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Immunohistochemical Analysis of p53 Protein Expression in Benign and Malignant Skin Tumors Using a Panel of Anti-p53 Antibodies

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The expression of p53 in a variety of benign and malignant skin lesions has been first assessed in frozen sections and then compared with the results obtained in corresponding paraffin-embedded sections using various immunohistochemical staining methods with a panel of anti-p53 antibodies. Of the 48 benign and malignant skin lesions studied, 46(96%) had corresponding paraffin sections and immunohistochemical results obtained with DO7 on frozen and paraffin sections were concordant in 97%, qualitatively. Using streptavidin-biotin complex method, p53 was identified in 33% of dysplastic squamous lesions, 50% of squamous cell carcinomas(SCCs) and 36% of basal cell carcinomas(BCCs) on frozen section, whereas 25% of dysplastic squamous lesions, 40% of SCCs, and 32% of BCCs showed p53 positivity on paraffin-embedded sections. In frozen sections, the same regions of each specimen exhibited similar topographic patterns of positive immunoreactivity with both monoclonal antibodies, PAb 1801 and DO7. In contrast, immunohistochemical staining with polyclonal antibody, CM-1, gave poor morphologic resolution, although effective in paraffin-embedded sections.

Key Words: *Immunohistochemistry, p53, skin tumors*

INTRODUCTION

The skin affords an excellent model of human carcinogenesis because a variety of lesions from benign tumors to invasive malignancy, with or without metastatic potential, are commonly found, and are easily accessible to biopsy. However, to date, few genetic alterations have been observed in skin neoplasia. Previous immunohistochemical studies of p53 have been restricted to frozen sections, because the epitope is destroyed by formaldehyde fixation. The only anti-p53 antibody that is effective in paraffin-embedded sections was CM-1, although the sensitivity and the specificity are believed to be reduced somewhat because the antibody is polyclonal in nature. In this regard, the development of anti-p53 antibody reactive in routinely processed, paraffin-embedded specimens will provide further possibilities to investigate old and new cases, thereby extending the applicability of this antibody.

In our previous study(Ro et al., 1993), using a new monoclonal antibody, DO7, on paraffin-embedded sections, we found that 27% of dysplastic squamous lesions, 56% of squamous cell carcinomas (SCCs), and 42% of basal cell carcinomas(BCCs) stained positively for p53. These results are at variance with those of other study(Gusterson et al., 1991) using the polyclonal antibodies CM-1 and JG-8, and monoclonal antibody PAb 1801, where p53 was detected in 8% of dysplastic lesions, 0% of BCCs, and 15% of SCCs.

The purpose of this study was to assess the expression of p53 in a wide variety of benign and malignant skin lesions using a panel of anti-p53 antibodies and to compare the results between

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frozen and paraffin-embedded sections. The results of different immunohistochemical staining methods were also investigated.

MATERIALS AND METHODS

Specimens and fixations

Biopsy specimens were obtained from patients with defined dermatologic diseases. Of the 48 benign and malignant skin lesions studied, 46(96%) had corresponding paraffin-embedded sections : actinic keratosis (3cases), Bowen's disease(6), SCC(6), BCC(25) and a variety of benign skin lesions(8). The specimens were cut into two parts : one was snap-frozen in liquid nitrogen and the other fixed in normal-buffered formalin followed by mercuric chloride post-fixation, and was embedded in paraffin.

Antibodies

Three antibodies to the p53 protein were used : (1) rabbit polyclonal antibody CM-1, raised against pure human p53 (Midgley et al., 1992) ; (2) mouse monoclonal antibody PAb 1801, which recognizes an epitope on p53 protein from amino acids 32-79 (Banks et al., 1986) ; (3) mouse monoclonal antibody DO7, which recognizes an epitope on p53 protein from amino acids 1-45(Vojtesek et al., 1992). DO7 is effective on both frozen and paraffin-embedded sections, while CM-1 is effective only on paraffin-embedded sections. PAb 1801 can be used on frozen sections as well as methacarn or formol saline-fixed paraffin sections. All antibodies were obtained from Novocastra, Newcastle upon Tyne, UK.

