Receptor protein tyrosine kinase *DDR* is up-regulated by p53 protein

Shirou Sakuma[a,*], Hideyuki Saya[b], Mitsuhiro Tada[a], Mitsuyoshi Nakao[b], Toshiyoshi Fujiwara[c], Jack A. Roth[d], Yutaka Sawamura[a], Yumiko Shinohe[a], Hiroshi Abe[a]

[a]*Department of Neurosurgery, University of Hokkaido School of Medicine, North 15, West 7, Kita-ku, Sapporo 060, Japan*
[b]*Department of Tumor Genetics and Biology, Kumamoto University School of Medicine, Kumamoto 860, Japan*
[c]*First Department of Surgery, Okayama University Medical School, Okayama 700, Japan*
[d]*Section of Thoracic Molecular Oncology, Department of Thoracic and Cardiovascular Surgery, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030 USA*

Received 13 September 1996; revised version received 18 October 1996

Abstract We have previously reported on radiation-induction of *ptk-3* in rat astrocyte culture [Sakuma et al. (1995) Radiat. Res. 143, 1–7]. Ptk-3 was considered to be a rat version of human DDR (discoidin domain receptor). We cloned and analyzed genomic DNA of the DDR and its promoter region. We discovered that the promoter region contained a consensus sequence of the p53 tumor suppressor binding site. Adenovirus-mediated p53 transfection induced a high level of DDR mRNA in SAOS2 human osteosarcoma cells. These results indicate that *DDR* is up-regulated by the p53 protein.

Key words: Genomic DNA structure; Human *DDR* gene; p53 regulatory element

1. Introduction

DNA damage caused by ionizing radiation effects many genes which exert fundamental cellular functions including DNA repair, cell cycle checkpoint, and apoptosis. Previously, we identified a new receptor tyrosine kinase gene *ptk-3* that was induced by ionizing radiation on rat astrocyte cultures using the mRNA differential display method [1]. Ptk-3 is considered a membrane-associated receptor which has a factor VIII-like extracellular domain, glycine proline-rich transmembrane domain, and a tyrosine kinase structure in the intracellular domain [2]. The human homologue of ptk-3 has been cloned by three independent groups, and named the discoidin domain receptor (DDR [3]), tyrosine receptor kinase E (TrkE [4]) and cell adhesion kinase (Cak [5]). Recently, Barker et al. [6] reported high level expression of DDR in human breast cancer cells as compared with more benign or normal cells, thus suggesting that DDR is involved with oncogenesis. However, at present, neither the ligand of this unique receptor nor its function is known.

In order to determine the biological functions of the receptor, we examined the genomic DNA structure of the human DDR gene and analyzed its promoter region. Here, we show that the DDR promoter region contains a p53 regulatory element, and that the gene is up-regulated by adenovirus-mediated p53 expression in the SAOS2 osteosarcoma cell line.

2. Materials and methods

2.1. Cloning and sequencing of genomic DNA of DDR

An *Sau*3A partially digested human genomic DNA library (Clontech, CA, USA) was used as a PCR template to amplify each intron. PCR primers were designed inside each pair of two *Sau*3A restriction sites according to the cDNA sequence of DDR (accession no. L11315). The PCR reaction was run in 20 μl of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 0.25 mM dNTP, 0.4 μM each primer and 2.5 units of recombinant Taq DNA polymerase (Takara, Ootsu, Japan). PCR consisted of 30 cycles at 95°C for 45 s, 62°C for 1 min, 72°C for 2 min, and then 5 min elongation at 72°C. A PromoterFinder DNA Walking Kit (Clonetech) was also used to obtain clones having 5′ and 3′ ends, or longer introns. The first PCR was run in 20 μl of 1 × reaction buffer, 0.2 mM dNTP, 1.1 mM $Mg(OAc)_2$, 0.2 μM adaptor primer (AP-1), 0.4 μM PM142 R (5′-AAAGATGACAGGGCCTCTGGTCCCAT-3′), 32 U/ml recombinant Tth DNA polymerase XL (Perkin Elmer, NJ, USA), and 56 nM Tth Start Antibody (Clontech). PCR consisted of 95°C for 1 min, then 35 cycles at 95°C for 30 s, 68°C for 5 min, followed by 8 min elongation at 68°C. 1 μl of a 1/100 dilution of primary PCR products were used as a template for the second PCR reaction, under the same conditions as the primary one except for 0.2 μM adaptor primer (AP-2), 0.4 μM PM118R (5′-TGATCCCTCGGGCCTAAG-3′), and 20 PCR cycles. The PCR products were then cloned into the TA cloning vector pCRII (Invitrogen, CA, USA) and sequenced on an automated sequencer Model 373A (Applied Biosystems, Chiba, Japan) with the DyeDeoxy Terminator Cycle Sequencing kit (Perkin Elmer, Chiba, Japan).

