P53 But Not Cyclin E Acts in A Negative Regulatory Loop to Control HER-2 Expression in MCF-7 Breast Carcinoma Cell Line

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Abstract- Cyclin E, HER-2 and p53, are considered as major prognostic markers in breast cancer. As they are related in patho-clinical level, we aimed to check if they have any direct interaction on expression of each other. To study the effect of cyclin E on HER-2 expression, cell lines stably overexpressing cyclin E or its low molecular weight (LMW) isoforms were generated. To understand the results of p53 silencing either alone or in combination with cyclin E overexpression, we created three different p53 stably knocked down cell lines. Protein expression was analyzed by western blot, HER-2 expression in the established cell lines were determined using SYBR green real time PCR and data analyzed by REST software. Results indicate that HER-2 expression is only downregulated following p53 silencing and none of cyclin E isoforms can alter its expression. The presence of cyclin E isoforms in p53 silenced clones also does not altered HER-2 expression. Given the fact that p53 degradation is increased by HER-2 overexpression, these data can draw a regulatory loop in which a non-mutated functional p53 and HER-2 can bidirectionally regulate the expression of these two genes. This study improves our understandings of these pathways and these proteins can be introduced either as a marker or as a target in cancer treatment.

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Introduction

Breast cancer is the second leading cause of cancer death and the main cause of overall mortality on women between 35-55 years. Execution of adjuvant systemic therapy following mastectomy is mostly on the basis of traditional prognostic and predictive factors such as tumor size, axillary lymph involvement, steroid receptors status and HER-2 overexpression. However, not all patients who fall in the same category respond similarly to the treatment; besides many patients in the low-risk group die and many others seems to be treated unnecessarily (1). Thus identifying more reliable factor(s) that classify breast tumors more precisely are imperative, especially among cell cycle modulating factors.

Deregulated cell cycle is a conspicuous feature of cancer (2). Cell cycle progression is governed by cyclins and their corresponding cyclin dependent kinases (cdks).Cyclin E and cyclin D are G_1 phase cyclins, which authorize the cell to progress through G_1 and entering S phase. The most prominent mechanism of this function is cyclin E/D-CDK2 mediated phosphorylating of retinoblastoma (RB) protein and its subsequent activation of transcription factor E2F. Negative adjustment is exerted by CDK inhibitors (CKIs), which are $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ in this case (3-5).

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Improper expression and kinetics of cyclin E overexpression has been correlated with unfavorable prognosis in various types of cancers including breast carcinoma (6,7). Generation of tumor exclusive low molecular weight (LMW) isoforms of cyclin E which are five post-translational variants of the primary 50 kDa molecule (8,9), has also been associated with treatment refractory characteristics such as not being inhibited by CKIs and conferring resistance to anti-estrogens and aromatase inhibitors (10-12). These oncogenic effects of LMW forms are boosted by the absence of p53 regulatory control on cell cycle (13).

HER-2/neu receptor tyrosine kinase is a member of the epidermal growth factor receptor (EGFR) family and has gene amplification and/or overexpression in about 25% of invasive breast cancers (14). HER-2 exerts its tumor inducing effects through several mechanisms including shortening of the G_1 phase of the cell cycle and facilitating entry to S phase (15,16), a function similar to the oncogenic mechanism of cyclin E. Besides, in a study of 189 breast cancer patients, it was demonstrated that cyclin E overexpression was significantly associated with HER-2 presence (17).

In this study we aimed to examine the interplay between different isoforms of cyclin E, HER-2 and p53 in MCF-7 breast cancer cell line. Stable clones overexpressing either full length or LMW forms generated and subsequently HER-2 expression measured. We also established a p53 knocked down model of MCF-7 cells expressing cyclin E and its LMW isoforms to elucidate the consequences of p53 abrogation-either alone or in combination with cyclin E isoforms overexpression- on HER-2 expression. Data suggest that cyclin E lies downstream of HER-2 and HER-2 and p53 have bidirectional regulatory effects on each other. Taken together these data suggest that if cyclin E, HER-2 and p53 status considered simultaneously, a more precise treatment strategy and prognosis could be predicted.

