

New Monoclonal Antibodies Recognizing the p53 Tumour Suppressor Protein Homologue p73

P. ČEŠKOVÁ¹, R. NENUTIL², S. BRAY³, M. SVITÁKOVÁ¹, S. BABČANOVÁ¹, S. ULDRÍJAN¹, B. VOJTĚŠEK¹

¹Department of Experimental Oncology, Masaryk Memorial Cancer Institute, Brno, Czech Republic

²Department of Pathology, Faculty Woman's Hospital, Brno, Czech Republic

³Department of Molecular and Cellular Pathology, Ninewells Medical School, The University of Dundee, Scotland, UK

Background

The *p53* tumour suppressor gene is the most frequent target for genetic alterations in human tumours (Nigro et al., 1989; Hollstein et al., 1991) and encodes a nuclear phosphoprotein which plays a key role in cell cycle regulation. This protein protects cells from undergoing tumorigenic alterations by inducing either cell cycle arrest or programmed cell death in response to a variety of cellular stress signals (Ko and Prives, 1996; Levine, 1997) and also plays an important role in maintaining the integrity of the genome (Lane, 1992). Attempts to find *p53* homologues analogous to the *pRb* family of tumour suppressors were successful and a new gene, *p73*, has been identified on the short arm of chromosome 1 in a region frequently deleted in neuroblastoma (Kaghad et al., 1997; Yang et al., 1998). This gene encodes several proteins with structural and functional homology to *p53* (De Laurenzi et al., 1998) that can activate transcription of the *p21^{WAF1}* gene responsible for cell-cycle arrest, and can also induce apoptosis when overexpressed (Jost et al., 1997; Kaghad et al., 1997). There are several differentially spliced variants of mRNAs, which are translated into different *p73* proteins. This splicing occurs at the 3' end of the sequence and translated proteins have differing C-termini, in which *p73α* is a full-length protein and the *β* and *δ* isoforms are truncated forms of this protein. The *δ* isoform lacking the major part of the C-terminal

region is most similar to *p53*. The *γ* isoform contains a different 75-residue C-terminus compared to the *α* isoform due to a long alternative reading frame in this region (Kaghad et al., 1997; De Laurenzi et al., 1998; De Laurenzi et al., 1999; Ueda et al., 1999). *p73* as well as *p53* contains a transactivation domain, a DNA-binding domain and an oligomerization domain. The highest level of homology is exhibited in the DNA-binding domain (63% identity between the *p53* and *p73*), and this finding suggests that both these proteins can bind to the same DNA sequences and transactivate the same target genes. The conservation of high homology in the oligomerization domain suggests that the members of this family could form mixed oligomers. Although the existence of these mixed oligomers *in vivo* is still open to question, the availability of specific antibodies could help to address this problem.

The identification of a family of *p53*-related transcription factors that can be potentially redundant in their ability to activate the same cellular responses (i.e. cell-cycle arrest and apoptosis) has encouraged a study into the basis for their similarities and differences in terms of their physical and functional interactions with one another, their mechanism of activation and their regulation. Monoclonal antibodies specific to different forms of the *p73* protein therefore provide a powerful tool to study these proteins both *in vivo* and *in vitro*.

Description of the antibodies p73-1.1 and p73α-1.1

Production

The hybridoma cell lines producing *p73*-1.1 and *p73α*-1.1 monoclonal antibodies recognizing the *p73α* protein were selected after immunization of BALB/c mice with either the purified *p73α* protein expressed in bacteria for development of antibody *p73*-1.1 or peptide QDLKQGHDYSTAQQ coupled to keyhole limpet haemocyanin (KLH) for development of antibody

Received August 20, 2001. Accepted September 5, 2001.

This work was supported by the grant NS 6404-3/2000 from the Grant Agency of the Ministry of Health of the Czech Republic and by the grant A4004110/207/01 from the Grant Agency of Academy of Sciences of the Czech Republic.

