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Oncogene Interaction in Basal Cell Carcinomas of Human Skin

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The expression of the p53 protein (p53) was compared with those of several oncogenes including c-fos (Fos), c-jun (Jun), and epidermal growth factor receptor (EGFR1) using immunohistochemistry in frozen and paraffin-embedded sections of 25 basal cell carcinomas (BCCs) to find out any correlation between p53 and oncogenes in the pathogenesis of human BCC. In normal skin, positive reactions were obtained for EGFR1 and Fos, while p53 and Jun were negative in all cases. In the lesions, EGFR1 was observed in all cases and p53 was positive in 9 of 25 (36%). Fos was expressed in 21 of 25 (84%) and four negative cases were all p53-positive ; this negative correlation between p53 and Fos staining was statistically significant ($P < 0.01$). Jun was detected in 14 of 20 (70%) and no significant relationship was observed between the expression of Jun and Fos or p53. These data suggest the possibility of down regulation of Fos expression by high levels of p53 protein. Further work is necessary to determine the mechanism of this interaction.

Key Words : *Epidermal growth factor receptor, c-fos, c-jun, p53, Basal cell carcinoma*

INTRODUCTION

The role of cellular oncogenes as key regulators of cellular proliferation and differentiation has been well recognized and alterations of a wide variety of cellular oncogenes have now been implicated in the causation of many human cancers. These alterations include activation of cellular proto-oncogenes and inactivation of tumor suppressor genes (Bishop, 1987). Alterations to any of these could occur with or relate to malignancy. Recently, genetic studies have indicated that an abnormality of a single gene is usually insufficient to elicit the fully transformed phenotype and that two or more genetic lesions may be necessary for this to occur (Fearon and Vogelstein, 1990). For this reason, there has been increasing interest in the potential for co-operation between various cellular oncogenes in the process of carcinogenesis (Fearon and Vogelstein, 1990 ; Weinberg, 1991).

The most common single genetic change known so far is p53 gene mutation (Hollstein et al., 1991). p53 is a tumor suppressor gene encoding a 53 kD nuclear phosphoprotein which is capable of binding to DNA and acts as a transcriptional factor (Farmer et al., 1992). The wild-type p53 protein inhibits cell proliferation (Deppert et al., 1990) and loss of this activity leads to a neoplastic transformation of the cells (Finlay et al., 1989 ; Nigro et al., 1989). Activating mutations, changing the gene to a dominant oncogene, have also been described (Levine, 1992)

The epidermal growth factor receptor (EGFR1) encodes a 170 kD trans-membrane glycoprotein, exhibiting an extracellular ligand (EGF or TGF-α) binding domain, a trans-membrane region and an intracellular domain facing the cytoplasm and exhibiting tyrosine

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kinase function. Recent studies have reported that overexpression of the EGFR1 was associated both with enhanced matastatic potential of some breast cancer cell lines (Fitzpatrick et al., 1984 ; Roos et al., 1986) and with high risk of early recurrence and death in some clinical studies (Sainsbury et al., 1987 ; Toi et al., 1991). Although there is no unequivocal evience that amplification of EGFR1 is the initial transforming event in human cancer (Heldin and Westermark, 1984 ; Stoscheck and King, 1986), an autocrine mechanism is considered as an important step for the independent growth of tumor cells (Salomon et al., 1984 ; Lippman et al., 1986).

The c-fos oncogene appears to have an important role in the regulation of cellular proliferation and differentiation. The expression of c-fos has been studied in various cell lines, and is thought to be associated with the induction of DNA synthesis, as shown by its rapid and transient induction following growth factor stimulation (Kruijer et al., 1984 ; Muller et al., 1984). In other cell types, it is also known as a 'differentiation gene' and high specific levels of c-fos expression can be found in certain terminally differentiated cells (Gonda and Metcalf, 1984). In addition to this divergence, its protein product, Fos, forms a stable heterodimeric complex with Jun, the product of the cellular oncogene, c-jun. It is this complex which interacts with the DNA regulatory element known as the AP-1 binding site (Halazonetis et al., 1988 ; Rauscher et al., 1988).

