Increased Expression of Cyclin-Dependent Kinase-Interacting Protein p21 during Tamoxifen-Induced Hepatocarcinogenesis in Female Rats

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Female Sprague-Dawley rats were given a single dose of tamoxifen (20 mg/kg body weight) by gavage or the same dose at 24-hr intervals for 2, 12, or 52 weeks, and the altered expression of cyclin-dependent kinase-interacting protein p21 (p21), tumor suppression protein p53, and the placental form of glutathione S-transferase (GST-P) in the liver was comparatively examined during the process of tamoxifen-induced hepatocarcinogenesis. The development of hepatocellular carcinoma was histopathologically observed only in rats administered tamoxifen for 52 weeks, but not in any other experimental groups. Significant increases in levels of the mRNA and protein of p21 were first observed in rats administered tamoxifen for 2 weeks, and the levels increased with further long-term treatment. Immunohistochemical analyses of p21 and GST-P in the liver of rats administered tamoxifen for 12 and 52 weeks revealed that the localization of p21-positive cells did not necessarily coincide with that of GST-P-positive cells. In the 52-week group, p21-positive cells, rather than GST-P-positive cells, were observed preferentially in the region consisting of histopathologically malignant cells. The present study demonstrated that during the process of tamoxifen-induced hepatocarcinogenesis, expression levels of the mRNA and protein of p21 were increased. The significance of the increased expression of p21 during hepatocarcinogenesis is discussed.

Key words —— tamoxifen, cyclin-dependent kinase-interacting protein p21, glutathione S-transferase, hepatocarcinogenesis, rat liver

INTRODUCTION

The hepatocarcinogenicity of tamoxifen in female rats has been reported,[1–4] although it is widely used not only as an anti-breast cancer drug[5,6] but also as a chemopreventive agent for breast cancer.[7] Recently, we have demonstrated the alteration of the gene expression of the tamoxifen-metabolizing enzymes,[8] DNA repair proteins/enzymes, and cell proliferation-associated proteins during the process of tamoxifen-induced hepatocarcinogenesis in female rats and discussed the correlation of those alterations with the development of hepatocarcinoma.[9]

In tumor biology, cyclin-dependent kinase-interacting protein p21 (p21) has received attention because it suppresses the cell cycle progression by inhibiting the activity of cyclin-dependent kinases[10,11] and by interacting with a proliferation cell nuclear antigen (PCNA).[12] Considering these functions of p21, it appears to be closely associated with the prevention of carcinogenesis.[12,13] However, the relationship between the expression of p21 and chemical carcinogenesis remains unclear.

In the present study, we investigated the altered expression of p21 and a placental form of glutathione S-transferase (GST-P), a marker enzyme for precancerous liver cells, during the process of tamoxifen-induced hepatocarcinogenesis in female rats.

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Table 1. Primer Sets Used for PCR for the Synthesis of DNA Standards and Real-Time PCR

Target gene	Primer set	Location of primer sequence in gene
	For synthesis of DNA standard	
p21		
Forward primer	5-AGCAAAGTATGCCGTCGTCT-3	124-143
Reverse primer	5-AGGCAGAAGATGGGGAAGAG-3	644-625
p53		
Forward primer	5-ATCTGGACGACAGGCAGACT-3	791-810
Reverse primer	5-AGGCAGTGAAGGGACTAGCA-3	1475-1456
β-Actin		
Forward primer	5-GACTTCGAGCAAGAGATGGC-3	664-683
Reverse primer	5-ACATCTGCTGGAAGGTGGAC-3	1066-1047
	For real-time PCR	
p21		
Forward primer	5-GACATCTCAGGGCCGAAAAC-3	489-508
Reverse primer	5-CGGCGCTTGGAGTGATAGAA-3	551-532
p53		
Forward primer	5-CCAAGAAGGGCCAGTCTACGT-3	1289-1309
Reverse primer	5-GCCCCACTTTCTTGATCATTG-3	1345-1325
β-Actin		
Forward primer	5-TACTGCCCTGGCTCCTAGCA-3	951-970
Reverse primer	5-GCCAGGATAGAGCCACCAATC-3	1040-1020