Corresponding author: Bořivoj Vojtěšek, Masaryk Memorial Cancer Institute, Žlutý kopec 7, 656 53 Brno, Czech Republic. Tel.: +420 (5) 43133303; Fax: +420 (5) 43211696; e-mail: vojtesek@mou.cz.

p73 α -1.1. The mouse splenocytes were fused with non-producing myeloma cell line Sp2/0 using polyethylene glycol as a fusogen. The dot-blot using specific and non-specific peptides coupled to bovine serum albumin (BSA) was used as a screening method for a panel of antibodies raised against the peptide derived from the p73 α protein sequence. Monoclonal antibodies developed against the p73 α protein were screened by Western blotting of the partially purified p73 α protein expressed in bacteria. Hybridoma cell lines were prepared using limiting-dilution cloning and re-cloning (Harlow and Lane, 1988). Monoclonal antibodies were more precisely characterized using different isoforms of the p73 protein over-expressed in a cell line transfected with plasmids carrying the cDNA coding the p73 isoforms α , β , γ and δ .

Specificity

Initially the specificity of p73-1.1 and p73 α -1.1 MABs was tested by Western blotting against p73 α expressed in *E. coli* performed under denaturing conditions. To test the specificity of MABs to different isoforms of the p73 protein, human lung carcinoma cell line H1299 expressing detectable levels of only the γ isoform of endogenous p73 protein was transfected with the plasmid pcDNA3 carrying the cDNA for p73 α , p73 β , p73 γ and p73 δ using EffecteneTM Transfection Reagent (Qiagen, Hilden, Germany). Non-transfected H1299 cells were used as a negative control. To prove the high levels of p73 isoform expression in transfected cells, the extracts were immunoblotted with the p73 (H-79) rabbit polyclonal sera (Santa Cruz Biotechnology, Santa Cruz, CA) specific to the N-terminus (amino acids 1–80) of p73, and recognizing all known p73 protein isoforms (data not shown). Western blot analysis employing p73-1.1 and p73 α -1.1 MABs detected the purified p73 α protein from the bacteria as well as the transfected p73 α protein in H1299 cells (Figs. 1 and 2). The p73-1.1 antibody also binds the γ isoform with

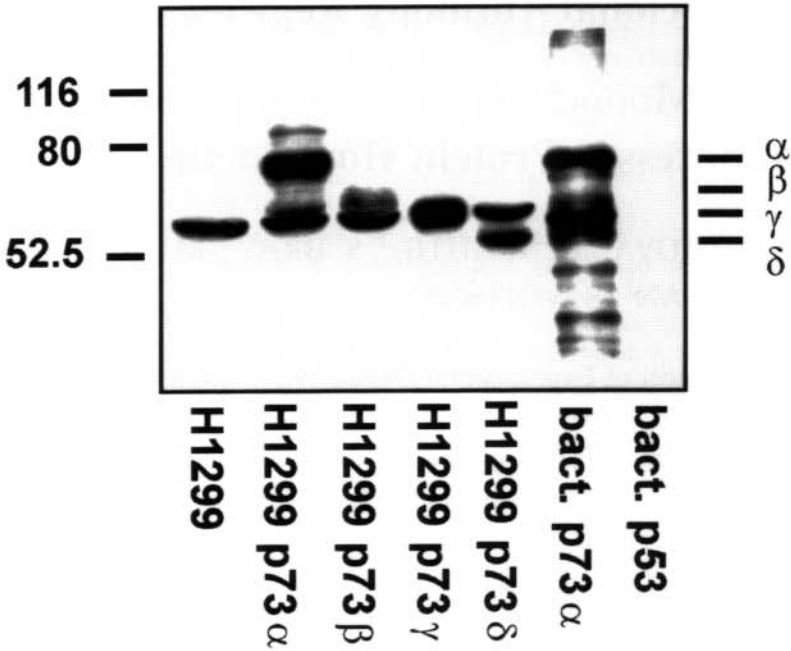


Fig. 1. Characterization of p73-1.1 MAB specificity using Western blot analysis. The H1299 cells were transfected with pcDNA3 plasmid vectors carrying p73 α , β , γ , or δ isoform cDNA using EffecteneTM Transfection Reagent (Qiagen). Non-transfected cells as well as purified p53 protein expressed in bacteria were used as negative controls. Partially purified p73 protein expressed in bacteria served as a positive control, antibody concentration was 1 μ g/ml.