Materials and Methods

Vectors construction

The FLAG-tagged Full length cyclin E (EL) and two of its truncated forms (Trunc 1 and Trunc 2 encoding 371 and 341amino acid respectively) constructs (18) were a kind gift from Prof. K.Keyomarsi (The University of Texas MD Anderson Cancer Center, Houston, TX). Flag tagged Trunc Mid (T_{mid}) encoding 365 amino acid of cyclin E was generated by using primers with restriction enzyme recognition sites for directional cloning and Kozak ribosome binding site in forward primer for successful expression. The forward and reverse primers (Eurofins MWG Operon, Germany) sequences were 5′ -GGACACCATGGCCAAAATCGACAGG-3′ and 5′-TTTCACTTGTCATGTCGTCCTTGTAGTCCG-3′, respectively. The product was then cloned into the mammalian vector pCDNA3.1 (Invitrogen, Carlsbad, CA).

For creating p53 silencing construct, we used pSUPER.puro system (OligoEngine, Seattle, WA, USA) that harbors human H1polymerase-III promoter. The sense and anti-sense oligos resuspended in water (100pmol) and 5 µl of each added to 40 µl of annealing buffer (20 mM Tris pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$), warmed up to 95°C for 10 minutes, cooled down to room temperature and used for ligation. The sequence of sense and anti-sense oligos were 5′-GATCCCCGACTCCAGTGGTAATCTACTTCAAGA GAGTAGATTACCACTGGAGTCTTTTTAAGCTTA T-3 ′and 5′-CGATAA GCTTAAAA AGACTCCAGTG GTAATCTACTCTCTTGAAGTAGATTACCACTGG AGTCGGG-3′, respectively.

The products of ligation reactions were then transformed in to the XL1Blue E.coli. Colonies picked following an overnight incubation on antibiotic plates and clones were confirmed by restriction analysis and sequencing.

Cell culture and transfection

MCF-7 human breast cancer cell line obtained from German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). Cells were cultured in RPMI 1640 medium containing 10% FBS, 2mM L-glutamine, 100 units/ml penicillin and 100 µg/ml streptomycin (all from PAA, Austria) at 37°C with 5% CO2. To make stably clones, MCF-7 cells were seeded in 6-well plates at ~ 70% confluency and transfected with either FLAG-tagged cyclin E isoforms coding vectors or p53 shRNA silencing construct using Polyfect transfection reagent (Qiagen, Valencia, CA).The transfected cells were then selected by G418 (500ug/ml) for pcDNA3.1 constructs or puromycin (1ug/ml) for pSUPER.puro based vectors. For each transfection thirty distinct colonies were picked and subjected for expression or silencing screening.

Western Blotting

Proteins were extracted in SDS-containing Laemmli buffer. Just before loading, samples were boiled for 10 minutes and 50µg of each sample was subjected to a 10% sodium dodecyl sulfate-polyacrylamide gel (SDS-

PAGE). The proteins were electrobloted to PVDF membrane (Roche, Germany) and the blots were blocked in casein blocking buffer (10% casein in TBS and 0.1% tween 20). Following blocking, blots were incubated with anti p53(Cell Signaling Technology, USA), anti DYKDDDDK Flag (Cell Signaling Technology, USA) or anti β-Actin antibodies (Santa Cruz, USA) as primary antibodies at 4°C overnight. Thereafter, the blots were incubated for an hour with goat anti mouse/rabbit horse radish peroxidase conjugate (Biorad, USA) and the bands were detected by BM chemiluminescence western blotting kit (Roche, Germany). The blots were digitized and the band intensity was quantified using ImageJ software. In all the experiments β-Actin served as a house keeping control.

