Endocr (2009) 35:118–122
DOI 10.1007/s12020-008-9124-4

ORIGINAL PAPER

Gene and protein expression of p53 and p21 in fibroadenomas and adjacent normal mammary tissue

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Received: 2 September 2008 / Accepted: 21 October 2008 / Published online: 11 November 2008

Abstract The aim of this study was to compare p53 and p21 mRNA, and proteins levels between fibroadenomas and adjacent normal mammary tissue of women in reproductive age. A transversal study was performed. Fourteen patients who attended the Breast Service of the Hospital de Clínicas de Porto Alegre were assessed and submitted to surgical resection of fibroadenomas. Fragments of the central area of the fibroadenoma and adjacent normal mammary tissue were obtained. mRNA expression for genes p53 and p21 was evaluated by RT-PCR, and protein expression by the western blot. Paired analyses showed higher gene expression of *p53* ($P = 0.017$) and *p21* ($P = 0.003$), and a higher protein expression of p53 ($P = 0.001$) in fibroadenomas as compared to normal breast tissue. p21 protein expression was not different ($P = 0.97$) between the fibroadenoma and the adjacent normal mammary tissue samples. These results suggest the participation of p53 in the formation of fibroadenomas. The role of p21 in fibroadenomas remains to be defined.

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Keywords Fibroadenoma · Normal breast tissue · p21 · p53

Introduction

Fibroadenomas are common benign mammary lesions originating in the stroma and epithelium of the lobular-duct unit, usually occur in breasts of young women, present self-limited growth, and remain unaltered or regress spontaneously [1]. The mechanisms involved in the development of fibroadenomas are poorly understood [2]. Besides the role of estrogen and progesterone receptors expressed by epithelial cells [3], growth factors and their receptors may play a role in the pathogenesis of benign breast diseases, including fibroadenomas, suggesting that multiple signaling routes may be involved in the growth and differentiation of benign breast disorders [4].

The cell cycle consists of a series of strictly controlled events which guide DNA replication and cell division [5]. Cell proliferation, motility, and survival are regulated by multiple factors, and the changes occurring in tumor cells are the result of multiple alterations in the cell signaling machinery [6].

The gene *p53* codes for a protein which plays an essential role in the regulation of the cell cycle, particularly in the transition from G0 to G1 [3]. Protein p53 acts preventing cell proliferation after DNA damage and triggering apoptosis in case of irreparable damage [7, 8]; its wild-type appears to be related to an inhibitory effect on cell transformation, while the mutated form would be related to tumor formation [9]. One of the routes of p53 action to promote cessation of the cell cycle occurs through the transactivation of p21 dependent on p53 [10].

Gene *p21* codes for an important cyclin-dependent kinase inhibitor, which is a key regulator of cell cycle arrest after DNA damage [3, 11]. This gene presents binding sites for *p53,* indicating that *p21* transcription may be directly regulated by *p53* [12, 13]. Several other factors can also induce *p21* expression and block cell cycle through pathways independently of p53 [14]. p21 is a key protein that determines whether a cell will proliferate or differentiate itself [15].

The aim of this study was to compare the gene and protein expression of p53 and p21 between fibroadenomas and normal adjacent mammary tissue of women in reproductive age.

Results

Patients' mean age was 25.1 ± 7.9 years, the mean age at menarche was 12.7 ± 1.8 years, and BMI was 22.3 ± 4.7 kg/cm^2. Of the 14 patients, 3 had a full term pregnancy and 7 were taking oral contraceptives. Patient consent and clinical protocols met approval of the Institutional Review Board of Hospital de Clínicas de Porto Alegre. Eleven patients presented only one fibroadenoma, three patients two or more nodules. In these cases, only the biggest nodule was sampled and analyzed. The mean diameter of the fibroadenomas was 2.4 ± 0.7 cm.

Samples of fibroadenomas were paired with adjacent normal mammary tissue were analyzed for expression of p21 and p53. *p53* mRNA gene expression was higher in fibroadenomas (0.87 ± 0.04) than in normal mammary tissue (0.8 ± 0.03) (P = 0.017) (Fig. 1).

p53 protein expression was also higher in fibroadenomas (1.4 ± 0.17) than in normal mammary tissue (1.15 ± 0.15) (P = 0.001) (Fig. 2).