To date, very few reports have adressed the role of oncogenes in the development of basal cell carcinomas (BCCs). In this study, we have assessed the expression of p53, Fos, Jun, and EGFR1 in 25 BCCs and examined the possibility of oncogene interaction which might be involved in the pathogenesis of human BCC as well.

MATERIALS AND METHODS

Tissue specimens

Twenty-five patients with previously untreated BCCs were investigated. Following surgical removal, 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

The monoclonal antibodies used and their specificities are listed in Table 1. For detection of the gene product of c-erbB1, the epidermal growth factor receptor (EGFR1), we used the mouse monoclonal antibody NCL-R1(Novocastra Laboratories, Newcastle upon Tyne, UK). It is an IgG2b antibody, the immunogen for which was the epidermoid carcinoma line A431. Two monoclonal antibodies to the p53 protein, PAb 1801 and DO7(Novocastra Laboratories, Newcastle upon Tyne, UK), were used as described previously (Ro and Kim, 1993). For detection of c-fos protein (Fos), we used mouse IgM-class monoclonal antibody NCL-FOS(Novocastra Laboratories, Newcastle upon Tyne, UK). c-jun protein (Jun) was detected using a mouse monoclonal antibody DK-4 which was kindly provided by Dr. Dina G Tiniakos, Royal Victoria Infirmary, Newcastle upon Tyne, UK (Tiniakos et al., 1994).

Immunohistochemistry

In the following protocol, sections were rinsed twice with Tris buffered saline (TBS) after each change of solution, up to dehydration steps ; all incubations were done at 25℃ unless otherwise specified, and all stated concentrations are final.

Methods for EGFR1, p53, and Fos.

Serial five micron cryostat sections were cut and placed on slides coated with poly-L-lysine solution (Sigma Chemicals, St Louis, MO, USA). The specimens were then fixed in cold acetone for 10 minutes and briefly air-dried. To quench any endogenous peroxidase activity that may be present in the tissue, the sections were reacted for 10 minutes with 0.5%

Table 1. The antibodies used, sources, and concentrations in this study

Oncogene	Clone	Working Dilution	Staining Pattern	Corporation
p53	DO-7/PAb 1801	1 : 100/1 : 50	Nuclear	Novocastra, UK
Fos	NCL-Fos	1 : 40	Nuclear	Novocastra, UK
Jun	DK-4	1 : 40	Nuclear	Novocastra, UK
EGFR1	NCL-R1	1 : 40	Cytoplasmic/Membrane	Novocastra, UK

hydrogen peroxide in methanol. Non-specific binding was blocked by incubating the slides in normal rabbit serum diluted 1 : 5 in TBS (NRS/TBS) for 10 minutes.

In the immunostainings the streptavidin-biotin complex method was used. The primary antibodies were applied separately to serial cryostat sections and incubated in a humidified chamber as follows : EGFR1 diluted 1 : 40 in NRS/TBS for one hour ; PAb 1801 diluted 1 : 50 for 30 minutes ; and DO7 diluted 1 : 100 for 30 minutes. With Fos, sections were incubated in c-fos diluted 1 : 40 in NRS/TBS overnight at 4℃. Subsequently, the slides were exposed to biotinylated rabbit anti-mouse secondary antibody (Dako Ltd., UK) at a dilution of 1 : 500 for 30 minutes, followed by streptavidin peroxidase conjugate(Dako Ltd., UK) for 30 minutes. Finally, the peroxidase reaction was developed using diaminobenzidine with 1% hydrogen peroxide. After 1-2-minute incubation in this solution, sections were thoroughly washed, counterstained with Mayer's hematoxylin, and mounted.

Method for Jun.

Corresponding paraffin sections were cut and mounted on lysine-coated slides. The specimens were then dewaxed in xylene and rehydrated in graded alcohol. Endogenous peroxidase was blocked by immersing the sections in 0.5% hydrogen peroxide in methanol for 10 minutes. Sections were then preincubated in 0.1% trypsin solution for 10 minutes, followed by blocking serum for 10 minutes. After washing in TBS, the sections were incubated overnight at 4℃, with the primary antibody at 1 : 40 dilution. Biotinylated anti-mouse and peroxidase-labeled streptavidin (Dako Ltd., UK) were added in sequence. The peroxidase reaction was developed using diaminobenzidine as chromogen.

