Cybernetics, Buckinghamshire, UK) by sampling an equal portion of 2–4 representative labeled cells at every time.

Transient tranfection

SKOV3 cells were transfected with p53 construct using the calcium-phosphate method essentially as described.[22] Briefly, transfection was carried out in Dulbecco's modified Eagle's medium (Sigma-Aldrich) supplemented with L-glutamine, antibiotics and 10% heat-inactivated FBS. $CaPO_4$-DNA precipitate containing 0.75 μg of p53 expression plasmid (pC53-SN3)[21] or empty vector (SKOV3/mock) was added. After 16 h, cells were gently washed with RPMI 1640, incubated with fresh RPMI 1640 containing 10% FBS, and treated as described for non-transfected cells.

Apoptosis assay

Apoptosis in transfected SKOV3 cells was evaluated by blinded counting apoptotic cells in the same samples prepared for confocal analysis and observed with an inverted fluorescence microscope. Percent apoptotic nuclei was calculated based on analysis of 100 transfected cells for each determination.

Results

Expression of HER2, p53 and p27 was investigated in 52 breast carcinoma archival specimens already characterized for p53 molecular status.[23] A total of 35% of samples were scored by Herceptest as 0, 15% as 1+, 19% as 2+ and 31% as 3+; cases 2+ were further analyzed by FISH and none resulted amplified and therefore considered as HER2 negative. In summary, 16/52 cases (31%) are HER2 positive and 36/52 (69%) HER2 negative. 13/52 (25%) of samples were scored as p53 positive and 33/52 (64%) as p27 high expressors. In our IHC series, p27 in the cytoplasm was observed at the same frequency in both the low and high p27 expressors, in keeping with a previous report of cytoplasmic p27 in 44% and 36%, respectively.[15]

Expression of p27 was significantly associated with HER2 expression: 9 of 19 low p27-expressing tumors (47%) were HER2-positive, whereas 7 of 33 tumors (21%) with high p27 expression were HER2-positive ($p = 0.05$).

Analysis of p27 immunostaining according to p53 status (Table 1), revealed a significant association between p27 positivity and elevated p53 protein expression (IHC-positive staining), since 93% of tumors that were p53-positive by IHC (either wild-type or mutated p53) were also positive for p27 expression; wild-type p53 but IHC-negative p53 tumors were positive for p27 expression in 59% of the cases, whereas only 29% of the mutated p53 tumors that were negative by IHC were found to express p27. These data suggest that high levels of p27 depend on p53 expression. It is noteworthy that 6 of 7 mutated p53 samples that were p53-negative by IHC were HER2-positive.

Table 1 Evaluation of p27 in relation to HER2 and p53.

p27-high cases/total (%)				
p53		HER2-pos. group	HER2-neg. group	Total
IHC	Mol. status*			
Pos.	WT	1/2 (80)	6/6 (100)	7/8 (87)
	Mut	1/1 (100)	4/4 (100)	5/5 (100)
	Total	2/3 (67)	10/10 (100)	12/13† (93)
Neg.	WT	3/7 (43)	16/25 (64)	19/32† (59)
	Mut	2/6 (33)	0/1 (0)	2/7† (29)
	Total	5/13 (40)	16/26 (61)	21/39 (53)
	Total	7/16 (44)	26/36 (72)	33/52 (64)

IHC analysis of HER2, p53 and p27 expression in 52 breast carcinoma archival specimens.

*Evaluated by APEX assay.

†$p = 0.01$ by Chi-square test.

To determine whether activation of the HER2 pathway involving p53 induction leads to variations in p27 expression levels, cells were treated with HRG in a time course experiment and analyzed by Western blot. HER2 activation was demonstrated by means of anti Phospho-HER2 antibody, which reaches its highest value in the first 30 min. Levels of p53 and p27 expression were increased 2.7- and 4.4-fold, respectively, after 30 min of HRG exposure in IGROV1 cells carrying wild-type p53 and transfected with HER2 (IGROV1/HER2) (Fig. 1, panel A).