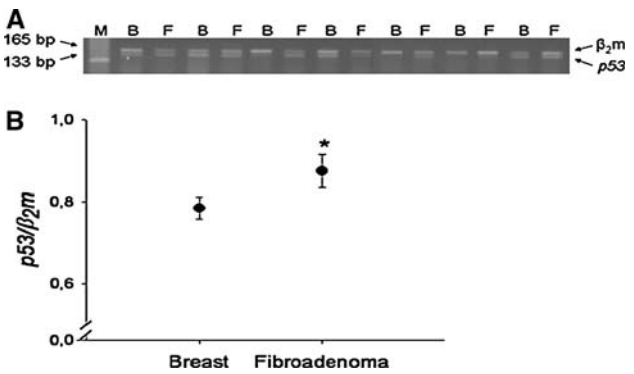

Fig. 1 Gene expression of *p53.* **a** Representative gel showing the amplified products by RT-PCR. *p53* with 133 bp (base pairs) and β_2m with 165 bp. *M* molecular weight marker, *B* normal breast tissue, *F* fibroadenoma. **b** Expression of *p53* mRNA in relation to β_2m in arbitrary units. *P = 0.017

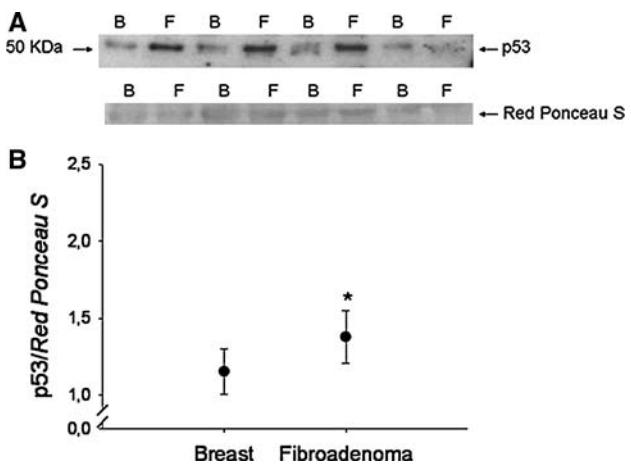

Fig. 2 Analysis of protein p53. **a** Representative autoradiogram with the bands of 53 kDa protein of *p53* and normalization with *Red Ponceau S.* **b** Densitometric analysis of bands expressed as the p53/*Red Ponceau S* ratio *P = 0.001. *M* molecular weight marker, *B* normal breast tissue, *F* fibroadenoma

p21 gene expression was higher in fibroadenomas (0.9 ± 0.08) than in normal mammary tissue (0.5 ± 0.12) (P = 0.003) (Fig. 3).

However, p21 protein expression was similar in fibroadenoma (1.0 ± 0.04) and normal mammary tissue (1.0 ± 0.07) (P = 0.97) (Fig. 4).

Discussion

In the present study, we have demonstrated an increase of gene expression of *p53* and *p21* in fibroadenomas compared to normal adjacent mammary tissue. p53 protein

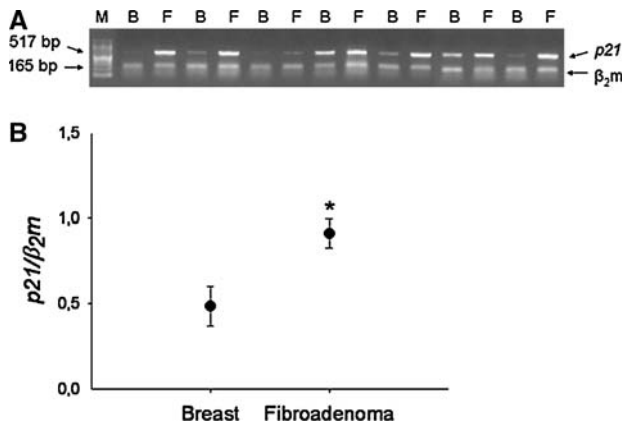

Fig. 3 Gene expression of *p21.* **a** Representative gel showing the amplified products by RT-PCR. *p21* with 517 bp (base pairs) and β_2m with 165 bp. *M* molecular weight marker, *B* normal breast tissue, *F* fibroadenoma. **b** Expression of *p21* mRNA in relation to β_2m in arbitrary units. *P = 0.003