2.2. Determination of chromosomal localization of DDR by radiation hybrid mapping

In order to determine the chromosomal localization of the *DDR*, we used a new PCR-based technique designated as 'Radiation Hybrid Mapping' [7]. Two PCR primers were designed according to the nucleotide sequence of the 5′ end of the genomic *DDR* sequence: PM82F (5′-TTTCTGCCATTGAGCATG-3′, corresponding to nt 1489–1506) and PM75R (5′-CGGCTTAGAACAAACAAC-3′, complementary to nt 1795–1812, accession no. U48705), and were used to amplify the DNA templates of the GeneBridge 4 panel (Research Genetics, Inc., Huntsville, AL). The PCR was run in a total reaction volume of 20 μl: 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.2 mM dNTP, 2 mM $MgCl_2$, 0.4 μM PM82F, 0.4 μM PM 75R, 2.5 U Taq DNA polymerase (Perkin Elmer, NJ, USA) and 2 μl of each DNA template. The PCR cycle consisted of 94°C for 5 min, then 35 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min, followed by 5 min of after-extension at 72°C. 93 DNA templates of the GeneBridge 4 panel were amplified, and the resultant 324-bp PCR products were analyzed on 1% agarose gel electrophoresis with ethidium bromide staining. The PCR results were coded into binary form (10000 00000 00100 00000 21000 10000 10010 10100 02020 10000 10110 00001 01101 00010 00102 01100 01210 00000 001) according to the instruction manual and sent to the Whitehead Institute/ MIT Center for Genome Research (E-mail: http://www-genome.wi.mit.edu/).

2.3. Adenovirus-mediated p53 transfer and Northern blotting

Human osteosarcoma cell line SAOS2, which does not express WT p53 protein [8], was transfected with p53- or luciferase-expression adenovirus vector as described elsewhere [9]. Satisfactory expression of the p53 gene was confirmed with a reverse-transcription PCR (data not shown). 24 h or at the indicated times after transfection, total RNA was isolated by using RNazol (Cinna/Biotecx Laboratories, Friendswood, TX) and electrophoresed through formaldehyde/agarose gels, and transferred to Hybond-N membranes (Amersham, Bucks, UK). In order to detect the *DDR* mRNA signal, the partial

*Corresponding author. Fax: (81) (11) 706-7878.
E-mail: ssakuma@med.hokudai.ac.jp

PII S0014-5793(96)01234-3

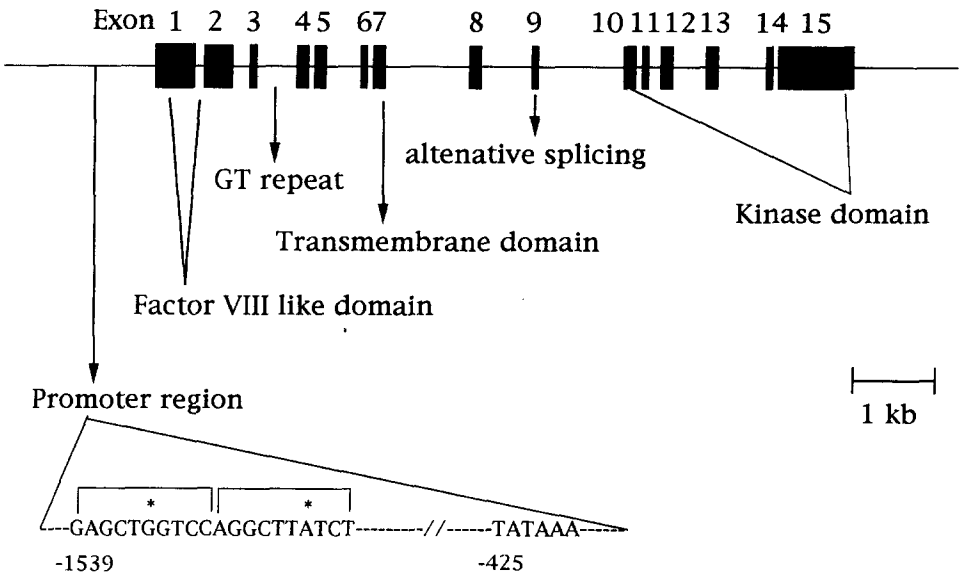

Fig. 1. Exon-intron organization of the genomic *DDR* gene. Filled boxes represent each exon. The tandem sequence of p53 protein binding consensus in the promoter region (1539 bp upstream of the translation initiation site) is shown. The TATA binding sequence is also shown (425 bp upstream of the translation initiation site). The asterisk indicates mismatched bases compared with the p53 consensus sequence.