Immunohistochemistry

In the following protocol, sections were rinsed twice with TRIS-buffered saline(TBS, pH 7.6) after each change of solution, up to the dehydration steps ; all incubations were done at 25°C unless otherwise specified, and all stated concentrations are final. Sections(4μm) were cut and mounted on lysin-coated slides. The paraffin of the paraffin-embedded sections was removed and sections were rehydrated in graded alcohol according to the standard procedures. For frozen tissue, cryostat sections were fixed in acetone for 10 minutes and air-dried. Then sections were reacted for 10 minutes with 0.5% hydrogen peroxide in methanol to quench any endogenous peroxidase activity that may be present in the tissue. Non-specific binding sites were blocked by incubation in normal serum from species supplying the secondary antibody, diluted 1:5 in TBS. Then, sections were incubated in each anti-p53 antibody at a dilution of 1:50 as follows: 30 minutes incubation for frozen sections and 1 hour incubation for paraffin-embedded sections.

In an attempt to achieve the best specific staining and minimal background activity, the following immunohistochemical staining methods were evaluated with each anti-p53 antibody : (1) two-step indirect method ; (2) peroxidase-antiperoxidase (PAP) method ; (3) streptavidin-biotin-peroxidase (SAB) method. Finally, the peroxidase reaction was developed using diamino-benzidine (DAB) with 1% hydrogen peroxide. After a 1-2-minute incubation in this solution, sections were thoroughly washed, counterstained with haematoxylin, and mounted. Tissue from a breast carcinoma and a squamous cell carcinoma which were known to be positive for p53 mutation on sequencing were used as positive controls, and omission of the primary antibody served as a negative control.

RESULTS

Influence of fixatives

Especially with PAb 1801, methacarn-fixed paraffin materials were used but the intensity of staining was considerably reduced or even lost in comparison with frozen sections which gave reproducibly good results with all antibodies.

Effects of trypsination

We found that the pre-incubation of the sections with trypsin for 5 or 10 minutes did not improve the quality of staining, regardless of the primary antibody and staining method used in this study.

Differences between primary anti-p53 antibodies

There was complete concordance between two anti-p53 antibodies in frozen sections, as well as in paraffin-embedded sections. The same region of the specimen exhibited similar topographic patterns of positive immunoreactivity with PAb 1801 and DO7 in serial frozen sections (Fig. 1A,B,C,D).

The immunoperoxidase staining of monoclonal antibody PAb 1801, DO7 was very specific and background-free, in comparison with polyclonal antibody, CM-1, showing poor morphologic resolution, such as non-specific or background staining (Fig. 2A,B).

The results obtained with DO7 on frozen and paraffin-embedded sections were concordant in