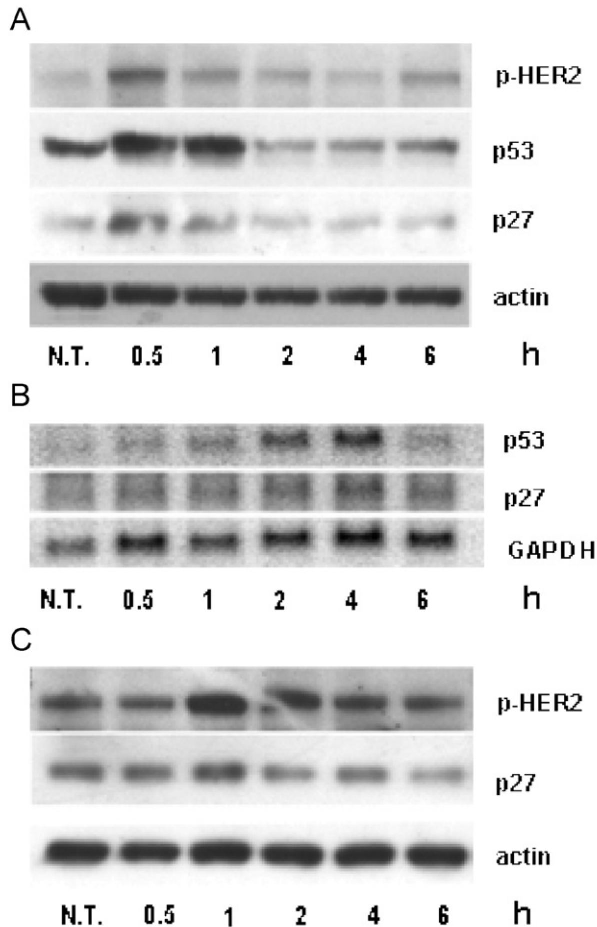

Figure 1 p53 and p27 expression over time in HRG-treated cells. (A) Western blot analysis of p-HER2, p53 and p27 protein expression in IGROV1/HER2 cells treated with HRG for the indicated times or left untreated (N.T.). Total protein extract (30 μg) was loaded in each lane. (B) Total cellular RNA was isolated from IGROV1/HER2 cells treated with HRG in complete medium for the indicated times, or left untreated (N.T.), and 10-μg aliquots were analyzed by Northern blotting, using ^{32}P-labeled full-length p53 cDNA or ^{32}P-labeled PCR product of a 613-bp region of p27 cDNA as probes, or [^{32}P]dCTP-GAPDH cDNA for RNA loading normalization. (C) Western blot analysis of p-HER2 and p27 protein expression in SKOV3 cells treated with HRG for the indicated times or left untreated (N.T.).

Northern analysis to determine whether the protein induction of both molecules corresponded to increased transcriptional activity in HRG-stimulated IGROV1/HER2 cells revealed no increase in p53 or p27 mRNA after 30 min of treatment, a small increase of 1.5-fold after 1 h for p27 mRNA, and a marked 2.8-fold increase for p53 after 4 h of treatment (Fig. 1, panel B). By contrast, Western analysis of protein in HER2-expressing but p53-null SKOV3 cells revealed no significant increase in p27 expression during the time course of HRG treatment (Fig. 1, panel C) although HER2 activation was achieved after 1 h of treatment.

Indirect immunofluorescence and confocal microscopy of HRG-stimulated IGROV1/HER2 cells indicated a nuclear localization of p53 throughout the treatment time, with a clear increase in p53 staining after 30 min of HRG treatment (Fig. 2, panel B) raising from 80.39125$\pm$13.93736 pixel mean$\pm$SE at $t=0$ to 161.0816$\pm$20.09902 at $t=30$ min, and observed at all times analyzed (133.1177$\pm$19.05844 pixel mean$\pm$SE at 6 h). Levels of p27 expression were also increased upon HRG stimulation, both at the nuclear and cytoplasmic levels, with no evidence of cytoplasmic relocalization even after 6 h of HRG stimulation. Fluorescence intensity was peak at 30 min (Fig. 2, panel E) (176.1583$\pm$24.50325 pixel mean$\pm$SE), decreasing to levels comparable to those in untreated cells (75.79888$\pm$15.4249 pixel mean$\pm$SE) or after 6 h of HRG treatment (panels D and F, respectively) (50.28491$\pm$12.46262 pixel mean$\pm$SE).