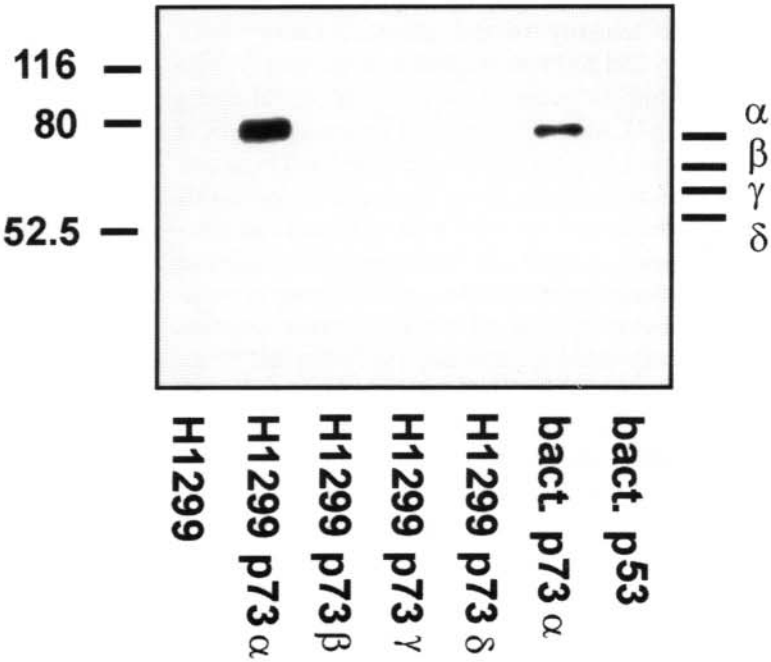


Fig. 2. Characterization of p73 α -1.1 MAB specificity using Western blot analysis. The H1299 cells were transfected with pcDNA3 plasmid vectors carrying p73 α , β , γ , or δ isoform cDNA using EffecteneTM Transfection Reagent (Qiagen). Non-transfected cells as well as purified p53 protein expressed in bacteria were used as negative controls. Partially purified p73 protein expressed in bacteria served as a positive control, antibody concentration was 1 μ g/ml.