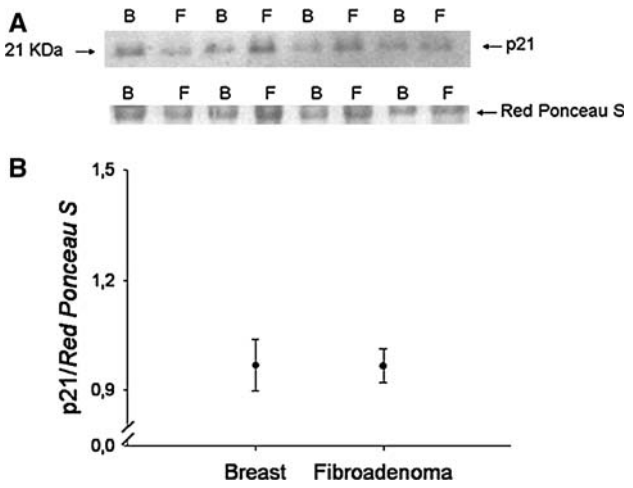

Fig. 4 Analysis of protein p21. **a** Representative autoradiogram with the bands of 21 kDa protein of *p21* and normalization with *Red Ponceau S*. **b** Densitometric analysis of bands expressed as the p21/*Red Ponceau S* ratio, *B* normal breast tissue, *F* fibroadenoma

expression was also significantly higher in fibroadenomas, while p21 protein showed similar expression in both tissues.

In the presence of elevated *p53* and *p21*, human breast epithelial cells in culture change spontaneously from senescence to proliferation [16]. This suggests that genomic alterations may be associated with neoplastic transformation, and it may also be involved in the formation of benign breast lesions.

Benign breast diseases showed an overexpression of *p53* compared to malignant disorders [9]. These differential expressions of p53 may be useful to identify a subset of benign breast diseases with a potentially different clinical behavior [9].

The tumor-suppressing gene *p53* is activated by several stress signals through mechanisms resulting in the stabilization and accumulation of p53 protein [17]. The tumor-suppressing gene *p53* acts protecting cells from malignant transformation, and the development of most tumors is associated with the loss of p53 function [18]. Increased expression of wild-type p53 protein can prevent the process of transformation, while inactivation of p53 predispose the cells to lose their differentiation [18–20]. Through their function as transcriptional activator or repressor, a number of genes controlling the cell cycle, cell death, and other cell functions are target sequences of p53, and p21 is one among them.

The cyclin $p21^{waf1/cip1}$-dependent kinase inhibitor has a key role in the control of the cell cycle and is mainly regulated at the transcriptional level. While induction of p21 leads predominantly to the interruption of the cell cycle, its repression can lead to diverse responses [21]. Cdk p21 inhibitor is often responsible for inducing the interruption of the cell cycle dependently or independently of p53. Cell cycle interruption enables the cells to repair damages and then resume cell division. p21 function as inhibitor of cell proliferation can contribute to its ability to act as a tumor suppressor gene. On the other hand, the ability of p21 to induce the interruption of the cell cycle after stress can protect the cell from stress-induced apoptosis. The anti-apoptotic activity of p21 can contribute for it to act as an oncogene. This confers to p21, a duality of opposing actions in which it often inhibits apoptosis (pro-cancer) contrary to its anti-proliferative (anticancer) effects [11]. These contrasting actions of p21 may contribute to explain the results of our study, in which the increased *p21* mRNA did not reflect an increase in the expression of p21 protein.

In conclusion these results suggest the participation of p53 in the formation of fibroadenomas. The role of p21 in fibroadenomas remains to be defined. Thus, our results contribute to the knowledge of the mechanisms of cell cycle control in breast cells, of how proliferation is regulated and how it is deregulated in benign breast disorders like fibroadenoma.

Benign breast disorders are under a complex control system by local and systemic hormonal factors. Improving the knowledge of the biological behavior and hormonal dependence of these tumors may help to characterize subsets of patients with potentially different clinical behaviors and consequently decrease the number of invasive procedures such as biopsies and surgeries.

Patients and methods

Patients

The study population included premenopausal women undergoing surgical removal of breast nodules suggestive of fibroadenomas, seen consecutively in the Serviço de Mastologia of the Hospital de Clínicas de Porto Alegre (HCPA). Fourteen patients whose ages ranged from 15 to 41 years were selected for the study.

The sample size was calculated from a previous study [22], in order to detect a difference of 0.5 between the means of arbitrary units of gene expression of p21 and p53, for a sample power 90% and a significance level of 0.05.

Study protocol

All patients were submitted to a routine preoperative evaluation. Clinical and reproductive history was recorded, as well as use of sex steroids-containing drugs and parity. Body weight, height, and body mass index (BMI) were measured. Fragments of the central area of the

fibroadenoma and normal adjacent mammary tissue were obtained during the surgery, identified, and immediately frozen in liquid nitrogen and transferred to a −80°C freezer for posterior RNA extraction. The diagnosis of fibroadenoma was confirmed by histopathologic examination.

