PAPERS

Expression of the p53 homologue p63 α and Δ Np63 α in the neoplastic sequence of Barrett's oesophagus: correlation with morphology and p53 protein

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Abstract

Background—While loss of p53 function is a key oncogenic step in human tumorigenesis, mutations of p53 are generally viewed as late events in the metaplasia-dysplasia-adenocarcinoma sequence of Barrett's oesophagus. Recent reports of a series of genes (p63, p73, and others) exhibiting close homology to p53 raise the possibility that abnormalities of these p53 family members may exert their influence earlier in the sequence.

Aim—Following recent characterisation of expression of p63 and a major isoform Δ Np63 by generation of an antiserum that recognises p63 isoforms, but not p53, our aim was a comparative study of expression of p63 protein and p53 protein in a morphologically well defined biopsy series representative of all stages of the metaplasia-dysplasia-carcinoma sequence in Barrett's oesophagus.

Methods—A series of 60 biopsy cases representing normal oesophagus through to invasive adenocarcinoma were stained, using immunohistochemistry, with antibodies to p63 and p53. All biopsies derived from patients with endoscopic and histopathological substantiation of a diagnosis of traditional/classical Barrett's oesophagus.

Results—There was exact concordance in p53 and p63 expression in more advanced forms of neoplasia, high grade dysplasia, and invasive adenocarcinoma, while p63, but not p53, was detected in the proliferative compartment of some non-neoplastic oesophageal tissue, in both squamous mucosa and in the non-neoplastic metaplastic glandular epithelium.

Conclusions—In neoplastic Barrett's oesophagus there is upregulation of both p63 and p53 while p63 isoforms may well have an important role in epithelial biology in both non-metaplastic and metaplastic mucosa of the oesophagus. While abnormalities of p53 function represent an indisputable and critical element of neoplastic transformation, other closely

linked genes and their proteins have a role in both the physiology and pathophysiology of the oesophageal mucosa.

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Keywords: p53; p63; immunohistochemistry; Barrett's oesophagus; dysplasia; adenocarcinoma

A considerable body of data places mutational inactivation of the p53 tumour suppressor as a critical element in the oncogenic pathways that lead to cancer in humans and other vertebrates.^{1–3} In addition to the plethora of observational data, there is now convincing experimental evidence that loss of p53 function (by whatever means) is one of the key oncogenic steps in human cells.⁴ Much is known about the function of the p53 tumour suppressor which acts as a transcription factor in a homo-tetrameric complex to regulate expression of downstream target genes after activation by a range of cellular stresses including DNA damage. The products of p53 target genes appear to act in a diverse set of processes that facilitate adaptive responses: these include (but are not restricted to) apoptosis and growth arrest.^{5–7} It would appear that loss of p53 function, whether through inactivation by viral oncoproteins or allelic loss as a result of mutation, disables cellular homeostasis such that mutant cells derive considerable selective advantage over the wild type. As a result, in combination with other molecular events, the neoplastic phenotype can eventuate.^{3–4} Many studies have shown that abnormalities of p53 can be frequently observed in human cancer and these include adenocarcinomas arising in Barrett's oesophagus.^{8–10}

It is now well recognised that Barrett's oesophagus, or columnar lined oesophagus (CLO), is a product of chronic gastro-oesophageal reflux disease¹¹ and that the cancer complicating it demonstrates one of the most rapidly increasing incidence rates in the Western world.¹² There is advancing understanding of the molecular events that underpin

Abbreviations used in this paper: CLO, columnar lined oesophagus; PBS, phosphate buffered saline.

Table 1 Immunohistochemical expression of p63 and p53 in columnar lined oesophagus

	p63 expression				p53 expression			
	-	+/-	+	++	-	+/-	+	++
Oesophageal squamous mucosa	0	0	9	1	10	0	0	0
Barrett's mucosa without IM	4	2	4	0	10	0	0	0
Barrett's mucosa with IM	4	0	5	1	10	0	0	0
Low grade dysplasia	6	0	2	2	7	0	1	2
High grade dysplasia	2	1	1	<u>6</u>	4	0	0	<u>6</u>
Adenocarcinoma	3	1	1	<u>5</u>	5	0	0	<u>5</u>

Cases italicised and underlined showed complete concordance for p63 and p53 expression.

Scoring of staining pattern: -, no staining observed; +/-, non-nuclear (cytoplasmic or extracellular) staining; +, unequivocal nuclear staining in 10% < x < 50% of cells, usually in the proliferative zone of non-neoplastic mucosa; ++, unequivocal nuclear staining of >50% of cells.

IM, intestinal metaplasia.

the metaplastic-dysplasia-carcinoma sequence of CLO.^{13 14} Studies have demonstrated that allelic deletions of 17p, the region where the p53 gene locus is situated, and mutations within the p53 gene itself are frequent events in oesophageal adenocarcinoma.^{10 15-17} In general, p53 gene mutation appears to be a late event in the metaplasia-dysplasia-carcinoma sequence, usually only being demonstrable in high grade dysplasia rather than in low grade dysplasia or in the adjacent non-dysplastic metaplastic mucosa.^{10 16} Certainly, p53 protein overexpression is a consistent feature of high grade dysplasia and adenocarcinoma in CLO.⁸⁻¹⁰ While p53 gene mutation and p53 protein overexpression appear to be specific to more advanced neoplasia in CLO, there is no evidence that these correlate with clinical, pathological, or staging factors of CLO adenocarcinoma or with survival.^{18 19}