Controls

Positive control stains for EGFR1 and c-fos were performed on sections of normal human skin and those for p53 and c-jun were performed on sections of squamous cell carcinoma and breast carcinoma, respectively. Negative controls were performed by substituting these antibodies by non-immune serums or by omitting the primary antibodies.

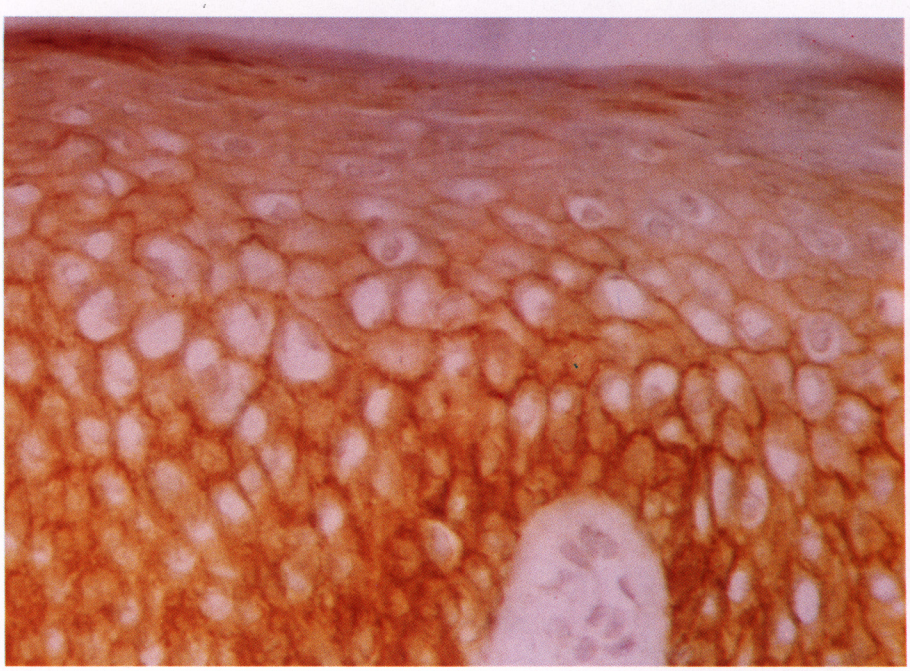

Fig. 1. Normal skin immunostained with EGFR1 showing mixed membrane and cytoplasmic staining of all layers of the epidermis except the stratum corneum.

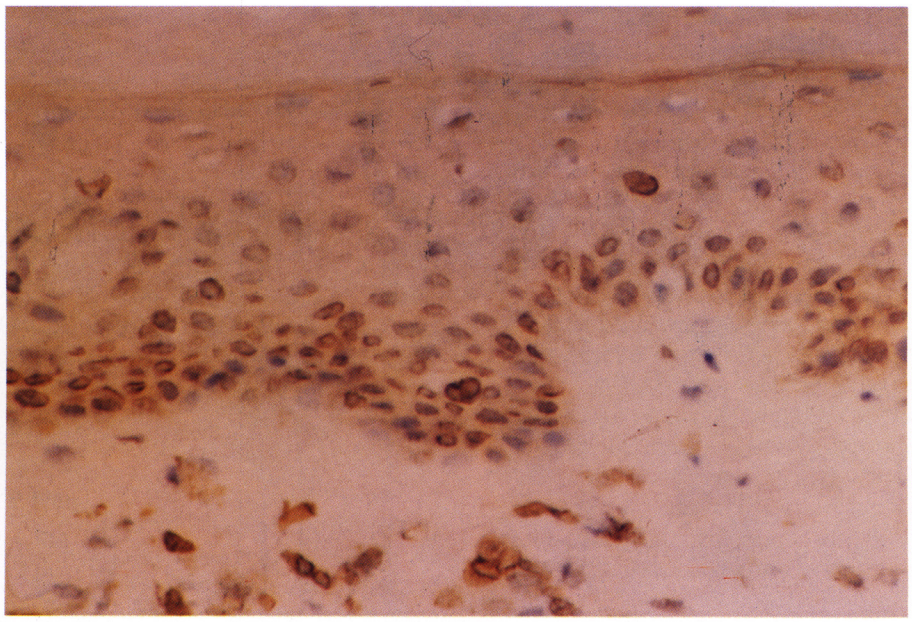

Fig. 2. Normal skin immunostained with Fos showing strong scattered nuclear staining at all layers of the epidermis, especially the lowest few layers.