To verify the role of p53 in induction of p27, SKOV3 cells were transiently transfected to express wild-type p53, stimulated with HRG, and analyzed by indirect immunofluorescence/confocal microscopy (Fig. 3). The analysis of SKOV3/mock-transfected cells (panels A–C) revealed no variation either in quantity or in localization of p27 after treatment. When p53-transfected SKOV3 cells were stained for p53 (panels D–I) (134.474$\pm$20.99084 pixel mean$\pm$SE at $t=0$, a marked fluorescence increase was observed at 30 min of HRG treatment, which was maintained throughout the observation times. Figure 3E shows two representative fields of p53-transfected SKOV3 cells treated for 2 h with HRG (252.9798$\pm$0.109903 pixel mean$\pm$SE at $t=2$ h and 248.4162$\pm$9.248038 pixel mean$\pm$SE at $t=6$ h). Analysis of p27 revealed an increase that paralleled the p53 increase (panel H) (53.18497$\pm$12.2324 pixel mean$\pm$SE at $t=0$, 212.262$\pm$12.72438 pixel mean$\pm$SE at $t=2$ h and 70.32755$\pm$15.51854 pixel mean$\pm$SE at $t=6$ h). Stimulation of the HER2 receptor led not only to p53 increased expression, but also to p53 activation, as indicated by an increase in percent of

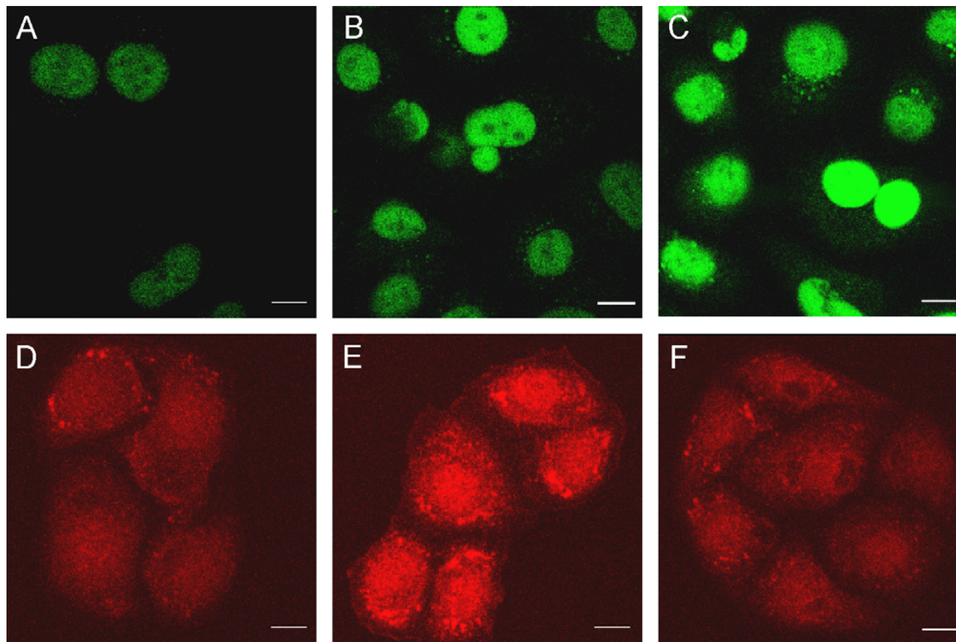

Figure 2 Indirect immunofluorescence and confocal microscopy of p53 and p27 expression in HRG-stimulated IGROV1/HER2 cells. Localization of p53 (green) and p27 (red) was examined in cells grown on glass coverslips and treated for 30 min (panels B and E) or for 6 h (panels C and F) with HRG or left untreated (panels A and D). Bar = 10 μm.

apoptotic cells observed during the time, i.e., from 22% after 24 h from wild-type p53 transfection to 77.5% after 6 h of HRG treatment (Fig. 4).

Discussion

The HER network is a powerful mechanism controlling cell fate through subtle regulation. Deregulation of this network is a common finding in human cancers, and alterations in the expression of the cell cycle inhibitor p27 might be one of the factors involved. Consistent with previous data,[17,24] we observed a significant inverse correlation between p27 and HER2 expression in archival breast tumor specimens.