cDNA sequence of *DDR* was amplified by PCR using two primers; PM1501 (5′-GTGTTGGAAGAGGAGCTGACGGTTCA-3′ corresponding to nt 1501–1526), and PM2880 (5′-CACCGTGTTGAGTGCATCCTCTGCCA-3′, complementary to nt 2855–2880, GenBank accession no. L11315), subcloned into the TA cloning vector (Invitrogen), and verified by sequencing. The membranes was prehybridized in Hybrisol I (Oncor, Gaithersburg, MD), followed by hybridization with the ^{32}P-labeled *Eco*RI-digested fragment at 45°C overnight. After washing, the membranes were exposed to Kodak X-omat AR film at −70°C for 4 h to 2 days. As a control, the membranes were also probed with a p21 probe and G3PDH (glyceraldehyde-3-phosphate dehydrogenase, Clontech). The p21 probe contained a partial cDNA sequence which was amplified by PCR with two primers: PM21F (5′-ATGTCAGAACCGGCTGGGGATGTCCG-3′, corresponding to nt 76–101), and PM21MR (5′-TTAGGGCTTCCTCTTGGAGAAGATCA-3′, complementary to nt 545–570, GenBank accession no. U03106), subcloned into the TA cloning vector (Invitrogen), and verified by sequencing.

2.4. Gel shift assay

The oligonucleotides 5′-CAACAGGGTGAGCTGGTCCAGGCTTATCTGATGT-3′ (oligomer DDR-34F) and 5′-TTAAACATCAGATAAGCCTGGACCAGCTCACCCT-3′ (oligomer DDR-34R), corresponding to the DDR promoter sequences from −1548 to −1511 bp, were annealed to produce a double-stranded DNA probe. The wt-p53 protein was generated and purified according to [10]. For gel shift assay, 0.1 μg wt-p53 protein was pre-incubated with monoclonal anti-p53 antibodies Pab 421 or DO-1 (Oncogene Science, Inc.), 2 μg of poly(dI·dC) and binding buffer (10 mM Tris-HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA, 4% glycerol, 2 mM $MgCl_2$, 1 mM dithiothreitol, 0.5 mM PMSF and 1 μg/ml pepstatin A) on ice for 15 min. 0.5 ng of ^{32}P-end-labeled probe (1×10^5 cpm) was then added. After 90 min, the samples were subjected to electrophoresis in 6% non-denaturing polyacrylamide gels using 0.25×TBE (25 mM Tris-HCl, 22 mM borate, 0.25 mM EDTA). The gels were then dried and exposed to Kodak X-omat AR film at −70°C for a few hours.

3. Results

3.1. Genomic DNA sequence of DDR

A total of 12010 bases were determined for the genomic DNA sequence of the *DDR* and deposited in GeneBank (accession no. U48705). Fig. 1 shows a schematic representation of the structure of the gene, which consists of 15 exons and 14 introns. Analysis of the promoter region revealed a tandem repeat of the consensus binding sequence of the p53 tumor suppressor protein with one base mismatch (5′-PuPuPuC(A/T)(T/A)GPyPyPy-3′, [11]) at 1114 bases upstream of the TATA box and 1539 bp upstream of the translation-initiation site.

Analyzing the intron sequences revealed a 46-base long GT repeat in the third intron, which has not previously been reported as a dinucleotide repeat marker.

3.2. Chromosomal localization of DDR

Fig. 2 shows the results of radiation hybrid mapping. The chromosomal assignment of the *DDR* was within 2.33 cR (centiRay) from D6S478 (Lod score > 3.0). The D6S478 marker is located between D6S258 and D6S273 [12]. Although certain difficulties remain because of its rather incomplete correlation with the cytological and physical genetic maps, both D6S258 and D6S273 are known to be mapped to human chromosome 6p21.3 [12], which is consistent with the results of FISH analysis by Edelhoff et al. [13].