Assessment

The immunostaining was assessed in the lesional skin by two observers and discrepant results were reevaluated using a double-headed microscope. Statistics were carried out using Fisher's exact test.

RESULTS

Normal tissue

EGFR1 produced strong, granular membrane staining and some cytoplasmic staining of all layers of the epidermis except the stratum corneum (Fig. 1). There was similiar staining of sweat and sebaceous glands. Endothelial cells and inflammatory cells were negative. Fos produced strong staining of scattered nuclei at all levels of the epidermis, the lowest few layers showing the greatest proportion of positive cells (Fig. 2). Sweat gland, endothelial and inflammatory cells were also strongly positive. Normal epidermis, sweat gland, endothelial cells and inflammatory cells were negative in all cases for p53 and Jun.

Basal cell carcinomas

Results of immunohistochemical stains are shown in

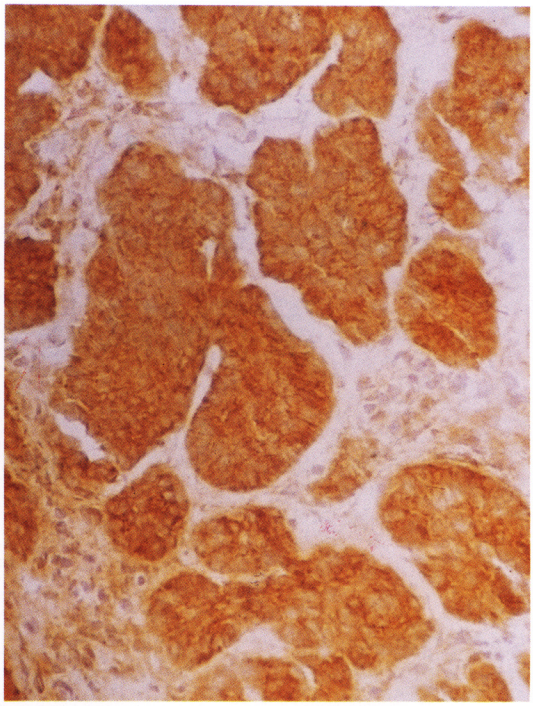

Fig. 3. A basal cell carcinoma showing less membranous and more cytoplasmic staining for EGFR1, compared with those of normal epidermis.