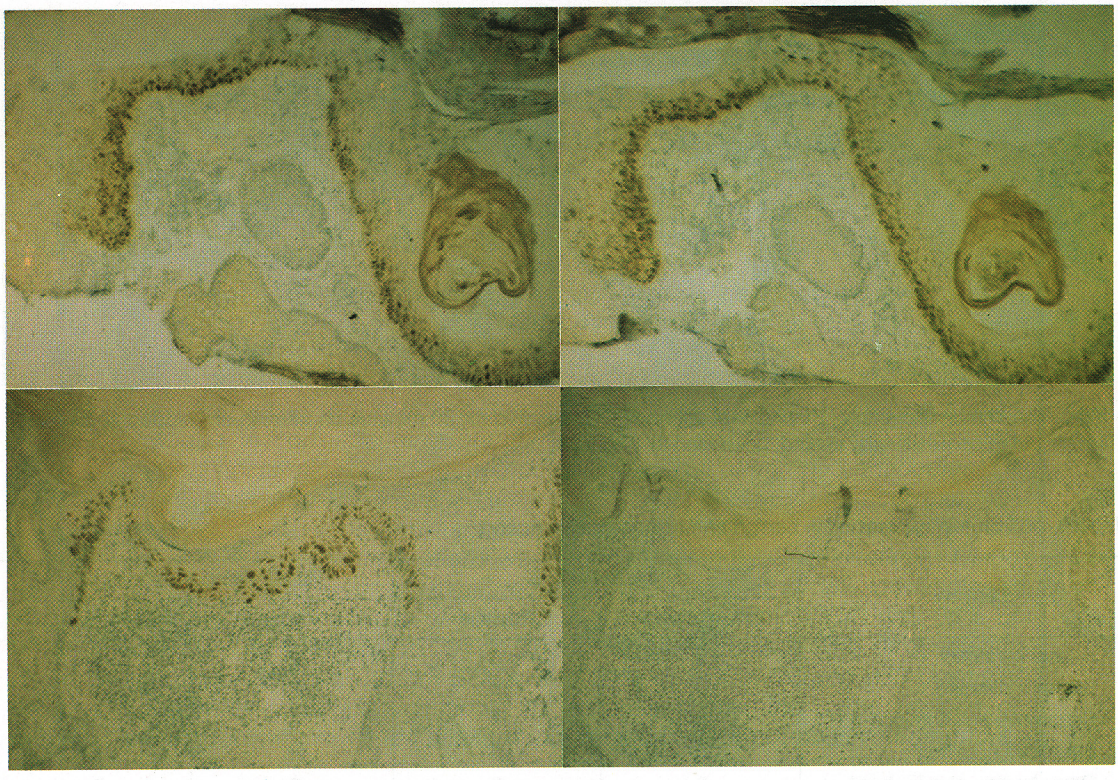

Fig. 1. The immunostaining of actinic keratosis using PAb 1801 (A), DO7 (B) on frozen sections and DO7 (C), CM-1 (D) on corresponding paraffin-embedded sections. Note colocalization of immunoreactivity with a panel of ant-ip53 antibodies in similar topographic pattern.

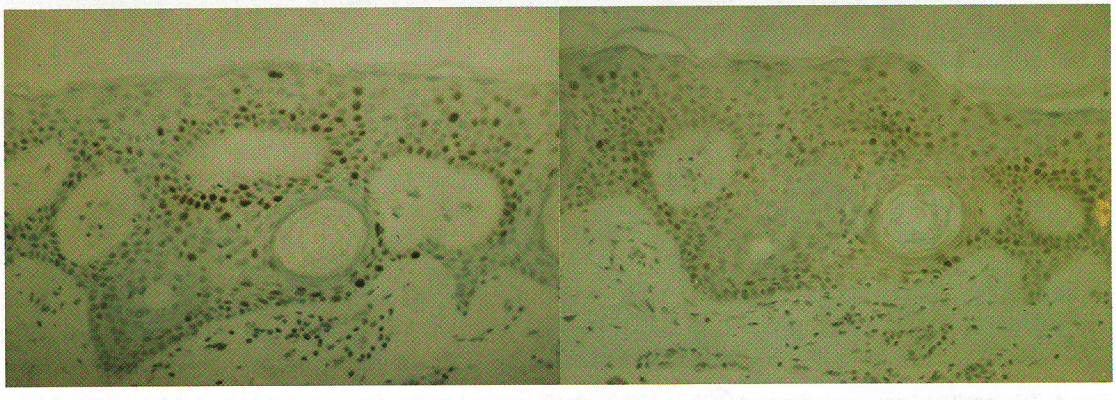

Fig. 2. The immunostaining of monoclonal antibody PAb 1801 was very specific and background-free (A), in comparison with polyclonal antibody, CM-1, showing poor morphologic resolution (B).

97%, qualitatively (i.e. with regard to simple positivity or negativity). Some cases positive with PAb 1801 on frozen section, but negative with DO7 on paraffin-embedded section turned out to be positive by setting incubation time from 30 minutes to 1 hour with the same primary antibody dilution of frozen section(Fig. 3A, B).

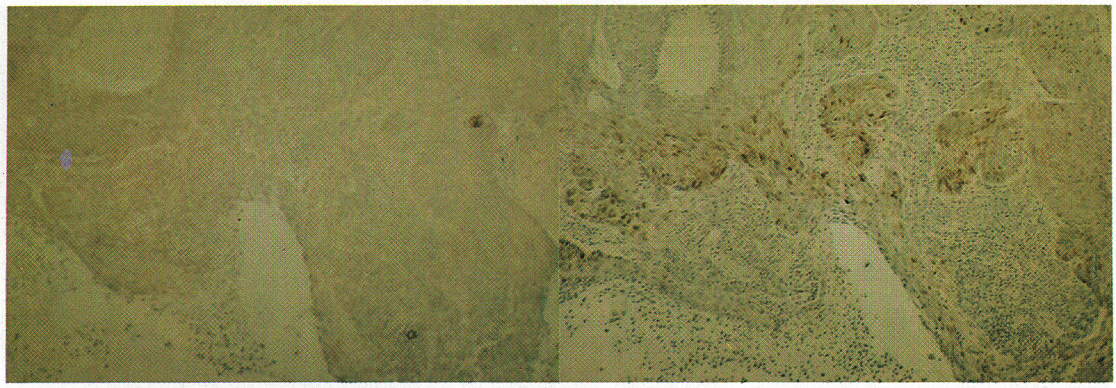

Fig 3. The immunostaining of monoclonal antibody DO7 applied for 30 minutes on paraffin-embedded sections (A) turned out to be positive by setting incubation to 1 hour (B).