We also observed a direct correlation between p27 expression and the presence of the p53 protein, i.e., a low frequency of p27 expression (29%) in tumors with IHC-negative mutated p53, but intense IHC reactivity for p27 in almost all samples with p53 protein overexpression. These data suggest that the presence of p53, independently of the mutational status, plays a role in p27 regulation.

Our in vitro experiments showing that p53 upmodulation by HRG-mediated HER2 activation correlates with increased p27 expression support this notion. Accordingly, this increase was not observed in p53-deficient SKOV3 cells, but transfection of these cells with wild-type p53 led to p27 induction after HRG stimulation. The increase in both p53 and p27 protein levels upon HER2 stimulation of cells harboring wild-type p53 suggests a tight link between p53 and p27 in response to growth factor receptor activation. This finding raises the possibility that p27 upregulation requires p53 protein accumulation in the nucleus. During the development, the HER family of tyrosine kinase receptors mediates different activities including proliferation, differentiation and apoptosis. The ability to promote different cellular responses appears to seat in a complex protein network, which acts through and activates several pathways, and might underlie the multifaceted role of this receptor family in physiological cellular regulation and in carcinogenesis. In our experimental model a "*near-physiological*" HER2-p53 cross-talk has been reproduced since in both IGROV1/HER2 and SKOV3 cells, bearing endogenous and transfected wild-type p53, respectively, HER2 stimulation with HRG led to inhibition of proliferation and/or induction of apoptosis. The majority of p53 mutations in HER2-positive tumors showed IHC-negative feature. This might be the result of selection of tumor cells carrying both HER2 amplification and p53 stop codon mutation, since other mutations leading to accumulation of p53 may impair tumorigenicity by upregulating p27. However, the existence of tumors overexpressing HER2 with p53 accumulation in the