MATERIALS AND METHODS

Liver Tissue —— Rat liver tissues examined in the present experiments were the samples prepared in our previous experiments.[8,9] Briefly, female Sprague-Dawley rats were purchased from Charles River (Atsugi, Japan) and used at 6 weeks of age. Tamoxifen citrate (Sigma Chemical Co., St. Louis, MO, U.S.A.) was suspended in 0.5% carboxymethyl cellulose and used as tamoxifen. Female rats were administered a once-daily dose (20 mg/kg body weight) by gavage for the indicated periods. The numbers of animals used were 14 for the 52-week administration period and 5 for other periods. After 52-week administration, hyperplastic nodules were observed macroscopically in 12 of the 14 rats, and we selected five livers from those12 animals to be used in further experiments. In addition, livers with hyperplastic nodules were divided into nodular and nodule-surrounding sections, and those were used as separate samples. The control group of 5 rats was treated with vehicle (0.5% carboxymethyl cellulose solution) alone. Twenty-four hours after the final administration of tamoxifen or vehicle alone, the rats were killed by exsanguination under anesthesia. Their livers were quickly removed and stored in liquid nitrogen until use.

Preparation of RNA and cDNA —— Total hepatic RNA was prepared by the method as described previously.[9] Briefly, synthesis of complementary DNA (cDNA) from total liver RNA (2 μg) was performed in a reaction mixture (40 μl) containing a Not I-(dT)18 primer (Amersham Pharmacia Biotech, Buckinghamshire, U.K.) and an Omniscript RT Kit (Qiagen GmbH, Hilden, Germany).

Preparation of Standard DNAs for Real-Time PCR —— Standard DNAs for the quantitative determination of target mRNAs using real-time PCR were amplified with the cDNAs synthesized from total RNAs of untreated rat liver with the Omniscript RT Kit. The primer sets for standard DNAs were designed using primer 3 software (http://frodo.wi.mit.edu/cgibin/primer3/primer3_www.cgi) to interleave sequences amplified by real-time PCR and are summarized in Table 1. Amplification of each cDNA (1 μl of 1/10 diluted sample) examined was carried out with a thermal cycler (Perkin-Elmer Cetus, Norwalk, CT, U.S.A.). The PCR program used was: pretreatment at 94°C for 2 min; denaturation at 94°C for 20 sec; annealing at 57°C for 45 sec; and extension at 72°C for 45 sec. Numbers of the PCR cycle used were: 30 cycles for β-actin; and 40 cycles for p21 and tumor suppression protein p53 (p53). The PCR reaction mixture was subjected to 2% low-

melting agarose gel-electrophoresis (FMC BioProducts, Rockland, ME, U.S.A.), and separated DNA bands were visualized with ethidium bromide under UV light. Each DNA band corresponding to a target gene was isolated from the agarose gel using Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, U.S.A.), and the concentration of each DNA was determined spectrophotometorically based on absorbance at 260 nm: 1 optical density (OD) = 50 μg/ml. To determine the molecular weight (MW) of double-stranded DNA (standard DNA), we counted the numbers of adenine (A), guanine (G), cytosine (C), and thymine (T) in the sequence of the corresponding single-stranded DNA and assigned the number of nucleic acids using the formula: MW (single strand) = (nA $\times$ 313.21) + (nC $\times$ 289.18) + (nG $\times$ 329.1) + (nT $\times$ 304.29) – 61.96. Using the weight concentration obtained from the absorbance and MW of standard DNA, the mole concentration was calculated and copy concentration (copy number/μl) was determined by multiplying this value times the Avogadro constant. Preparations were adjusted to the range from 10^8 to 10 molecules with serial dilutions and used in the experiments.

Real-Time PCR —— All real-time PCRs were performed using the quantitative real-time method with the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, U.S.A.). Briefly, real-time PCR was performed in 50 μl of reaction mixture containing 25 μl of SYBR Green PCR Master Mix (Applied Biosystems), 100 nM of each primer, 5 μl of each cDNA preparation (1/10 dilution) or appropriately diluted standard DNA, and DEPC-water in 0.2-ml MicroAmp optical tubes (Applied Biosystems). Thermal cycling conditions comprised initial denaturation (at 95°C for 10 min) and extension (40 cycles at 95°C for 15 sec and 60°C for 1 min). Copies of mRNA were quantified and normalized to that of β-actin, an internal standard. Primer sets for p21, p53, and β-actin shown in Table 1 were designed using Primer Express (Applied Biosystems).