3.3. Northern blotting

Fig. 3 shows the results of Northern blot analysis of the SAOS2 cell line, transfected with the adenovirus-mediated p53 expression vector (Ad-p53) in comparison with those with luciferase expression vector (Ad-Luc) as a control. Fig. 3 also shows the time course after transfection. A strong signal of *DDR* mRNA was detected 24 and 36 h after transfection with the p53 expression vector in comparison with those with the luciferase expression vector, indicating a transcriptional activation of *DDR* gene by the p53 protein. In order to confirm that the p53-mediated transcriptional activation actually occurred due to adenovirus transfection, the same blot was also hybridized with a p21 probe, since p21/waf-1 is well known as a downstream mediator of p53 [14]. The p21 signal in the p53 expression vector transfected SAOS2 cells was markedly stronger than that of the luciferase control vector.

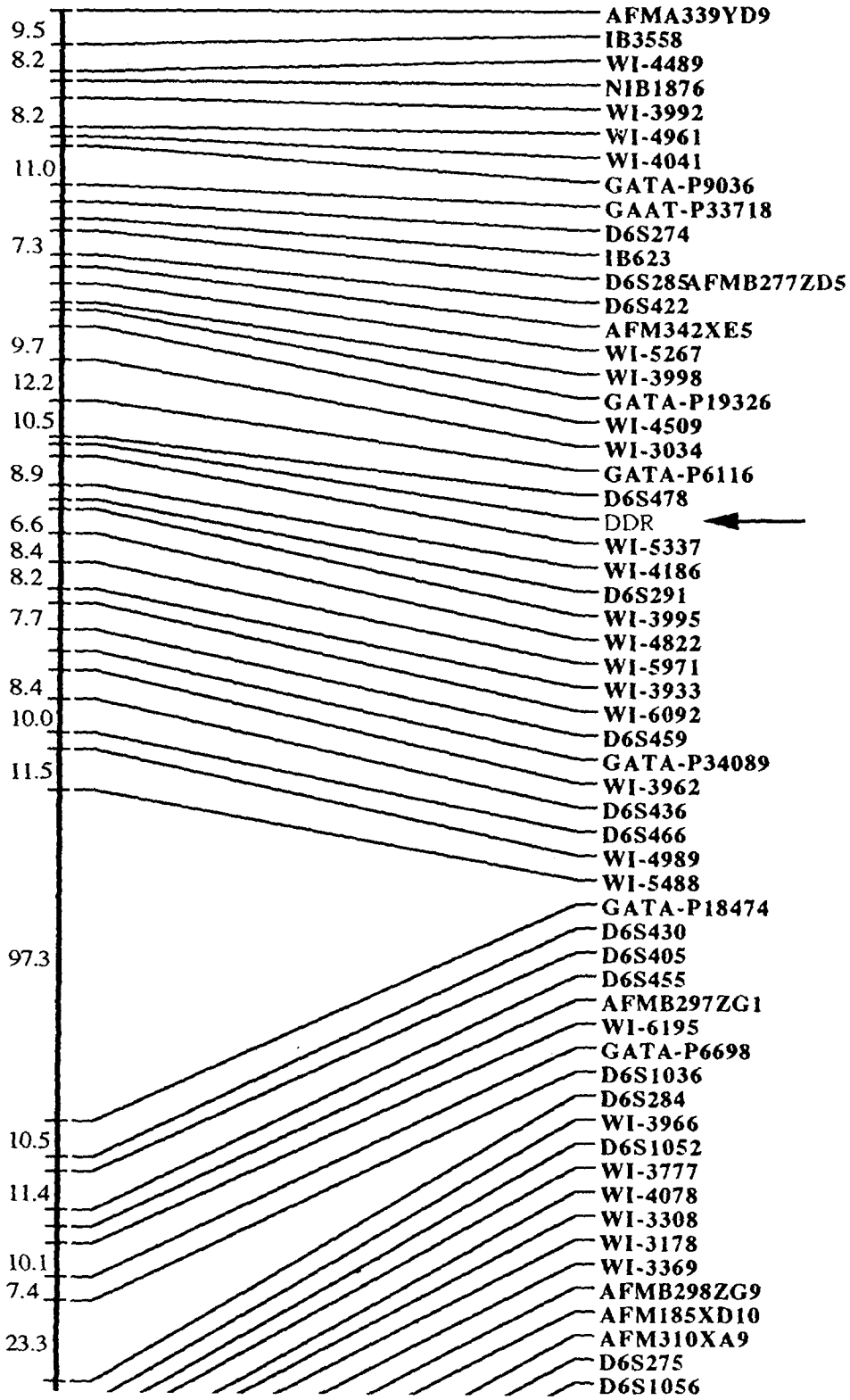

Fig. 2. Results of radiation hybrid mapping. Chromosomal assignment of the DDR was within 2.33 cR (centiRay) from D6S478 (Lod score > 3.0), which is consistent with the results of Edelhoff et al. [13], 6p21.3.