RNA extraction and transcript analysis

Total RNA was extracted from cell lines by using Tripure reagent (Roche, Germany) according to the manufacturer's instructions. Suitable RNA quality was confirmed by the A260/A280 absorbance ratio and by electrophoresis on 1% agarose formaldehyde gel. Then 1ug of total RNA was reverse transcribed to single stranded cDNA using random hexamer primers and M-MuLV reverse transcriptase (Fermentas, Lithuania). Quantitative SYBR green PCR reactions were performed in a total volume of 20 µl using Precision-R MasterMix (Primerdesign, UK) by StepOnePlus Real-Time PCR system (Applied Biosystems, USA). All the reactions were carried out in duplicate and 2ul of reverse transcriptase products was used as template. Quantitative values determined as C_t numbers and fold changes in gene expression were determined by using the formula $2^{-\Delta\Delta Ct}$ and analyzed by REST software (19). Results stated as N-fold change (decrease or increase) of genes of interest. HPRT and GAPDH were used as housekeeping genes. Primers specifications are depicted in table 1.

Results

Establishment of stable cell lines overexpressing full length (EL) and LMW forms of cyclin E

The MCF-7 human breast cancer cell line has been used as an acceptor of mammalian plasmid expression vectors to reveal the effects of oncogenes overexpression on the estrogen-dependent and poorly invasive phenotypes exhibited by this line. Cell lines overexpressing the EL and its LMWs generated by transfection of MCF-7 cells with either the pcDNA3.1 or any of C-terminal FLAG-tagged cyclin E isoforms vectors named EL, T1, T2 and T_{mid}. The EL construct expresses the 50kDa full length cyclin E, the T1 construct expresses the 44 and 45 kDa forms, the T2 construct expresses the 33 and 34 kDa forms, and the T_{mid} construct expresses the 40 kDa form (20). For any of the coding constructs, thirty clones chosen and screened for producing of the desired protein using FLAG antibody. Ultimately, two clones having the highest level of expression (HE) selected and propagated for further experiments (Figure 1).

Table 1. The target genes primer sequences, position and product size.

Primer	Forward	Position	Reverse	Position	Product size
HER-2	AGCCGCAGTGAGCACCATGG	223-242	GTGCCGGTGCACACTTGGGT	305-324	102
HPRT	CTGGCGTCGTGATTAGTGATGATGA	184-208	TCGAGCAAGACGTTCAGTCCTGTC	297-320	137
GAPDH	ACACCCACTCCTCCACCTTG	968-988	TCCACCACCCTGTTGCTGTAG	1059-1079	112

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HPRT, hypoxanthine phosphoribosyltransferase; HER-2,Homo sapiens v-erb-b2 erythroblastic leukemia viral oncogene homolog

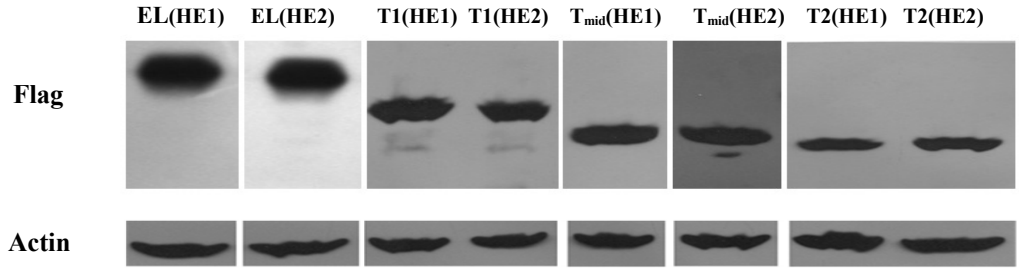

Figure 1. Western blot analysis. Overexpression of cyclin E Flag-fused isoforms in MCF-7. Stable transfectants grew in selective medium containing 500 µg/ml G-418. High expressing clones identified by western blotting.