Histopathologic and Immunohistochemical Analyses —— Serial paraffin sections (3 μm thick) of the liver obtained from rats in each experimental group were cut and placed on poly-L-lysine-coated slides. After the sections were immersed in xylene and dehydrated with alcohol, they were stained with hematoxylin and eosin (H&E) and used for histopathologic analysis.

Immunohistochemical analysis of GST-P-positive foci was performed using the method described previously.[9] Immunohistochemical analysis of p21 was performed according to the following protocol. After endogenous peroxidase in a liver section was quenched by treatment with 3% hydrogen peroxide in phosphate-buffered saline (PBS) solution for 10 min, the section was immersed in 5% normal goat serum (IBL Co., Ltd., Gunma, Japan) at room temperature for 30 min, rinsed with Tris-buffered saline solution containing 0.1% Tween20 (TBS-T), and then incubated with a mouse anti-human $p21^{WAF1/Clip1}$ monoclonal antibody (DakoCytomation Co., Ltd., Glostrup, Denmark) overnight at 4°C. After the section was washed with TBS-T, it was incubated with K4000 EnVision+ peroxidase Mouse (DakoCytomation Co., Ltd.), a secondary antibody, at room temperature for 30 min. After washing three times with TBS-T, the liver section was stained with the 3,3-Diaminobenzidine Tetrahydrochloride Liquid System (DakoCytomation Co., Ltd.) at room temperature and then counterstained with hematoxylin. p21-Positive cells were expressed as the number per cm^2 of a tissue section using an image analyzer (LUZEX III; Nireco, Tokyo, Japan).

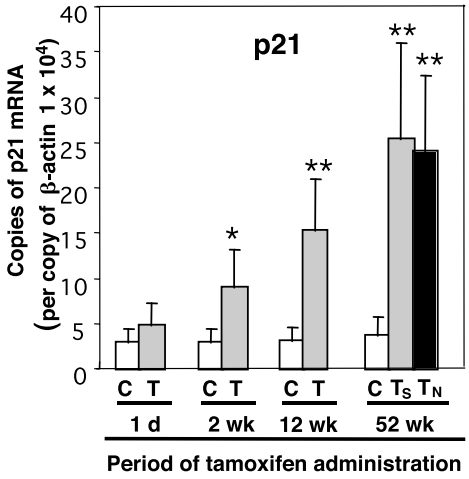

Fig. 1. Change in Expression Level of the p21 Gene during Tamoxifen-Induced Hepatocarcinogenesis

Copies of the p21 genes expressed were calculated based on that of β-actin 1×10^4, an internal control. C, control; T, tamoxifen treatment; T_S and T_N in the 52-week administration mean the nodule-surrounding tissues and hyperplastic nodules, respectively. The amount of each RT-PCR product was assayed in 5 individual rats in each experimental group, and the data shown are expressed as mean ± S.D. (n = 5). *,**Significant differences from the corresponding controls assessed by Student's t-test; *$p < 0.05$, **$p < 0.01$.

RESULTS

A significant increase in the gene expression level of hepatic p21 was first observed in rats administered tamoxifen for 2 weeks, and the level was markedly increased with further long-term administration (Fig. 1). On the other hand, no significant change in the gene expression of p53 was observed in any experimental group (Fig. 2).