3.4. Gel shift assay

Fig. 4 show the results of the gel shift assay. The arrow indicates the presence of the shifted bands which are thought to represent a complex of the DDR probe and wt-p53 protein. The shifted bands disappear on incubation with excess cold probe, however, the supershifted bands which are thought to be a complex of the DDR probe, wt-p53 protein and the monoclonal antibodies Pab421, were not detected. The same results were obtained by using monoclonal antibodies DO-1 instead of Pab421 (data not shown).

4. Discussion

Evidence is accumulating that tumor suppressor protein p53 functions as a transcriptional factor and mediates induction of various genes following DNA damage by ionizing radiation. Genes so far known to contain the p53 regulatory element include *p21/WAF-1* [14,15], *Gadd-45* [16], *Bax* [17], and the retinoblastoma susceptibility (*Rb*) gene [18]. Some of these (*p21, Gadd45, Rb*) are related to cell cycle arrest (checkpoint) and repair of damaged DNA. However, their precise function or role in radiation-induced DNA damage are unknown.

Among these p53 regulatory genes, the chromosomal localization of *p21* was reported to be 6p21.2 by El-Deiry et al. [14]. Edelhoff et al. [13] reported the chromosomal localization of *DDR* to be 6p21.3. We confirmed this result by using radiation hybrid mapping, since Shelling et al. [19] reported that the chromosomal localization of *EDDR1* (epithelial-specific receptor kinase 1), which is considered to be a short transcript form of the *DDR*, was 6q16 as determined via FISH analysis. In addition to the p53-mediated induction of *DDR*, the near chromosomal localization of *DDR* to *p21* suggests that *p21* and *DDR* may share intimate or related functions downstream of the p53 cascade. Besides *p21*, *HLA* (human leukocyte antigen) associated genes and *TNF* (tumor necrosis factor)-α/β genes are also known to be located at 6p21.3 (NCBI, OMIM Gene Map).

The *ptk-3* gene, the rat homologue of human *DDR*, is induced by ionizing radiation in normal rat astrocyte culture [1]. Taken together with the present results concerning the up-regulation of *DDR*, *DDR/ptk-3* is considered to be a conserved gene which is transcriptionally activated by the induced p53 protein subsequent to DNA damage. Interestingly, Di Marco et al. [4] reported the expression of a short transcript form of *DDR* named *trkE* in normal human keratinocytes, and speculated from several lines of evidence that nerve growth factor (NGF) might be a candidate ligand for trkE.

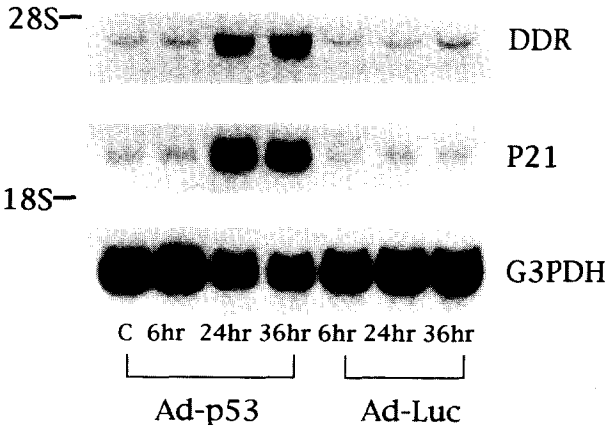

Fig. 3. Northern blot analysis of SAOS2 cell line; time course after the transfection with adenovirus-mediated p53 expression vector (Ad-p53) in comparison with cells transfected with the luciferase expression vector (Ad-Luc). A strong signal of *DDR* mRNA was detected 24 and 36 h after transfection with p53 expression vector as compared with those with the luciferase expression vector. In order to confirm the successful transfection of Ad-p53, the same blot was also hybridized with a p21 probe. The p21 signal in Ad-p53 transfected SAOS2 cells was markedly stronger than that of Ad-Luc. G3PDH was used as a loading control.