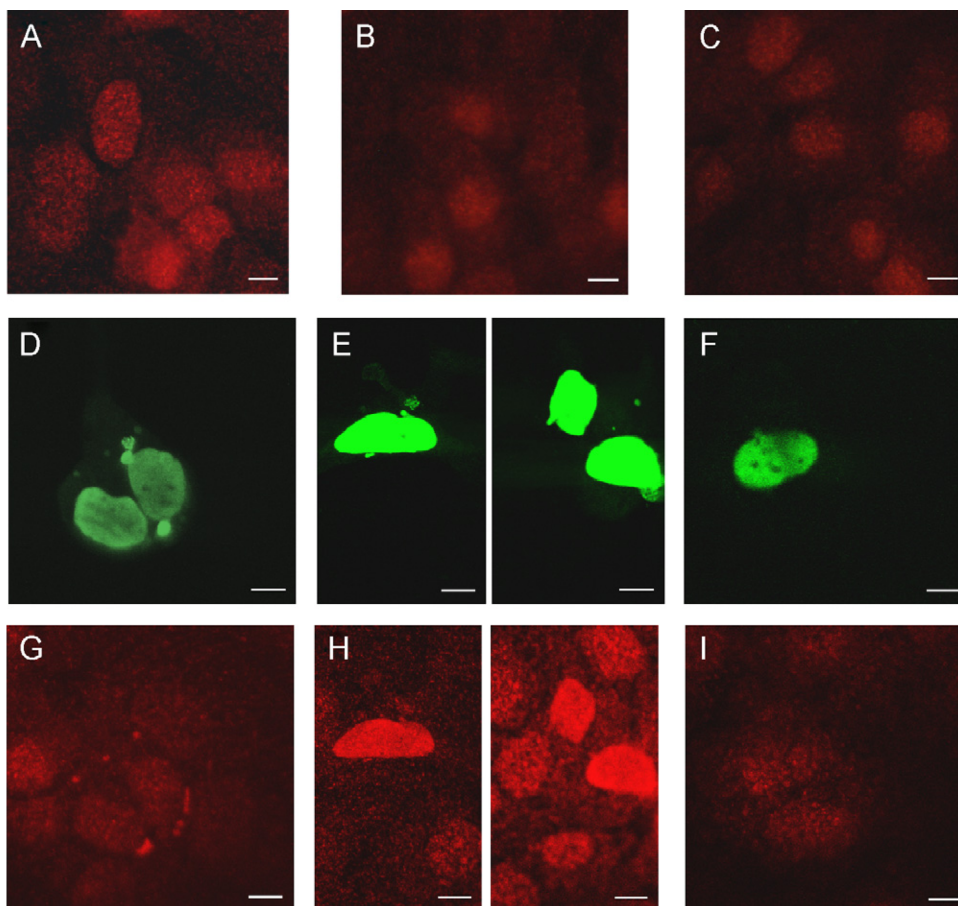

Figure 3 Indirect immunofluorescence and confocal microscopy of p53 and p27 expression in HRG-stimulated SKOV3 cells transfected with wild-type p53. Co-localization of p53 (green) and p27 (red) was examined in SKOV3 cells grown on glass coverslips and transiently transfected with empty vector and left untreated (A) or treated with HRG for 2 h (B) or for 6 h (C), or grown on glass coverslips and transiently transfected with wild-type p53 and left untreated (panels D and G) or treated with HRG for 2 h (panels E and H) or 6 h (panels F and I). Bar = 10 μm.

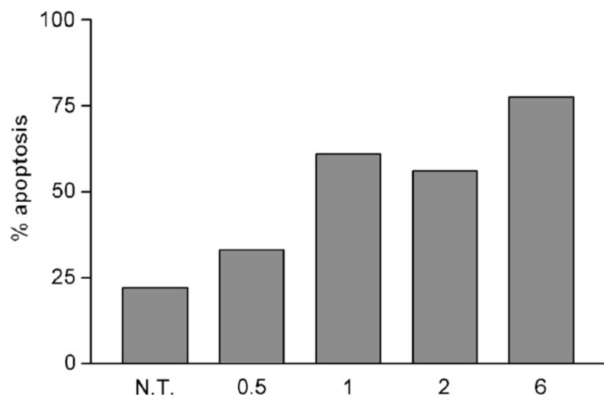

Figure 4 Apoptosis of SKOV3 cells transfected with wild-type p53. SKOV3 cells were grown on glass coverslips and transiently transfected with wild-type p53, and treated with HRG for 30 min, 1, 2, 4 or 6 h. Indirect immunofluorescence with anti-p53 MAb DO7 was carried out and apoptotic nuclei were counted under a fluorescence microscope.

nucleus, either wild-type or mutated, suggests the presence of other alterations related to the apoptotic pathway in tumor cells allowing the shift from apoptosis to proliferation related to HER-2 oversignaling.

Upmodulation of the p27 protein observed in our models, occurred without a substantial increase in p27 mRNA levels (1.5-fold mRNA increase versus 4.4-fold protein increase), suggesting either increased translation or decreased degradation. Indeed, previous reports indicate that downregulation of p27 mRNA is not frequently observed in human cancer[16] and that p27 is mainly regulated post-transcriptionally.[25] The findings that ectopic p53 driven by a non-physiologic promoter is increased by HER2 activation, and that p53 protein levels increase before mRNA strongly suggest protein stabilization, which might in turn determine stabilization of p27.

The mechanism through which anti-HER2 antibody Trastuzumab exerts its effects involves upmodulation of p27.[18,19] Based on our data, the ability of this reagent to upregulate p27 might be limited to tumors expressing p53, wild-type or carrying mutations that do not impair the ability of this oncosuppressor to activate p27. The 6 of 26 HER2-positive tumor samples displaying a mutated p53 that was undetectable by IHC staining might represent tumors resistant to Trastuzumab.

Conclusions

There is a direct association between p53 and p27 expression in human breast tumors. In vitro analyses indicate the crucial role of p53 in determining p27 upmodulation following HER2 activation. This could have implications in the response to Transtuzumab therapy, since upregulation of p27, mechanism through which this MAb exerts its effect, might be limited to tumors able to express p53, either mutated or wild-type.

Both alteration of p53 and low expression of the Cdk inhibitor p27 have been reported as separate prognostic indicators in human breast cancer. Their reciprocal interdependence in breast cancer suggests that they identify the same poor-prognosis tumor subset.

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