Table 1. p53 protein expression in benign and malignant skin tumors

Lesions	Frozen tissue		Paraffin-embedded tissue	
	No. of cases	Positive	No. of cases	Positive
Actinic keratosis	3	0	2	0
Bowen's disease	6	3	6	2
Squamous cell carcinoma	6	3	5	2
Basal cell carcinoma	25	9	25	8
Other benign skin lesions*	8	0	8	0

* Psoriasis(2), neurofibroma(1), intradermal nevus(1), ichthyosis(1), trichilemmoma(1), epidermal cyst(1), trichoepithelioma(1).

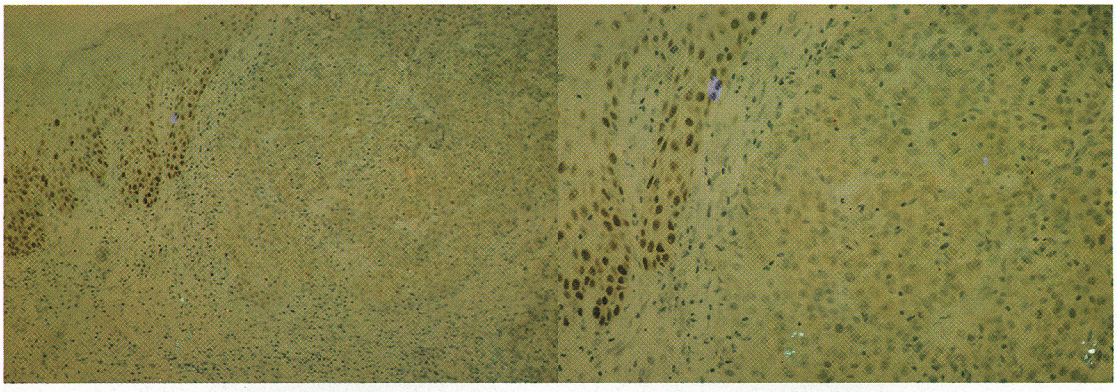

Fig. 4. The positive immunostaining of DO7 in histologically normal epidermis adjacent to the p53-negative SCC ; low-power view (A) and high-power view (B).

Comparison of results using SAB method

Preliminary experiments indicated that the SAB method gave the best quality of staining comparing with other methods. The evaluation of the results considered both the specific staining and the background activity in order to achieve the best combination. No evidence of p53 positivity was found in 8 cases of benign skin lesions. p53 was

Table 2. Comparison of results with other studies

Author	Reference number	Staining method	Antibody used	Staining tissue	p53 positivity(%)		
					Dysplastic lesion	SCC	BCC
Ro	1	SAB	DO7	Paraffin	27	56	42
Gusterson	2	ABC	JG8, CM1	Paraffin	8	15	0
		Indirect	1801	Frozen			
Shea	10	ABC	1801	Frozen	ND	ND	83
			240				58
Present study		SAB, PAP	DO7, 1801	Frozen	33	50	36
		Indirect	DO7, CM1	Paraffin	25	40	32

SAB, streptavidin-biotin-peroxidase complex method; ABC, avidin-biotin-peroxidase complex method; PAP, peroxidase-antiperoxidase method; ND, not done.

identified in 0/3(0.0%) cases of actinic keratosis, 3/6(50%) of Bowen's diseases, 3/6(50%) of SCCs, and in 9/25(36%) of BCCs on frozen sections, whereas 0/2(0.0%) of actinic keratoses, 2/6(33.3%) of Bowen's diseases, 2/5(40%) of SCCs, and 8/25(32%) of BCCs showed p53 positivity on paraffin-embedded sections (Table 1). In positive tumors the pattern of immunostaining was not uniform. The greatest proportion of positive cells was found around the advancing margins of tumor cells. Variations in the intensity of staining between nuclei of the same tumor were also observed. Interestingly, in one patient with SCC, positive nuclear staining for p53 was noted in histologically normal epidermis adjacent to the p53-negative tumor cells(Fig. 4A, B).