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA extraction was performed according to the method of guanidine thiocyanate [23]. The optical density ratios obtained (260/280 nm) of the RNA preparations were greater than 1.6. cDNA synthesis was performed from 2 μg of total RNA. After RNA denaturation together with *primer* Oligo$(dT)_{12–18}$ and 10 mM dNTPmix at 65°C for 5 min, a mixture of Tris–HCl 200 mM (pH 8.4), KCl 50 mM, $MgCl_2$ 25 mM, 10 mM dithiothreitol (DTT), and 0.1 M RNaseOUT was added and incubated for 2 min at 42°C, followed by addition of reverse transcriptase and incubation at 42°C for 50 min. The mixture was denatured at 70°C for 15 min, and incubated with *E. coli* Rnase H for 20 min at 37°C to destroy untranscribed RNA, as described by Brum et al. [24].

Two microliters of cDNA (with an expected cDNA yield of 4 ng) were denatured at 94°C for 2 min, in the presence of 200 mM Tris–HCl (pH 8.4), 500 mM KCl, and 50 mM $MgCl_2$. After this hot start, the reaction was cooled in ice and a mixture of 10 μl of the same Tris–HCl buffer and 50 mM $MgCl_2$ with dNTP mix, sense and antisense primers, and Taq polymerase were added, and submitted to amplification. Table 1 shows the characteristics of synthesized oligonucleotides for the amplification of specific cDNA fragments. All reagents were from Invitrogen (*SuperScript® Preamplification System for First Strand cDNA Synthesis*).

Of the final product of PCR, 10 μl were separated by electrophoresis in 1.5% agarose gel containing ethidium bromide. The bands were quantified by densitometric analysis through an image capturing system (ImageMaster VDS, Pharmacia Biotech, Uppsala, Sweden), and the result of normalization of the gene versus $\beta_2 m$ was expressed as arbitrary units (AU).

Western blots

To obtain whole tissue lysate, 0.2 g of fibroadenoma and normal adjacent mammary tissue were individually homogenized in 500 μl of 50 mM HEPES, pH 7.5, 1 mM PMSF, 10 μg/ml aprotinin, 100 mM NaF, 10 mM $Na4P_2O_7$, 2 mM NaVO4, 1% Triton X-100. Samples were shaken overnight at 4°C and centrifuged at 12,000×*g* for 30 min at 4°C. Whole tissue lysate was applied to a 10% or 15% SDS-PAGE and transferred to nitrocellulose by electroblotting. The nitrocellulose was washed with block solution (NET) containing NaCl (150 mM), EDTA (5 mM), Tris (50 mM), Triton X-100 (0.025%), and gelatin (0.25%), pH 7.4, and incubated with specific rabbit anti-p53 (Chemicon) or mouse anti-p21 (Upstate Biotechnology) diluted in NET. The bands were detected by a Western blotting detection system (chemoluminescence reaction; ECL, Amersham) with film (Kodak X-Omat) exposure for 15–60 s. The optical density (OD) of the bands obtained by chemoluminescence was measured by means of densitometric analysis with an image-processing system (ImageMaster VDS, Pharmacia Biotech). NC staining with Red Ponceau S was used to normalize the p21 and p53 protein amounts.

Statistical analysis

Results are presented as mean ± standard error of mean (SEM). Comparisons were analyzed by paired Student's *t* test. $P < 0.05$ was considered statistically significant.

Table 1 Characteristics of synthesized oligonucleotides for amplification of specific cDNA fragments

Gene	Synthesized sequence	Fragment (bp)	Reference
$\beta_2 m$			
Sense	5′ CTATCCAGCGTACTCCAAAG 3′	165	http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi
Antisense	5′ ACAAGTCTGAATGCTCCACT 3′		
p21			
Sense	5′ CTCAG7AGGAGGCGCCATG 3′	517	[25]
Antisense	5′ GGGCGGATTAGGGCTTCC 3′		
p53			
Sense	5′ AGGTGACCCAGGCTTGGAAG 3′	133	[26]
Antisense	5′ TCCTGACTCAGAGGGGGCTC 3′		

Acknowledgments This work was supported by Conselho Nacional de Desenvolvimento Científico e Tecnológico, Pró-Reitoria de Pesquisa Universidade Federal do Rio Grande do Sul, Grupo de Pesquisa e Pós-Graduação do Hospital de Clínicas de Porto Alegre, Fundação de Amparo à Pesquisa do Rio Grande do Sul.

