

ORIGINAL ARTICLE

Deregulation of p53-MDM2 auto-regulatory pathway in breast carcinoma

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Summary

Purpose: p53 tumor suppressor protein (17p13.1) regulates critically the cell cycle and thus it is involved in cancer initiation and prevention. The gene is frequently mutated in breast cancer patients and the mutations have been associated with poor prognosis and response rates to chemotherapy. The purpose of this study was to correlate p53 expression with MDM2, a proto-oncogene (12q14.3), which acts as a major negative regulator in p53-MDM2 auto-regulatory pathway.

Methods: Seventy breast adenocarcinoma cases were included in the study. Sixty tumors were pathologically categorized as invasive ductal adenocarcinomas, whereas the rest of them were diagnosed as pure in situ carcinomas. Immunohistochemistry (IHC) was applied using anti-p53 and anti-MDM2 antibodies in the corresponding tissue sections.

Results: Overexpression of p53 protein was observed in 39/60 (65%) invasive cases, while 40/60 (66.7%) expressed MDM2 protein. Interestingly, in 26/60 (43%) cases a combined p53/

MDM2 co-expression was detected, whereas in 7/60 (11%) a combined loss of expression was identified (overall co-expression: $p=0.999$). Concerning in situ carcinomas, co-expression of p53/MDM2 was observed in 7/10 (70%) cases.

Conclusions: MDM2 oncogene overexpression - predominantly due to gene amplification - is a frequent and critical genetic event in both in situ and invasive breast adenocarcinomas. Accumulation of p53 protein in the nucleus of tumor cells harboring mutant p53 - as the result of its overexpression - does not mean necessarily decreased expression of MDM2. MDM2 directly binds to p53 and represses its transcriptional activity promoting p53 degradation. So targeting the molecule, p53's crucial tumor suppressor function is normally regulated.

Key words: breast, carcinoma, immunohistochemistry, MDM2, p53.

Introduction

Breast carcinogenesis is a multistep process characterized by a series of proliferative and pre-invasive alterations. However, the mechanisms of development from non-invasive to invasive lesions have not yet been determined with precision [1]. Identification of specific molecular profiles regarding these lesions provides improved knowledge for diagnostic and potentially thera-

peutic aspects. Among genes that critically modify pathological and molecular characteristics in patients with breast adenocarcinoma, p53 and MDM2 are of high significance [2]. p53 is a key regulator of the genome stability and function. The gene is located on the short (p) arm of chromosome 17 at position 13.1 (17p13.1), encoding a nuclear phosphoprotein with a molecular weight

of 53 kDa acting as a transcription factor that negatively regulates cell proliferation. It is also involved in a significant number of cell-signaling pathways including cell cycle, programmed cell death, and DNA repair [3]. The protein is expressed at a low level in malignant epithelia. Due to point mutations p53 overexpression is frequently detected by immuno-histo-cytochemistry assays in about 60% of malignancies of different histogenetic origin, including also breast adenocarcinoma [4].

Additionally, MDM2, a proto-oncogene (12q14.3) encoding a nuclear-localized E3 ubiquitin ligase, acts as a major negative regulator in p53-MDM2 auto-regulatory pathway. MDM2 directly binds to p53 and represses its transcriptional activity and promotes p53 proteasomal degradation [5]. Gene amplification is the major mechanism of MDM2 deregulation and overexpression in breast carcinoma correlated with aggressive phenotype [6]. In the current study we co-analyzed p53/MDM2 at the protein level in breast adenocarcinoma (invasive and *in situ*) cases for determining the significance of their expression in the corresponding lesions.

Methods

Study group

For the purposes of this study, 70 archival, formalin-fixed and paraffin-embedded tissue specimens including 60 histologically confirmed invasive ductal breast adenocarcinomas and also 10 *in situ* carcinomas were selected. All of the patients were female with average age 56.9 years (range 34-86). The initial diagnosis was performed by fine needle aspiration biopsy (FNAB). The Ethics Committee consented to the use of these tissues in the 1st Department of Pathology, Medical School, University of Athens for research purposes, according to World Medical Association Declaration of Helsinki. The tissue samples were fixed in 10% neutral-buffered formalin. Hematoxylin and eosin (H&E)-stained slides of the corresponding samples were reviewed for confirmation of the initial histopathological diagnoses. All lesions were classified according to the histological typing criteria of World Health Organization (WHO) [7]. The clinicopathological parameters of the examined cases were age, tumor size (diameter), tumor grade and lymph node metastasis.