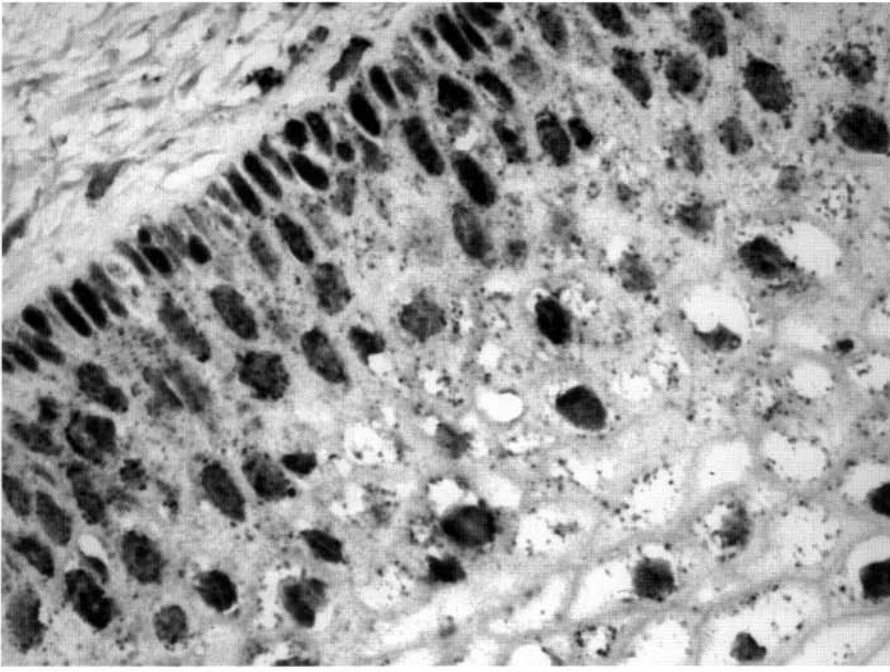


Fig. 3. Staining of the squamous epithelium of the uterine cervix using p73-1.1 antibody. Note the nuclear and granular cytoplasmic positivity accented in the basal - suprabasal layers.

Formalin-fixed, paraffin-embedded tissue, antigen retrieval in 0.001 M EDTA-NaOH buffer, pH 8.0. Concentration of antibody 1 μ g/ml, detection Vector Elite ABC, DAKO DAB+. Magnification 400x.

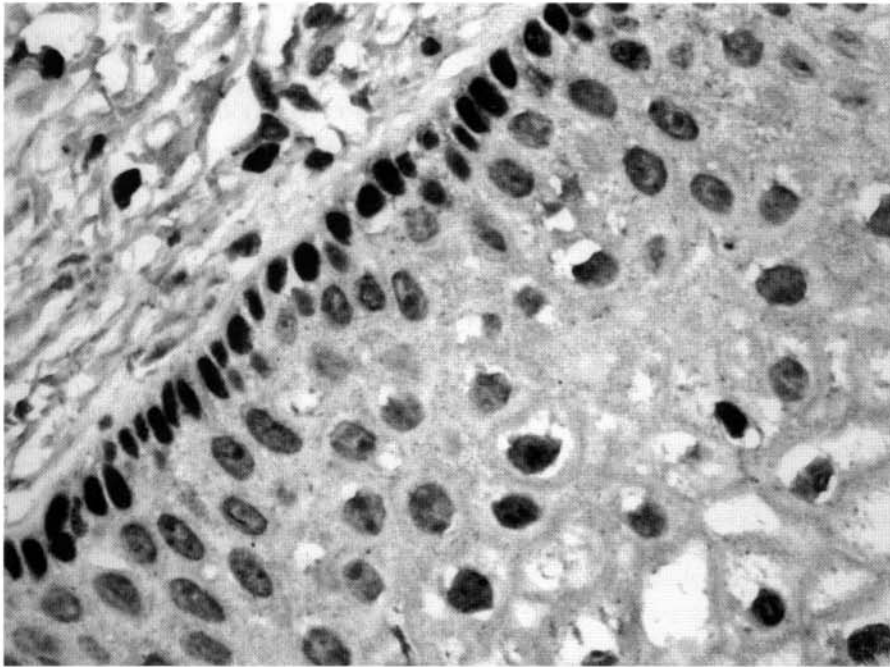


Fig. 4. Staining of the squamous epithelium of the uterine cervix using p73 α -1.1 antibody. Note the nuclear positivity, confined mostly to the basal layer. Formalin-fixed, paraffin-embedded tissue, antigen retrieval in 0.001 M EDTA-NaOH buffer, pH 8.0. Concentration of antibody 1 μ g/ml, detection Vector Elite ABC, DAKO DAB+. Magnification 400x.

high affinity and β and δ isoforms with lower affinity (Fig. 1).

Both monoclonal antibodies p73-1.1 and p73 α -1.1 are useful for immunohistochemistry with detection of p73 protein isoforms in the formalin-fixed, paraffin-embedded tissue samples upon antigen retrieval (Figs. 3 and 4).

Our data suggest that for construction of MAb against only one specific isoform of the p73 protein it is better to use a synthesized isoform-specific epitope rather than the full-length protein, to exclude the possibility of obtaining an antibody reacting with all other isoforms of the p73 protein.