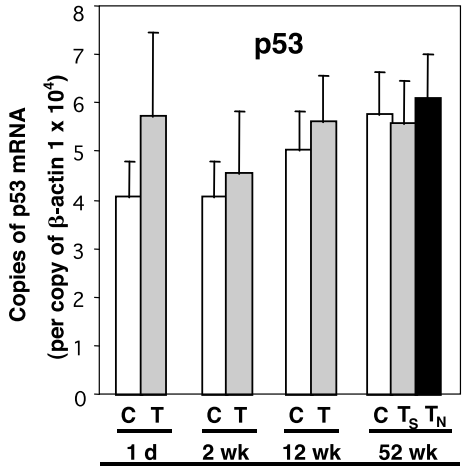

Fig. 2. Change in Expression Level of the p53 Gene during Tamoxifen-Induced Hepatocarcinogenesis

Copies of the p53 gene expressed were calculated based on that of β-actin 1×10^4, an internal control. C, control; T, tamoxifen treatment; T_S and T_N in the 52-week administration mean the nodule-surrounding tissues and hyperplastic nodules, respectively. The amount of each RT-PCR product was assayed in 5 individual rats in each experimental group, and the data shown are expressed as mean ± S.D. (n = 5). Significant differences from the corresponding controls assessed by Student's t-test.

Immunohistochemical analyses revealed that the number of p21-positive cells was significantly increased upon administration of tamoxifen for 12 weeks, but not for 2 weeks. The 52-week administration led to further increases in p21-positive cells. These results are summarized together with the previous results on GST-P-positive cells in Table 2. Briefly, GST-P-positive foci developed in the livers of rats administered tamoxifen for more than 12 weeks, and the GST-P-positive area in liver sections occupied greater than 60% of the total area after 52-week administration.

Immunohistochemical and histopathologic analyses (Fig. 3) revealed that the locations of p21-positive and GST-P-positive cells in a liver section differed. Most p21-positive cells (upper portions of Fig. 3A–3C) had more basophilic cytoplasms, a high nuclear cytoplasmic ratio, and severe structural atypisms as compared with GST-P-positive cells (lower portions of Fig. 3A–3C).

DISCUSSION

In the present experiments, we demonstrated an increase in the expression of the mRNA and protein of hepatic p21 without gene activation of p53 during the process of tamoxifen-induced hepatocarcinogenesis in female rats. To best of our knowledge, this is the first report of the overall change in

Table 2. Development of p21- and GST-P-Positive Cells in the Liver Tissue after Tamoxifen Administration in Female Rats

Treatment	p21-positive cells (no./cm^2)[a)]	GST-P-positive foci[b)] (no./cm^2)[a)]
1 day ($n = 5$)		
Control	0.92 ± 0.72	ND
Tamoxifen	13.62 ± 15.19	ND
2 weeks ($n = 5$)		
Control	0.92 ± 0.72	ND
Tamoxifen	39.64 ± 31.73	ND
12 weeks ($n = 5$)		
Control	2.41 ± 1.79	0.7 ± 0.7
Tamoxifen	59.41 ± 28.82*	10.9 ± 9.3**
52 weeks ($n = 5$)		
Control	9.79 ± 15.28	0.5 ± 0.7
Tamoxifen	2464.7 ± 2381.8*	NC

ND, not detected; NC, not counted (GST-P-positive area occupied more than 62% of a tissue section). *a*) Values are means ± S.D. *b*) Data in our previous report (Ref. 9) were used. Significant differences from the corresponding controls assessed using Student's t-test; *$p < 0.05$, **$p < 0.01$.

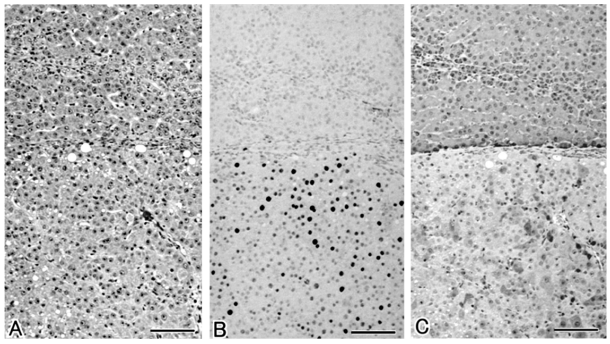

Fig. 3. Histologic Findings in the Liver of Female Sprague-Dawley Administered Tamoxifen for 52 weeks

A, H&E staining; B, immunodetection of p21; C, immunodetection of GST-P. Each bar indicates 50 μm.

gene expression of p21 during chemical-induced carcinogenesis. A significant increase in the level of p21 mRNA occurred with more than 2-week administration of tamoxifen, and the increases were not significantly different among rats administered tamoxifen for any period examined. However, no significant increase in the number of p21-positive cells was observed immunohistochemically up to 2 weeks of administration and thereafter the number of the p21-positive cells increased in a time–dependent fashion. Although this difference in the altered expression of the mRNA and protein of p21 indicates that the increase in p21 protein levels is not necessarily dependent on an increase in the corresponding mRNA, the reason for the difference remains unclear.