References

1. P. Tobias, L.A. De Luca, F.C.L. Schmitt, U. Frederigue Jr, G. Uemura, Revista Brasileira de Mastologia **9**, 68–74 (1999)
2. C. Courtillot, G. Plu-Bureau, N. Binart, C. Balleyguier, B. Sigal-Zafrani, V. Goffin, F. Kuttenn, P.A. Kelly, P. Touraine, J. Mammary Gland Biol. Neoplasia **10**, 325–335 (2005)
3. A. Gompel, D. Levy, M. Chaouat, P. Forgez, W. Rostene, Maturitas **38**, 39–44 (2001)
4. S. La Rosa, F. Sessa, L. Colombo, M.G. Tibiletti, D. Furlan, C. Capella, J. Clin. Pathol. **54**, 37–41 (2001)
5. C.E. Caldon, R.J. Daly, R.L. Sutherland, E.A. Musgrove, J. Cell. Biochem **97**, 261–274 (2006)
6. A.E. Gururaj, S.K. Rayala, R. Kumar, Breast Cancer Res. **7**, 5–12 (2005)
7. A.L. Borresen-Dale, Hum. Mutat. **21**, 292–300 (2003)
8. P.P. Osin, S.R. Lakhani, Breast Cancer Res. **1**, 36–40 (1999)
9. A. Kalogeraki, J. Panayiotides, D. Tamiolakis, M. Tzardi, V. Chaniotis, G. Chalkiadakis, J. Melissas, D. Stiftsis, P. Kanavaros, G.S. Delides, Anticancer Res. **20**, 1801–1805 (2000)
10. W.S. el-Deiry, T. Tokino, V.E. Velculescu, D.B. Levy, R. Parsons, J.M. Trent, D. Lin, W.E. Mercer, K.W. Kinzler, B. Vogelstein, Cell **75**, 817–825 (1993)
11. A.L. Gartel, A.L. Tyner, Mol. Cancer Ther. **1**, 639–649 (2002)
12. B. Haendler, R. Hofer-Warbinek, E. Hofer, EMBO J. **6**, 947–950 (1987)
13. M.H. Lee, I. Reynisdottir, J. Massague, Genes Dev. **9**, 639–649 (1995)
14. K. Somasundaram, H. Zhang, Y.X. Zeng, Y. Houvras, Y. Peng, H. Zhang, G.S. Wu, J.D. Licht, B.L. Weber, W.S. El-Deiry, Nature **389**, 187–190 (1997)
15. R.A. Steinman, B. Hoffman, A. Iro, C. Guillouf, D.A. Liebermann, M.E. el-Houseini, Oncogene **9**, 3389–3396 (1994)
16. S.R. Romanov, B.K. Kozakiewicz, C.R. Holst, M.R. Stampfer, L.M. Haupt, T.D. Tlsty, Nature **409**, 633–637 (2001)
17. M. Ashcroft, Y. Taya, K.H. Vousden, Mol. Cell. Biol **20**, 3224–3233 (2000)
18. A.J. Levine, Cell **88**, 323–331 (1997)
19. D. Eliyahu, D. Michalovitz, S. Eliyahu, O. Pinhasi-Kimhi, M. Oren, Proc. Natl. Acad. Sci. USA **86**, 8763–8767 (1989)
20. C.A. Finlay, P.W. Hinds, A.J. Levine, Cell **57**, 1083–1093 (1989)
21. A.L. Gartel, S.K. Radhakrishnan, Cancer Res. **65**, 3980–3985 (2005)
22. R. Cericatto, A. Pozzobon, D.M. Morsch, C.H. Menke, I.S. Brum, P.M. Spritzer, Steroids **70**, 153–160 (2005)
23. P. Chomczynski, N. Sacchi, Anal. Biochem. **162**, 156–159 (1987)
24. I.S. Brum, D.M. Morsch, A. Pozzobon, V.A. Boeri, G. Geib, P.M. Spritzer, Horm. Res. **60**, 209–214 (2003)
25. C.N. Robson, V. Gnanapragasam, R.L. Byrne, A.T. Collins, D.E. Neal, J. Endocrinol. **160**, 257–266 (1999)
26. C.H. Sundfors, Y.U. Collan, Faseb J. **11**, 897–903 (1997)