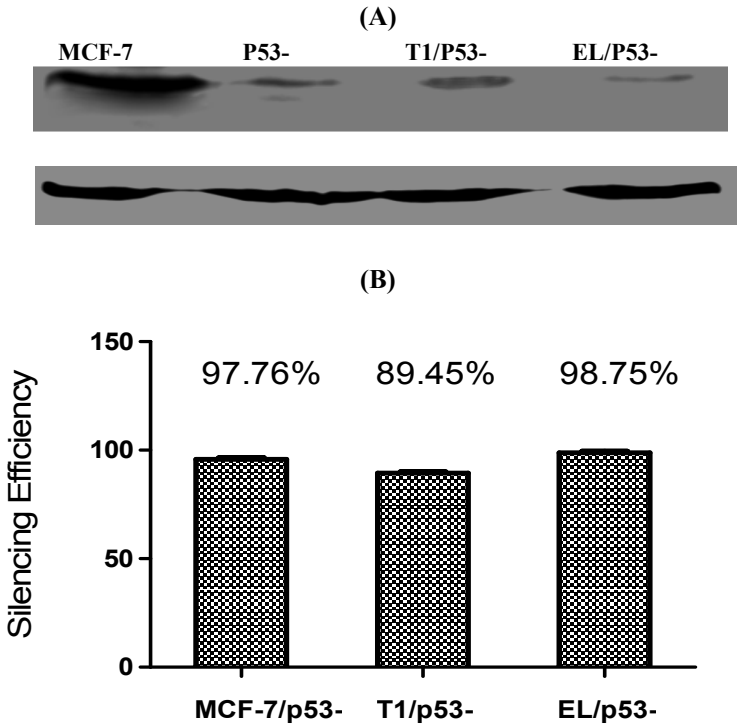

Figure 2. (A) Western blot analysis of p53 expression in stable knocked out cell lines. Stable knocked outs selected by cultivating in 1μg/ml puromycin medium. Clones with the highest degree of silencing chose for further interrogations. (B) The efficiency of p53 silencing was determined by western blotting and analyzed by ImageJ software. β-Actin used as internal control. Results represent the means ± SE of duplicates from three different experiments.

Establishment of MCF-7/p53-, T1/p53- and EL/p53- cell lines

To examine the effects of p53, we transfected the cells with pSUPER.puro.p53 plasmid to block p53 expression. For this purpose, we used MCF-7 cells to study the direct consequences of p53 silencing and the stably expressing clones of T1 and EL to study the interaction between these isoforms and p53. These clones were selected as one of them, EL, overexpresses the normal physiological form of cyclin E and the other one, T1, overexpresses the LMW form of cyclin E having the highest kinase activity (12). As shown in Figure 2.a, expression of a shRNA vector a gainst p53 (pSUPER.puro.p53) drastically decreased p53 expression in the selected clones. Efficiency of silencing is displayed in Figure 2.b.

HER-2 expression is not affected by full length or LMW isoforms of cyclin E

Cyclin E overexpression is often seen in tumors with high levels of HER-2 (17). However the detailed mechanism of this coinciding has not been understood yet. Here, we used MCF-7 cells engineered to overexpress full-length or LMW isoforms of cyclin E to find out if HER-2 expression is upregulated in these cell lines or not. Our results indicate that there are no significant changes in HER-2 expression in stably expressing cyclin E isoforms, suggesting that HER-2 expression is not directly regulated by cyclin E or its LMW isoforms (Table 2).

HER-2 expression is inhibited via p53 silencing

P53, the crucial cell cycle brake, is known to be degraded by HER-2 amplification or overexpression (21). Taken in account that cyclin E overexpression and LMW isoforms are found in tumors or cell lines with either deleted or mutated p53, we examined if the lack of p53 could alter HER-2 expression. Results indicate that there is a direct relation between HER-2 expression and p53 presence. Thus p53 depletion induces a significant decrease in HER-2 expression independent of cyclin E status (Table 2). Besides no statistically significant difference in downregulation has been observed among the three different p53 silenced cell lines.

Table 2. Relative gene expression of Her-2 in stable transfectants. Relative gene expression measured as the fold change of the target genes with regards to housekeeping genes (GAPDH and HPRT) and compared with the gene expression in the control group using REST 2009 software. P(H1) depicts the probability of alternate hypothesis that difference between sample and control groups is due only to chance. Data are representative of three independent experiments.