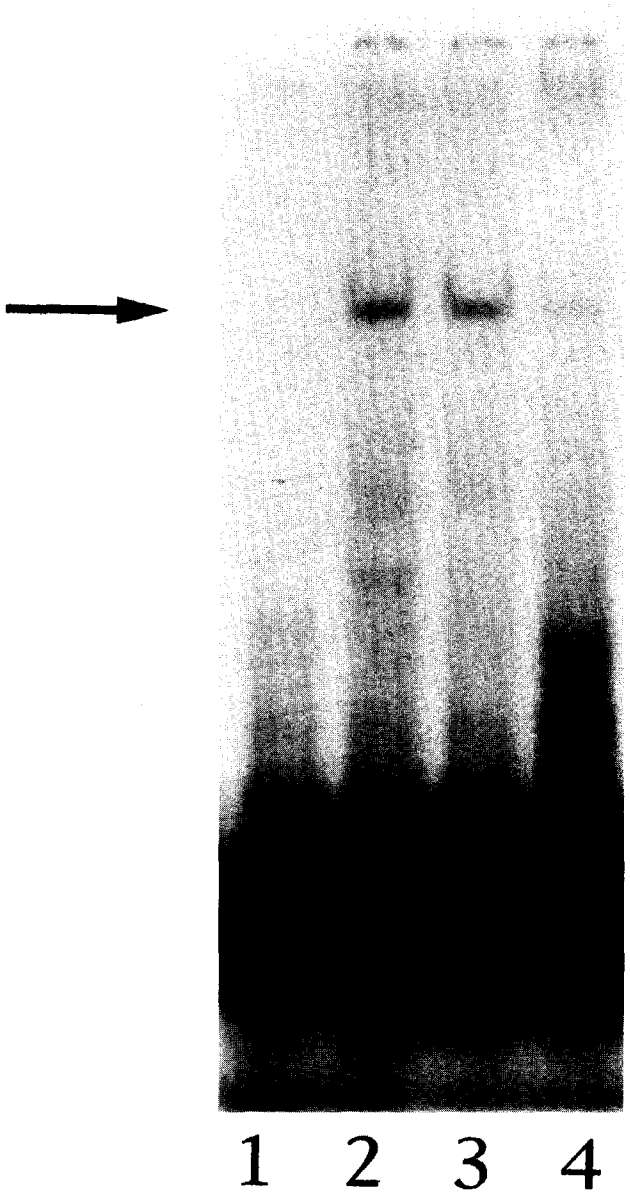

Fig. 4. Results of gel shift assay. Lane 1 shows the probe alone and the arrow indicates the presence of the shifted bands which are thought to be a complex of the DDR probe and wt-p53 protein (lane 2). The shifted bands disappeared on incubation with a 50-fold excess of cold probe (lane 4), however, supershifted bands which are thought to be a complex of the DDR probe, wt-p53 protein and monoclonal antibodies Pab421, were not detected (lane 3).

NGF is a neurotrophic factor necessary for the survival of neuronal cells; in situations where NGF is lacking, cells may lead to apoptotic death [20]. Thus, p53-mediated *DDR* induction may be related to a modification of apoptotic cellular process which occurs after exposure to DNA-damaging agents such as ionizing radiation. Further investigation is warranted concerning the physiological and pathophysiological roles of DDR/ptk-3.

References

[1] Sakuma, S., Saya, H., Ijichi, A. and Tofilon, P.J. (1995) Radiat. Res. 143, 1–7.