Antibodies and immunohistochemistry assay (IHC)

Ready-to-use anti-p53 (clone DO7-DAKO, UK, dilution 1:40) and also anti-MDM2 (clone IF2, ThermoFisher, USA, dilution 1:40) mouse monoclonal antibodies were applied in the corresponding cases. IHC for these

antigens was carried out on 5 µm serial tissue sections. The slides were deparaffinized and rehydrated. The Zytocem Plus HRP Polymer Kit (ZYTOMED Systems GmbH, Berlin, Germany) was used. Blocking solution was applied to all slides for 10 min, followed by incubation for 1 hr using the corresponding monoclonal antibodies at room temperature (25°C). Following incubation with the secondary antibody for 10 min, diaminobenzidine-tetrahydrochloride-DAB (0.03%) containing 0.1% hydrogen peroxide was applied as chromogen and incubated for 5 min. Sections were counterstained, dehydrated and cover-slipped. For negative control slides, the primary antibodies were omitted. IHC protocol was performed using an automated staining system (I 6000 Biogenex, CA, USA). Pre-analyzed cancer tissue sections expressing the proteins and normally appearing breast epithelia were used as control groups, respectively.

The assessment of IHC expression took into account two factors: the percentage of positive tumor cells and the intensity of the color reaction to specific antibodies. The staining intensity was characterized as weakly positive (+), moderately positive (++) or strongly positive (+++).

Statistics

Descriptive and inferential techniques were applied. Quantitative variables were presented as mean±standard deviation, while qualitative variables were presented in frequency Tables. Due to the small number of cases in the groups, evaluation of the relationship between qualitative and quantitative variables was done by the non parametric Mann-Whitney U test. To evaluate the relationship between independent qualitative variables, where appropriate, for χ^2 for linear trend and Fisher's exact test were applied. The relationship between dependent qualitative variables was assessed using McNemar test. Two-tailed p values <0.05 were considered statistically significant.

Results

According to expression analysis, overexpression of p53 protein was observed in 39/60 (65%) invasive cases, while 40/60 (66.7%) expressed MDM2 protein. Nuclear predominantly and cytoplasmic staining was considered acceptable for the markers, according to manufacturer's data sheets (Figure 1 a,b). Interestingly, in 26/60 (43%) cases a combined p53/MDM2 co-expression was detected, whereas in 7/60 (11%) a combined loss of expression was identified (overall p53 & MDM2 co-expression: $p=0.999$). In the rest of the cases (N=27/60) an inverse expression (negative/positive) of the two molecules was noted. Concerning *in situ* carcinomas, p53 overexpression was detected in 9/10 cases, whereas MDM2 in