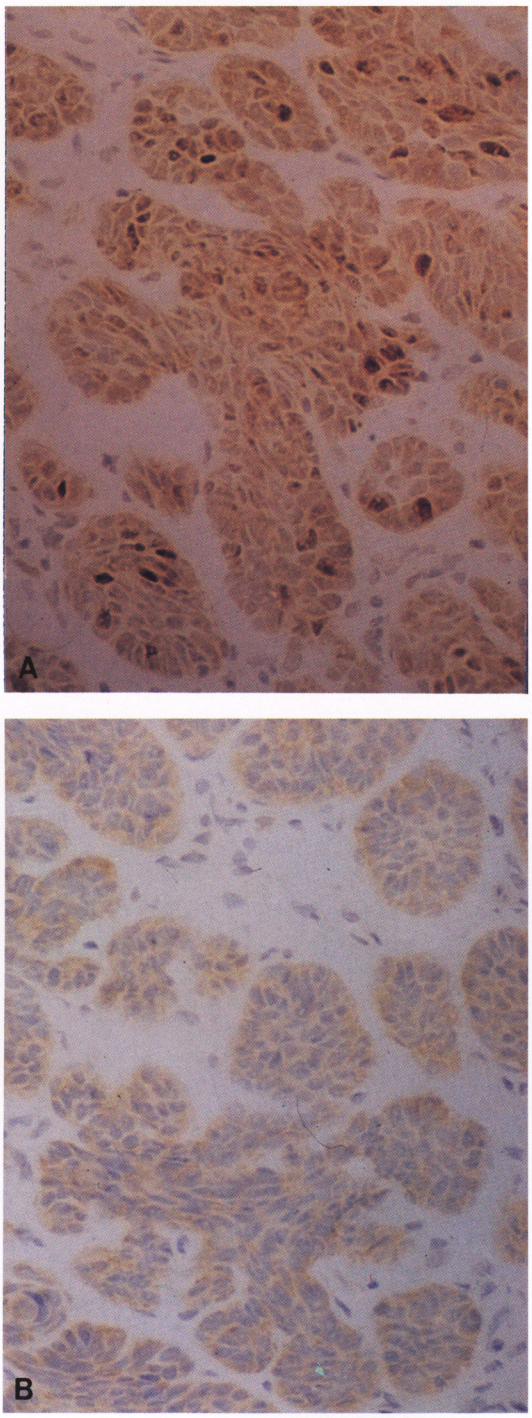

Fig. 4. A basal cell carcinoma showing positive staining for p53 protein(A), but negativity for c-fos protein(B). Note that nearby inflammatory cells stain positively for c-fos protein, providing an internal control.

Table 2. EGFR1 was observed in all of the specimens of basal cell carcinoma studied and the pattern of reactivity was less membranous and more cytoplasmic (Fig. 3), compared with those of normal epidermis. The expression of p53 protein was detected in 36%(9/25) and there was complete concordance between the two anti-p53 antibodies, PAb 1801 and DO7. Fos was expressed in 84%(21/25) and 4 negative cases were all p53-positive (Fig. 4A and 4B) ; this negative correlation between p53 protein and Fos staining was statistically significant ($p < 0.01$)(Table 3). Jun was detected in 70%(14/20) and no significant relationship was observed between the expression of Jun and Fos or p53.

Table 2. Expression of oncogenes in basal cell carcinoma

Case No	Sex/Age	p53	Fos	Jun	EGFR
1	F/68	−	+	+	+
2	M/70	−	+	−	+
3	M/68	−	+	+	+
4	M/71	−	+	ND*	+
5	M/67	+	+	ND	+
6	M/72	+	−	ND	+
7	M/66	−	+	+	+
8	M/77	+	+	+	+
9	M/71	−	+	+	+
10	M/70	+	+	−	+
11	M/68	+	−	+	+
12	M/65	−	+	−	+
13	M/66	−	+	+	+
14	M/66	−	+	+	+
15	M/76	−	+	+	+
16	M/76	−	+	−	+
17	M/76	−	+	+	+
18	F/60	−	+	ND	+
19	M/54	+	−	+	+
20	F/71	−	+	−	+
21	F/66	+	+	−	+
22	M/46	+	+	+	+
23	M/57	−	+	ND	+
24	F/63	+	−	+	+
25	F/63	−	+	+	+

* ND : Immunohistochemical stain was not performed with c-Jun.

Table 3. Negative correlation between p53 and Fos*

Oncogens	Fos(−)	Fos(+)	Total
p53(−)	0	16	16
p53(+)	4	5	9
Total	4	21	25

* : Negative correlation between p53 protein and c-for protein staining was statistically significant (Fisher's exact test, 2-tailed $P = 0.0096$).