[2] Sanchez, M.P., Tapley, P. Saini, S.S., He, B., Pulido, D. and Barbacid, M. (1994) Proc. Natl. Acad. Sci. USA 91, 1819–1823.
[3] Johnson, J.D., Edman, J.C. and Rutter, W.J. (1993) Proc. Natl. Acad. Sci. USA 90, 5677–5681.
[4] Di Marco, E., Cutuli, N., Guerra, L. Cancedda, R. and De Luca, M. (1993) J. Biol. Chem., 268, 24290–24295.
[5] Perez, J.L., Shen, X., Finkernagel, S., Sciorra, L., Jenkins, N.A., Gilbert, D.J., Copeland, N.G. and Wong, T.W. (1994) Oncogene 9, 211–219.
[6] Barker, K.T., Martindale, J.E., Mitchell, P.J., Kamalati, T., Page, M.J., Phippard, D.J., Dale, T.C., Gusterson, B.A. and Crompton, M.R. (1995) Oncogene 10, 569–575.
[7] Hudson, T.J., Stein, L.D. Gerety, S.S., Ma, J., Castle, A.B., Silva, J., Slonim, D.K., Baptista, R., Kruglyak, L., Xu, S., Hu, X., Colbert, A.M.E., Rosenberg, C., Reeve-Daly, M.P., Rosen, S., Hui, L., Wu, X., Vestergaard, C., Wilson, K., Bae, J.S., Maitra, S., Ganiatsas, S., Evans, C.A., DeAngelis, M.M., Ingalls, K.A., Nahf, R.W., Horton Jr, L.T., Anderson, M.O., Collymore, A.J., Ye, W., Kouyoumjian, V., Zemsteva, I.S., Tam, J., Devine, R., Courtney, D.F., Renaud, M.T., Nguyen, H., O'Connor, T.J., Fizames, C., Faure, S., Gyapay, G., Dib, C., Morissette, J., Orlin, J.B., Birren, B.W., Goodman, N., Weissenbach, J., Hawkins, T.L., Foote, S., Page, D.C. and Lander, E.S. (1995) Science 270, 1945–1954.
[8] Chandar, N., Billig, B., McMaster, J. and Novak, J. (1992) Br. J. Cancer 65, 208–214.
[9] Fujiwara, T., Grimm, E.A., Mukhopadhyay, T., Zhang, W., Owen-Schaub, L.B. and Roth, J.A. (1994) Cancer Res. 54, 2287–2291.
[10] Takenaka, I., Morin, F., Seizinger, B.R. and Kley, N. (1995) J. Biol. Chem. 270, 5405–5411.
[11] El-Deiry, W.S., Kern, S.E., Pietenpol, J.A., Kinzier, K.W. and Vogelstein, B. (1992) Nat. Genet. 1, 45–49.
[12] Chumakov, I.M., Rigault, P., Le Gall, I. Ballanne-Chantelot, C., Billaut, A., Guillou, S., Soularue, P., Guasconi, G., Poullier, E., Gros, I., Belova, M., Sambucy, J., Susini, L., Gervy, P., Gilbert, F., Beaufils, S., Bui, H., Massart, C., De Tand, M., Dukasz, F., Lecoulant, S., Ougen, P., Perrot, V., Saumier, M., Soravito, C., Bahouayila, R., Cohen-Akenine, A., Barillot, E., Bertrand, S., Codani, J., Caterina, D., Georges, I., Lacroix, B., Lucotte, G., Sahbatou, M., Schmit, C., Sangouard, M., Tubacher, E., Dib, C., Faure, S., Fizames, C., Gyapay, G., Millasseau, P., Nguyen, S., Muselet, D., Vignal, A., Morissette, J., Menninger, J., Lieman, J., Desai, T., Banks, A., BrayWard, P., Ward, D., Hudson, T., Gerety, S., Foote, S., Stein, L., Page, D.C., Lander, E.S., Weissenbach, J., Paslier, D.L. and Cohen, D. (1995) Nature 377, 189–238.
[13] Edelhoff, S., Sweetser, D.A. and Disteche, C.M. (1995) Genomics 25, 309–311.
[14] El-Deiry, W.S. Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzier, K.W. and Vogelstein, B. (1993) Cell 75, 817–825.
[15] Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K. and Elledge, S.J. (1993) Cell 75, 805–816.
[16] Hollander, M.C., Alamo, I., Jackman, J., Wang, M.G., McBride, O.W. and Fornace, A.J., Jr. (1993) J. Biol. Chem. 268, 24385–24393.
[17] Miyashita, T. and Reed, J.C. (1995) Cell 80, 293–299.
[18] Osifchin, N.E., Jiang, D., Ohtani-Fujita, N., Fujita, T., Carroza, M., Kim, S.-J., Sakai, T. and Robbins, P.D. (1994) J. Biol. Chem. 269, 6383–6389.
[19] Shelling, A.N., Butler, R., Laval, S., Boyle, J.M. and Ganesan, T.S. (1995) Genomics 25, 584–587.
[20] Pittman, R.N., Wang, S., DiBenedetto, A.J. and Mills, J.C. (1993) J. Neurosci. 13, 3669–3680.