Properties

Antibodies p73-1.1 (IgA) and p73 α -1.1 (IgG₁) can recognize the p73 α protein under denaturing and non-denaturing conditions with different degrees of cross-reactivity with p73 isoforms. p73 α -1.1 is more specific and recognizes only the α isoform of the p73 protein while the monoclonal antibody p73-1.1 recognizes the α , β , γ and δ isoforms. Both antibodies can be used for analysis of p73 expression by immunoblotting and immunohistochemistry. Monoclonal antibody p73 α -1.1 can also be used for immunoprecipitation and ELISA. The antibody p73 α -1.1 seems to be more suitable for immunocytochemistry or immunohistochemistry, since it does not display any cross-reactivity with isoforms other than p73 α . The results from immunohistochemistry suggest that both antibodies could be used for characterization of nuclear and cytoplasmic expression of the p73 homologues.

Acknowledgement

The authors thank Dr. D. Valik for critically reading the manuscript, Dr. C. A. Midgley for the p73 α plasmid for bacterial expression and Dr. Melino for the kind gift of a set of p73 plasmids for eukaryotic expression.

References

- De Laurenzi, V., Costanzo, A., Barcaroli, D., Terrinoni, A., Falco, M., Annicchiarico-Petruzzelli, M., Levrero, M., Melino, G. (1998) Two new p73 splice variants, gamma and delta, with different transcriptional activity. *J. Exp. Med.* **188**, 1763-1768.
- De Laurenzi, V. D., Catani, M. V., Terrinoni, A., Corazzari, M., Melino, G., Costanzo, A., Levrero, M., Knight, R. A. (1999) Additional complexity in p73: induction by mitogens in lymphoid cells and identification of two new splicing variants epsilon and zeta. *Cell Death Differ.* **6**, 389-390.
- Harlow, E., Lane, D. P. (1988) *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Hollstein, M., Sidransky, D., Vogelstein, B., Harris, C. C. (1991) p53 mutations in human cancers. *Science* **253**, 49-53.
- Jost, C. A., Marin, M. C., Kaelin, W. G. Jr. (1997) p73 is a human p53-related protein that can induce apoptosis. *Nature* **389**, 191-194.
- Kaghad, M., Bonnet, H., Yang, A., Creancier, L., Biscan, J. C., Valent, A., Minty, A., Chalon, P., Lelias, J. M., Dumont, X., Ferrara, P., McKeon, F., Caput, D. (1997) Monoallelically expressed gene related to p53 at 1p36, a region frequently deleted in neuroblastoma and other human cancers. *Cell* **90**, 809-819.
- Ko, L. J., Prives, C. (1996) p53: puzzle and paradigm. *Genes Dev.* **10**, 1054-1072.
- Lane, D. P. (1992) Cancer. p53, guardian of the genome. *Nature* **358**, 15-16.
- Levine, A. J. (1997) p53, the cellular gatekeeper for growth and division. *Cell* **88**, 323-331.
- Nigro, J. M., Baker, S. J., Preisinger, A. C., Jessup, J. M., Hostetter, R., Cleary, K., Bigner, S. H., Davidson, N., Baylin, S., Devilee, P., et al. (1989) Mutations in the p53 gene occur in diverse human tumour types. *Nature* **342**, 705-708.
- Ueda, Y., Hijikata, M., Takagi, S., Chiba, T., Shimotohno, K. (1999) New p73 variants with altered C-terminal structures have varied transcriptional activities. *Oncogene* **18**, 4993-4998.
- Yang, A., Kaghad, M., Wang, Y., Gillett, E., Fleming, M. D., Dotsch, V., Andrews, N. C., Caput, D., McKeon, F. (1998) p63, a p53 homolog at 3q27-29, encodes multiple products with transactivating, death-inducing, and dominant-negative activities. *Mol. Cell.* **2**, 305-316.