On the other hand, no significant change in the level of mRNA of p53, a positive transcription factor for *p21* gene,[14–16] was observed during the process of tamoxifen-induced hepatocarcinogenesis. During tamoxifen-induced hepatocarcinogenesis, an increase in the expression level of p21 is thought to occur in a p53-independent manner, although the mechanism of the increased expression remains unclear. In addition, such p53-independent expression of p21 has been reported to occur in muscle and other terminally differentiating cells.[17]

During the initiation-promotion process (~12 weeks) of tamoxifen-induced hepatocarcinogenesis, the expression pattern of p21, which promotes apoptosis and DNA repair by arresting the cell cycle in DNA-damaged cells,[18] was similar to those of tamoxifen-activating enzyme(s), especially the CYP3A subfamily enzymes and DNA repair enzyme,[9] suggesting that the increased gene expression of p21 was dependent on the development of tamoxifen-induced DNA damage in liver cells.

Immunohistochemical and histopathologic analyses of p21-positive and GST-P-positive cells in the liver tissue indicated that the localizations of these positive cells were not necessarily identical, and p21-positive cells, as compared with GST-P-positive cells, existed preferentially in the region with an abundance of cells with basophilic cytoplasms and severe structural atypisms as compared with GST-P-positive cells. Although GST-P-positive foci, a marker for hepatocarcinogenesis, often disappear in cancerous tissues,[19] p21-positive cells would developed even in cancerous tissues.[20]

Although p21-deficient mice show a greater tendency to develop spontaneous tumors in extrahepatic tissues (the lung, testis, skin, *etc*.) but not in the liver compared with wild-type mice,[21] p21 has an oncogenic function in some cases.[22] Previous reports and the present results suggest that there is tissue selectivity in the action of p21.

In the present experiments, the overall change in the expression of the mRNA and/or protein of p21 during tamoxifen-induced hepatocarcinogenesis was similar to those of cell proliferation-associated proteins, including c-myc, cyclin D1, and PCNA, as reported previously.[9] Thus the increased expression of p21 might be an important event in the development of hepatocarcinoma in female rats.

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REFERENCES

1) Greaves, P., Goonetilleke, R., Nunn, G., Topham, J. and Orton, T. (1993) Two-year carcinogenicity study of tamoxifen in Alderley Park Wistar-derived rats. *Cancer Res*., **53**, 3919–3924.
2) Hard, G. C., Iatropoulos, M. J., Jordan, K., Radi, L., Kaltenberg, O. P., Imondi, A. R. and Williams, G. M. (1993) Major difference in the hepatocarcinogenicity and DNA adduct forming ability between toremifene and tamoxifen in female Crl:CD (BR) rats. *Cancer Res*., **53**, 4534–4541.
3) Hirsimäki, P., Hirsimäki, Y., Nieminen, L. and Payne, B. J. (1993) Tamoxifen induces hepatocellular carcinoma in rat liver: a 1-year study with two antiestrogens. *Arch Toxicol*., **67**, 49–54.
4) Williams, G. M., Iatropoulos, M. J., Djordjevic, M.