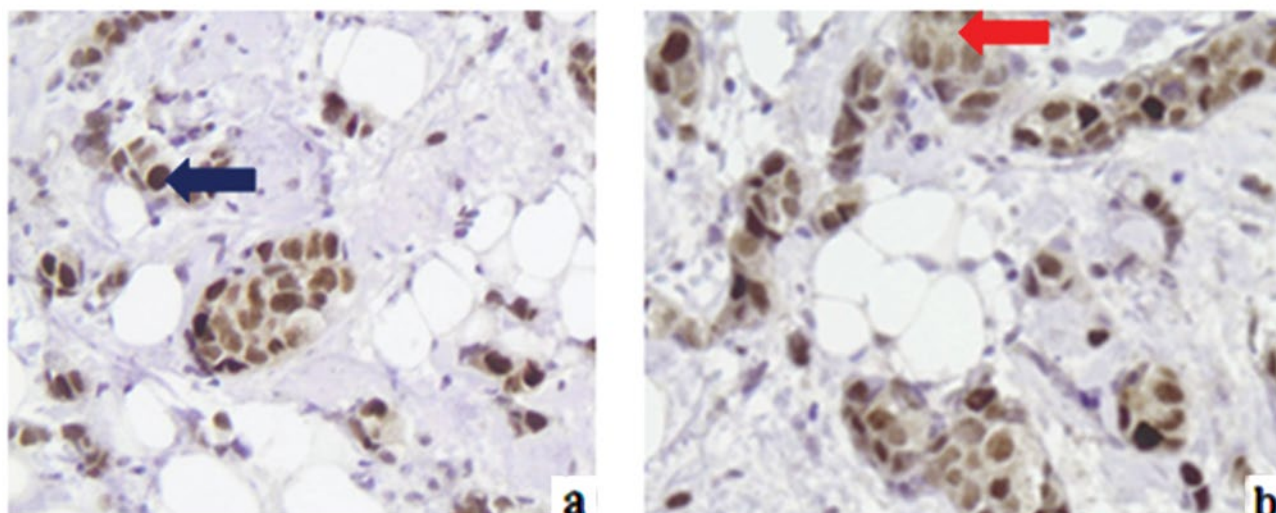


Figure 1. Overexpression of the two markers (**a** p53 and **b** MDM2). Note mainly nuclear (arrow) and cytoplasmic (arrow) brown staining pattern (DAB stain, original magnification 100x).

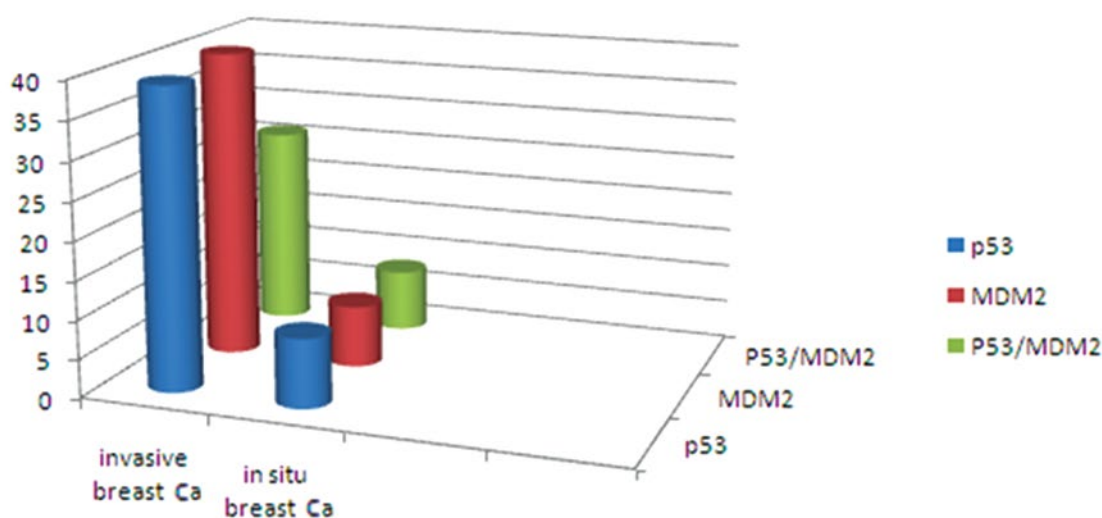


Figure 2. 3-D histogram of the combined p53 & MDM2 expression in invasive and *in situ* breast adenocarcinoma.

Table 1. Expression status of p53 & MDM2 in invasive and *in situ* breast adenocarcinomas

Invasive breast adenoCa, N=60			p53	
		N/LE-	OE	<i>p</i> value
MDM2	N/LE-	7	13	0.999
	OE	14	26	
In situ breast adenoCa, N=10			p53	
		N/LE-	OE	<i>p</i> value
MDM2	N/LE-	0	2	0.034
	OE	1	7	

N/LE: Negative/low expression (up to +)
 OE: overexpression (+/+++)

7/10. Co-expression of p53/MDM2 was registered in 7/10 (70%) cases (Figure 2). Overall, p53 and MDM2 expression was not statistically signifi-

cant in relation to age ($p=0.434$ and $p=0.098$, respectively), to tumor size/diameter ($p=0.783$ and $p=0.181$, respectively), to tumor grade ($p=0.886$ and $p=0.998$, respectively) or to lymph node metastasis ($p=0.987$ and $p=0.636$, respectively). IHC results and correlations are described in Tables 1 and 2.