DISCUSSION

The p53 gene encodes a nuclear phosphoprotein which has been implicated in the normal proliferation and neoplastic transformation of cells (Lane and Benchimol, 1990). The normal or 'wild-type' p53 protein acts as a potent suppressor of cell growth(Finlay et al., 1989) and present at very low concentration, amounting to only a few thousand molecules per cell. The very low concentration of the normal p53 protein means that it is extremely difficult to detect using conventional immunohistochemical staining methods. p53 detection could be a consequence of p53 gene mutation, as mutant p53 protein has a longer half-life ; it binds to wild p53 protein and other oncogenic or viral proteins to form stable complexes(Lane and Benchimol, 1990; Finlay et al., 1989). This has led to the interpretation of the immunohistochemical detection of p53 protein as synonymous with p53 mutations(Lane and Benchimol, 1990). However, there are a number of pit falls in the interpretation of immunohistochemical data which need to be considered, since there are several potential cases where loss of p53 activity may not be accompanied by the expected accumulation of the protein(false negative). And false positive results could also occur, if normal p53 protein accumulated in cells to a higher than normal concentration, for example because of a defect in the degradative pathway. There is as yet little data on the frequency of occurrence of such false negative and positive reactions(Wynford-Thomas, 1992).

Interestingly, in one patient with SCC, we found focal positive staining for p53 in histologically normal epidermis adjacent to the p53-negative tumor cell island. It is possible that this may be a technical artefact of immunohistochemical staining, but considering the rarity of this factor and the lack of p53 expression in adjacent tumor cells, it is likely that non-mutational mechanism for p53 stabilization could be responsible for the detection of these occasional p53-positive cells in benign tissues. p53 is thought to be important in limiting cellular DNA damage following exposure to noxious stimuli such as ultraviolet radiation(UVR). Exposure to UVR increases p53 protein expression by post-translational mechanisms which in turn block the cells in the G1/S step of the cell cycle allowing vital DNA repair to take place before DNA replication occurs prior to the onset of mitosis(Vogelstein and Kinzier, 1992). Shea et al(1992) reported that keratinocytes of chronically sun-exposed epidermis adjacent to BCCs also focally overexpressed p53 protein in the majority of cases and suggest-

ed a possible field effect from chronic UVR exposure.

In this study, p53 protein has been identified with PAb 1801 on frozen sections in 9(36%) of the 25 BCCs, and with DO7 on paraffin-embedded sections in 8(32%) of the 25 BCCs, which is similar to our previous results(Ro et al., 1993) using only one antibody, DO7, on paraffin-embedded sections. In contrast, previous study of p53 expression in premalignant and malignant squamous epithelium reported that none of the 10 BCCs studied was positive(Gusterson et al., 1991). The results of previous studies are summarized in Table 2. Several reasons may account for these differences. First, it is apparent from differences in frequency of p53-positive tumors between the methacarn-fixed and the frozen sections that p53 detection is very sensitive to fixation. Therefore it appears likely that these differences are due to the greater sensitivity of the monoclonal antibody, DO7, which overcome the problem of antigenic loss during the fixation procedure. However, as mentioned previously, the immunoreactivity of DO7 on paraffin-embedded sections was somewhat decreased, leading to more concentrated dilution of primary antibody or longer incubation time, up to 1 hour, in order to obtain the same immunoreactivity in frozen section. Second, the comparison of the various staining methods with the use of a panel of antibodies recognizing discrete epitopes on p53 clearly allows us to pick up more positive cases. We found some tumors did not react with CM-1 antibody at the dilution which gave positive reaction with both PAb 1801 and DO7. In this regard, the use of CM-1 alone will lead to an underestimation of the number of p53-expressing tumors. Cattoretti et al also found such variations in the incidence of p53 detection depending on the antibody used(Cattoretti et al., 1988). In keeping with our positive rate of BCC, recent study revealed that heterozygous mutations were detected in 7(50%) of the 14 BCCs using the polymerase chain reaction(PCR) followed by direct DNA sequencing(Rady et al., 1992).

The colocalized immunolabelling by PAb 1801 and DO7 strongly indicates a specific immunoreactivity directed against p53 protein, rather than fortuitous cross-reactivity to an irrelevant antigen, because both monoclonal antibodies recognize different epitopes on the p53 protein. However, although less likely, we can not exclude the possibility that there may be positive immunostaining without mutation or vice versa, since we were unable to perform both techniques on the same samples. Simultaneous immunohistochemical and molecular studies are needed, which aim to show more clearly the relationship between p53 protein detection and p53 gene mutation.

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