J. and Kaltenberg, O. P. (1993) The triphenylethylene drug tamoxifen is a strong liver carcinogen in the rat. *Carcinogenesis*, **14**, 315–317.
5) Jordan, V. C. (1998) The development of tamoxifen for breast cancer therapy: a tribute to the late Arthur L.Walpole. *Breast Cancer Res. Treat.*, **11**, 197–209.
6) Baum, M., Brinkley, D. M., Dossett, J. A., Mcpherson, K., Patterson, J. S., Rubens, R. D., Smiddy, F. J., Stoll, B. A., Wilson, A., Richards, D. and Ellis, S. H. (1985) Controlled trial of tamoxifen as single adjuvant agent in management of early breast cancer. *Lancet*, **13**, 836–840.
7) Fisher, B., Costantino, J. P., Wickerham, D. L., Redmond, C. K., Kavanah, M., Cronin, W. M., Vogel, V., Robidoux, A., Dimitrov, N., Atkins, J., Daly, M., Wieand, S., Tan-Chiu, E., Ford, L. and Wolmark, N. (1998) Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J. Natl. Cancer Inst.*, **90**, 1371–1388.
8) Kasahara, T., Hashiba, M., Harada, T. and Degawa, M. (2002) Change in the gene expression of hepatic tamoxifen-metabolizing enzymes during the process of tamoxifen-induced hepatocarcinogenesis in female rats. *Carcinogenesis*, **23**, 491–498.
9) Kasahara, T., Kuwayama, C., Hashiba, M., Harada, T., Kakinuma, C., Miyauchi, M. and Degawa, M. (2003) The gene expression of hepatic proteins responsible for DNA repair and cell proliferation in tamoxifen-induced hepatocarcinogenesis. *Cancer Sci.*, **94**, 582–588.
10) Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K. and Elledge, S. J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell*, **75**, 805–816.
11) Xiong, Y., Hannon, G. J., Zhang, H., Casso, D., Kobayashi, R. and Beach, D. (1993) p21 is a universal inhibitor of cyclin kinases. *Nature* (London), **366**, 701–704.
12) Li, R., Waga, S., Hannon, G. J., Beach, D. and Stillman, B. (1994) Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* (London), **371**, 371534–371537.
13) Li, R., Hannon, G. J., Beach, D. and Stillman, B. (1996) Subcellular distribution of p21 and PCNA in normal and repair-deficient cells following DNA damage. *Curr. Biol.*, **6**, 189–199.
14) El-Deiry, W. S., Harper, J. W., O'Connor, P. M., Velculescu, V. E., Canman, C. E., Jackman, J., Pietenpol, J. A., Burrell, M., Hill, D. E., Wang, Y., Wiman, K. G., Mercer, W. E., Kastan, M. B., Kohn, K. W., Elledge, S. J., Kinzler, K. W. and Vogelstein, B. (1994) WAF1/CIP1 is induced in p53-mediated G1 arrest and apoptosis. *Cancer Res.*, **54**, 1169–1174.
15) Noda, A., Ning, Y., Venable, S. F., Pereira-Smith, O. M. and Smith, J. R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell. Res.*, **211**, 90–98.
16) El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W. and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell*, **75**, 817–825.
17) Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W. and Elledge, S. J. (1995) p53-independent expression of p21Cip1 in muscle and other terminally differentiating cells. *Science*, **267**, 1024–1027.
18) Geske, F. J., Nelson, A. C., Lieberman, R., Strange, R., Sun, T. and Gerschenson, L. E. (2000) DNA repair is activated in early stages of p53-induced apoptosis. *Cell Death Differ.*, **7**, 393–401.
19) Iida, K., Yamamoto, M., Kato, M., Yoshida, K., Kurita, K. and Tatematsu, M. (1999) Strong expression of glutathione S-transferase placental form in early preneoplastic lesions and decrease with progression in hamster buccal pouch carcinogenesis. *Cancer Lett.*, **135**, 129–136.
20) Martens, U., Lennartsson, P., Hogberg, J. and Stenius, U. (1996) Low expression of the WAF1/CIP1 gene product, p21, in enzyme-altered foci induced in rat liver by diethylnitrosamine or phenobarbital. *Cancer Lett.*, **104**, 21–26.
21) Martin-Caballero, J., Flores, J. M., Garcia-Palencia, P. and Serrano, M. (2001) Tumor susceptibility of p21 (Waf1/Cip1)-deficient mice. *Cancer Res.*, **61**, 6234–6238.
22) Roninson, I. B. (2002) Oncogenic functions of tumour suppressor p21 (Waf1/Cip1/Sdi1): association with cell senescence and tumour-promoting activities of stromal fibroblasts. *Cancer Lett.*, **179**, 1–14.