Discussion

Approximately 30% of breast carcinomas of all pathological types harbor a p53 mutation. Interestingly, about 50% of HER2 amplified cases demonstrate also mutant p53 expression [8]. Mechanisms of p53 mutation include insertion/deletion polymorphisms, or simpler base substitutions dependent on the tumor histology [9]. Another mechanism of p53/MDM2 auto-regulatory

Table 2. p53 & MDM2 overall expression

Expression	Age	p values		
		Tumor size	Grade	Lymph node metastasis
p53	0.434	0.783	0.886	0.987
MDM2	0.098	0.181	0.998	0.636

pathway deregulation is the aberrant alternative splicing of mRNA precursors. This genetic process leads to abnormal protein expression that promotes cell growth, local invasion and metastasis by enhancing oncogenes and downregulating tumor suppressor genes [10]. Furthermore, the role of post-transcriptional regulators - termed microRNAs - seems to be significant for regulating positively or not the p53/MDM2 dependent pathway. In particular, positive feedback loops involving miR-192, miR-34a and miR-29a have been already identified [11].

Besides p53 mutations that lead to abnormal protein expression, wild-type p53 is influenced by two main inhibitors: MDM2 and MDM4 (also called MDMx). The role of MDM4 is critical because it inhibits the transcriptional activity of p53, enhancing also the ability of MDM2 to target p53 for degradation [12]. Overexpression of these molecules is mediated mainly by gene amplification although there are differences in the frequency of this genetic mechanism among breast carcinomas [13].

In the current study we analyzed p53/MDM2 complex at the protein expression level. A significant subset of the examined tumors demonstrated a co-overexpression, whereas in many cases elevated MDM2 expression inversely correlated with p53 levels. Interestingly, their upregulation was detected also in *in situ* carcinomas as an early genetic abnormality in breast carcinogenesis. Quite recently a study showed that AGR2 protein upregulates DUSP10 which subsequently inhibits p38 MAPK and prevents p53 activation by phos-

phorylation. This novel pro-oncogenic signaling pathway affects the p53 function independently of MDM2 expression [14]. Additionally, agents with potential anticancer activity, such as violacein, seem to upregulate apoptotic genes, including p53 and markedly reduce MDM2 expression levels [15]. Concerning also MDM2 regulation, novel molecules, such as transcription factor NFAT1, that induce their oncogenic function have been identified. Based on this observation, two study groups showed that JapA molecule inhibits NFAT1-mediated MDM2 at transcriptional and post-translational levels [16,17]. Another important study suggested that the cholesterol metabolite 27-hydroxycholesterol (27-OHC) regulates p53 activity and increases cell proliferation via MDM2 in breast cancer cells. This is a selective estrogen receptor modulator involved in ER-positive breast cancer progression by disrupting constitutive p53 signaling in an MDM2-dependent manner [18]. Finally, the development of novel anti-p53/MDM2 agents such as PRIMA-1, PRIMA-1MET and nutlin antagonists in cancers with a high prevalence of p53 mutations should be a very promising targeted therapeutic strategy [19,20].

In conclusion, MDM2 oncogene overexpression - predominantly due to gene amplification - is a frequent and critical genetic event in both *in situ* and invasive breast adenocarcinomas. Accumulation of p53 protein in the nucleus of tumor cells harboring mutant p53 - as the result of its overexpression - does not mean necessarily decreased expression of MDM2. Because MDM2 directly binds to p53 and represses its transcriptional activity promoting p53 degradation, targeting the p53 tumor suppressor function is normally regulated when mutations are absent.

Conflict of interests

The authors declare no conflict of interests.