Transcript	Type	Reaction efficiency	Expression	P(H1)	Result
EL (HE1)					
HER-2	TRT	0.95	1.26	0.238	NS
GAPDH	REF	1.1	0.99		
HPRT	REF	0.93	1.00		
EL (HE2)					
HER-2	TRT	0.95	1.066	0.953	NS
GAPDH	REF	1.1	0.890		
HPRT	REF	0.93	1.15		
T1 (HE1)					
HER-2	TRT	0.95	0.850	0.810	NS
GAPDH	REF	1.1	1.05		
HPRT	REF	0.93	0.952		
T1 (HE2)					
HER-2	TRT	0.95	0.950	0.145	NS
GAPDH	REF	1.1	0.910		
HPRT	REF	0.93	1.20		
T2 (HE1)					
HER-2	TRT	0.95	1.093	0.900	NS
GAPDH	REF	1.1	0.980		
HPRT	REF	0.93	1.02		
T2 (HE2)					
HER-2	TRT	0.95	1.62	0.610	NS
GAPDH	REF	1.1	0.084		
HPRT	REF	0.93	1.182		
T_{mid} (HE1)					
HER-2	TRT	0.95	1.17	0.220	NS
GAPDH	REF	1.1	0.934		
HPRT	REF	0.93	1.07		
T_{mid} (HE2)					
HER-2	TRT	0.95	1.23	0.370	NS
GAPDH	REF	1.1	1.02		
HPRT	REF	0.93	0.98		
P53-					
HER-2	TRT	0.95	0.026	0.025	DOWN
GAPDH	REF	1.1	1.28		
HPRT	REF	0.93	0.879		
P53-/T1					
HER-2	TRT	0.95	0.328	0.024	DOWN
GAPDH	REF	1.1	1.082		
HPRT	REF	0.93	0.924		
P53-/EL					
HER-2	TRT	0.95	0.148	0.018	DOWN
GAPDH	REF	1.1	0.966		
HPRT	REF	0.93	1.035		

TRT, target transcripts; REF, reference transcripts; NS, nonsignificant (comparing to parental MCF-7).

Discussion

In this article, we report a novel interaction between HER2 and p53 in breast cancer. We found that although highly positive HER-2 tumors also overexpress cyclin E, particularly the treatment resistance LMW forms, HER-2 is not the downstream target of cyclin E mediated signaling. Conversely, both cyclin E upregulation and generation of LMW forms are known to be inducible through overexpression of exogenous HER-2 in MCF-7 cells (22). This enhanced oncogenic activity is not only at transcriptional and modification but also at regulation level as it causes increased degradation and nuclear exclusion of p27 (15). Together, these data support the notion that cyclin E and HER-2 have unidirectional crosstalk and HER-2 is one of the molecules controlling cyclin E expression.

The consequence of tumor suppressor p53 silencing on HER-2 expression was also investigated in our study. To address this issue, we generated three different p53 silenced cell lines with different cyclin E expression status. To get a perception of the outcomes result from p53 silencing *per se*, we knocked down p53 in MCF-7 cells with unmodified cyclin E expression. To study the effect of both p53 silencing and cyclin E overexpression on HER-2 expression, two other cell lines generated; one overexpressing EL and the other T1isoform.The data indicate that HER-2 expression is only downregulated by p53 knock down and this effect cannot be altered by neither EL nor T1 expression. HER-2 overexpression is demonstrated to enhance MDM2-mediated ubiquitination and degradation of p53 (21). Combining the results from ours and previous study, a bidirectional relation between HER-2 and p53 can be postulated. According to this crosstalk, HER-2 expression regulates p53 expression and the level of p53 negatively regulates Her-2 expression. Any likely increase in HER-2 expression activate the compensatory response by means of increased p53 degradation and so as an outcome of this feedback loop HER-2 expression will be return back to a normal level. Thus if p53 mutation or deletion precede HER-2 upregulation in the oncogenesis process, HER-2 expression cannot be controlled anymore. Taken together, these data shed some light on how p53 is stated in the regulatory network of HER-2 and cyclin E as well as how much these markers can be treatment predictive or survival prognostic.

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