DISCUSSION

Basal cell carcinoma (BCC) is one of the most common human cancers of the skin, but its molecular-genetic pathogenesis has not been fully understood. Sun exposure is generally thought to play an important role in the development of BCC, since it occurs most frequently on the head, neck, and other chronically sun-exposed areas (Sober, 1983). p53 is thought to be important in limiting cellular DNA damage following exposure to noxious stimuli such as ultraviolet radiation (UVR) (Levine et al., 1991). Recent study suggests that within one cell type the pattern of p53 response to UVR is wavelength dependent (Campell et al., 1993). Due to its common association with human malignancy it has been extensively investigated during the last few years and data from many lines of research has recently led to the proposal that an important function of normal p53 is to act as a protector of the integrity of the genome (Lane, 1992). In this hypothesis p53 accumulates in response to DNA damage and switch off replication (G1 arrest) thus allowing extra time for DNA repair. Cells in which p53 is functionally inactivated by mutation are unable to carry out this replication arrest and so accumulate mutations at an increased rate, thus facilitating progression to a malignant phenotype. The replication arrest produced by normal p53 protein appears to be dependent on sequence specific DNA binding; as required by the hypothesis outlined above, this specific binding has been lost in the point-mutated p53 proteins analysed to date (Ginsberg et al., 1991). Furthermore, many of the mutated proteins are also able to inhibit the action of normal p53 protein, resulting in complete loss of normal p53 actions from the cell. In such cells, p53 degradation also appears to be abnormal, allowing (inactive) p53 to accumulate to high levels and to be detectable by immunohistochemistry.

In our study, the most striking observations concerned p53 and c-fos. As we have previously observed in paraffin-embedded tissue (Ro et al., 1993), p53 was positive in approximately half of the lesions. However, in view of the recent interest in the interaction between p53 and c-fos (Ginsberg et al., 1991), we were particularly interested to observe that a substantial number of p53 positive cases were negative for c-fos, in contrast to normal tissue, which was p53 negative and c-fos positive. A simple explanation for this finding might be that in these cases mitogen stimulation was inactive, for example due to lack of expression or downregulation of the receptor for EGF, one of the most important mitogens for epithelial cells. However, staining for EGFR1 was positive in these cases, eliminating the above hypothesis, although of course this dose not rule out functional inactivity of the mitogen stimulatory pathway.

Another possibility, which would not be straight forwardly consistent with the hypothesis outlined above, is that in some cases where p53 has apparently mutated (allowing its detection in immunohistochemistry), it has nevertheless not lost its normal ability to bind to DNA and cause G1 arrest. If this is the case, it would appear that under some circumstances accumulation of high levels of mutated p53 does cause down-regulation of c-fos, i.e. mutation has occurred in such a way that p53 accumulates (perhaps because of abnormal breakdown) but is still active in at least one important normal function. In keeping with this, a recent study has revealed that the transforming mutants of murine and human p53 displayed an overall ability to repress transcription of the IL-6, c-fos β-actin, and major histocompatibility complex (MHC) promoters, although less actively than the wild-type p53 (Santhanam et al., 1991).

It has been reported that the different properties which c-fos has are dependent on the cell type. Since c-fos induction relates to differentiation in addition to cell growth, another possibility is due to an altered, perhaps diminished functional differentiation of tumor cells, as compared with corresponding non-neoplastic tissues. A similar role for c-fos was suggested in renal cell carcinoma (Weidner et al., 1990) and salivary gland tumor (Birek et al., 1993), as its reduced level of expression correlated well with a higher grade of malignancy. Consistent with this is our finding that there was always strong staining within normal skin and all the p53 negative lesions, including benign skin lesions (unpublished observation), although half of the p53 positive cases were also positive for c-fos. Taken together, these findings suggest that the expression of c-fos protein and its relationship with p53 protein is diverse and complex. Furthermore, as previously noted, c-fos protein does not act alone. In growth factor-stimulated cells, Jun can bind DNA as a dimer with Fos as its natural partner (Halazonetis et al., 1988; Rauscher et al., 1988). And it is suggested that overexpression of c-jun in the absence of c-fos may result in formation of aberrant homodimeric transcription complexes,

which could abrogate the normal mechanisms controlling gene expression (Halazonetis et al., 1988). However, we could not demonstrate such a contention in our study.

In conclusion, this study has examined several oncogene products and has demonstrated the feasibility of immunohistochemical detection of oncogene interaction in BCCs of human skin. Although p53 appeared to have lost its normal role as regulatory early S phase protein in some lesions, in others high levels of p53 were associated with underexpression of c-fos, reflecting the diversity of c-fos oncogene and the possible down regulation of c-fos expression by high levels of p53 protein. The precise mechanisms underlying this interaction await additional study. Further examination and description of oncogene interaction in human cancer are likely to enhance our understanding of the pathogenesis of BCCs.

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