References

1. De Mascarel I, MacGrogan G, Mathoulin-Pelissier S et al. Breast ductal carcinoma in situ with micro-invasion: a definition supported by a long term study of 1248 serially sectioned ductal carcinomas. *Cancer* 2002;94:2134-2142.
2. Silwal-Pandit L, Vollan HK, Chin SF et al. TP53 mutation spectrum in breast cancer is subtype specific and has distinct prognostic relevance. *Clin Cancer Res* 2014;20:3569-3580.
3. Purvis JE, Karhohs KW, Mock C et al. p53 dynamics control cell fate. *Science* 2012;336:1440-1444.
4. Dumay A, Feugeas JP, Wittmer E et al. Distinct tumor

- protein p53 mutants in breast cancer subgroups. *Int J Cancer* 2013;132:1227-1231.
5. Wade M, Li YC, Wahl GM. MDM2 MDMX and p53 in oncogenesis and cancer therapy. *Nat Rev Cancer* 2013;13:83-96.
 6. Santarius T, Shipley J, Brewer D, Stratton MR, Cooper CS. A census of amplified and overexpressed human cancer genes. *Nat Rev Cancer* 2010;10:59-64.
 7. Sinn HP, Kreipe H. A Brief Overview of the WHO Classification of Breast Tumors (4th Edn). Focusing on Issues and Updates from the 3rd Edition. *Breast Care* 2013;8:149-154.
 8. Shah SP, Roth A, Goya R et al. The clonal and mutational evolution spectrum of primary triple-negative breast cancers. *Nature* 2012;486:395-399.
 9. Walerych D, Napoli M, Collavin L, Del Sal G. The rebel angel: mutant p53 as the driving oncogene in breast cancer. *Carcinogenesis* 2012;33:2007-2017.
 10. Inoue K, Fry EA. Aberrant splicing of the DMP1-ARF-MDM2-p53 pathway in cancer. *Int J Cancer* 2016;139:33-41.
 11. Moore R, Ooi HK, Kang T, Bleris L, Ma L. MiR-192-Mediated Positive Feedback Loop Controls the Robustness of Stress-Induced p53 Oscillations in Breast Cancer Cells. *PLoS Comput Biol* 2015;11(12):e1004653
 12. Lam S, Lodder K, Teunisse AF, Rabelink MJ, Schutte M, Jochemsen AG. Role of Mdm4 in drug sensitivity of breast cancer cells. *Oncogene* 2010;29:2415-2426.
 13. Haupt S, Buckley D, Pang JM et al. Targeting Mdmx to treat breast cancers with wild-type p53. *Cell Death Dis* 2015;6:e1821
 14. Hrstka R, Bouchalova P, Michalova E et al. AGR2 oncoprotein inhibits p38 MAPK and p53 activation through a DUSP10-mediated regulatory pathway. *Mol Oncol* 2015;S1574-7891(15)00239-2
 15. Alshatwi AA, Subash-Babu P, Antonisamy P. Viola-cein induces apoptosis in human breast cancer cells through up regulation of BAX, p53 and down regulation of MDM2. *Exp Toxicol Pathol* 2016;68:89-97.
 16. Qin JJ, Wang W, Voruganti S, Wang H, Zhang WD, Zhang R. Inhibiting NFAT1 for breast cancer therapy: New insights into the mechanism of action of MDM2 inhibitor JapA. *Oncotarget* 2015;6:33106-33119.
 17. Zheng J, Fang F, Zeng X, Medler TR, Fiorillo AA, Clevenger CV. Negative cross talk between NFAT1 and Stat5 signaling in breast cancer. *Mol Endocrinol* 2011;25:2054-2064.
 18. Raza S, Ohm JE, Dhasarathy A et al. The cholesterol metabolite 27-hydroxycholesterol regulates p53 activity and increases cell proliferation via MDM2 in breast cancer cells. *Mol Cell Biochem* 2015;410:187-195.
 19. Duffy MJ, Synnott NC, McGowan PM et al. p53 as a target for the treatment of cancer. *Cancer Treat Rev* 2014;40:1153-1160.
 20. Wang W, Qin JJ, Voruganti S et al. The pyrido[b]indole MDM2 inhibitor SP-141 exerts potent therapeutic effects in breast cancer models. *Nat Commun* 2014;5:5086.