Although there is increasing knowledge of the role of p53 in carcinogenesis, including that complicating CLO, the p53 field has recently become further complicated by the recognition that there exists a series of genes that are closely related to p53.⁷ A locus on human chromosome 1p36, encoding a protein termed p73, has been reported to have considerable homology with mammalian p53.²⁰ Containing an N terminal transactivation domain with considerable identity to the DNA binding domain of p53 including absolute conservation of the critical DNA contact residues, p73 also demonstrates identity and similarity in the oligomerisation domain. p73 differs from p53 in having a long C terminal extension which as a result of alternate splicing exists in two isoforms, a long form (p73 α) and a short form (p73 β). Recently, a second locus (at human 3q27-29) has been demonstrated encoding a p53 homologue that has been termed p63.²¹ This protein has been independently identified by other groups and variously named KET, p40, p51, p73L, and CUSP.²² Like p73, p63 can undergo alternate splicing with three C terminal variants (α , β , and γ), two N terminal variants (TA and Δ N), and an interstitial splice variant in which 12 bases (encoding four amino acids) may be deleted from exon 9.²¹ p73 and p63 have considerable homology at the amino acid level as well as similar genomic architecture, significantly in the regions homologous to p53. The existence of a family of p53 like genes

is perhaps not a surprise given that other critical cellular regulators exist in gene families. Indeed, elements of parallel tumour suppressor pathways (such as the retinoblastoma pathway) and many other molecular systems show this phenomenon, often with consequent functional redundancy. At present little is known about the biology and function of the newly reported p53 homologues.

We have recently begun to characterise expression of p63 and a major isoform Δ Np63 by generating an antiserum (SC1) that recognises p63 isoforms but not p53 nor the highly related p53 homologue p73.²² Western blot analysis has shown that both p63 α and the N terminal truncated form of p63 α (Δ Np63 α) are found in a range of cell lines. Similar immunoblot analysis of tissues reveals considerable complexity with at least four SC1 immunoreactive isoforms being identified. In immunohistological studies SC1 immunoreactivity is widely detectable, being predominantly associated with proliferative compartments in epithelia. This is in agreement with other studies which have reported that there may be a relationship between p63 expression and proliferative compartments in epithelia and that overexpression can be seen in neoplasia.^{23 24} The availability of the SC1 reagent allows examination of expression of p63 in pathological material. Consequently, we report the first study in which expression of p63 isoforms has been investigated, in parallel with studying p53 protein, in a morphologically well defined series representing various stages of the metaplasia-dysplasia-carcinoma sequence in Barrett's oesophagus.

Material and methods

ANTISERUM AND CHEMICALS

All chemicals and other reagents were from Sigma UK Ltd (Poole, UK) unless otherwise stated.

The 13 C terminal residues of rat KET (NH₂-DMDSRRNKQQRK) were conjugated to KLH prior to immunisation of rabbits using standard methods.²² The resultant hyperimmune serum was designated SC1 and its characterisation has been reported in detail.²² Expression of p53 protein was assessed using the monoclonal antibody DO-1 (Dako, High Wycombe, UK) as previously described.²⁵

PATIENTS, TISSUES, AND IMMUNOHISTOCHEMISTRY

All tissue samples used in this study derived from a retrospective survey of paraffin embedded archival biopsy material from patients with endoscopic and histopathological substantiation of a diagnosis of traditional/classical CLO treated at Gloucestershire Royal Hospital. Thus all patients had at least a 3 cm segment of CLO in the lower oesophagus. Ten cases were selected for each patient group identified as in table 1. Biopsies of oesophageal type squamous mucosa and of non-neoplastic CLO were derived from patients under routine surveillance for CLO. In cases with dysplasia and carcinoma, the diagnosis had been previously confirmed by independent review of one expert gastrointestinal pathologist (NAS).

All samples had been immediately fixed in 10% buffered formalin for 24 hours prior to processing to paraffin wax using standard procedures. Paraffin sections (4 µm) were cut, dewaxed, and rehydrated using standard procedures. After blocking endogenous peroxidase activity (hydrogen peroxide (3% v/v) in phosphate buffered saline (PBS) for 20 minutes), non-specific binding activity was reduced by incubation with normal goat serum (20% v/v in PBS) for 20 minutes at room temperature. Slides were then incubated overnight at 4°C with either primary antibody (SC1) (1:2000 (v/v) in neat goat serum) or preimmune sera (negative control; 1:1000 (v/v) in neat goat serum). Unbound antibody was removed and washed three times in PBS for five minutes. The antibody signal was then amplified by incubation for one hour at room temperature in a 1:100 (v/v) solution of goat antirabbit HRP (Dako, High Wycombe, UK) prepared in neat goat serum. Visualisation was achieved using the rabbit StriAvidin supersensitive immunohistochemistry kit (Biogenex, San Ramon, USA) according to the manufacturer's instructions. After immunostaining, sections were washed in distilled water, lightly counterstained with Mayer's haematoxylin, dehydrated, cleared, and mounted in DPX mounting medium (BDH, Poole, UK) according to the manufacturer's instructions.

The immunohistochemically stained sections were assessed and scored independently by two histopathologists (PAH and NAS) using the scoring system shown in table 1. While noting any gross staining patterns, p63 and p53 expression were only considered to be present if 10% of nuclei showed uniform staining. In all cases but two, there was complete agreement concerning the staining pattern. In the two cases, consensus agreement was achieved on discussion and review.

Results

In the studies described here we used the then available sequence of the C terminus of rat p63α (KET) to design a peptide (NH₂-DMDSRRNKQQRIK) to be employed for immunisation of rabbits by standard methods.²² Reports have shown that this sequence is identical to the analogous murine cDNA and the same except for one conservative substitution to the human sequence (NH₂-DMDARRNKQQRIK).^{21, 26} The resultant antiserum SC1 recognises KET (rat p63α) in *in vitro* transcription-translation reactions of KET cDNA but does not recognise either p73α or murine p53 in control experiments.²² In addition, SC1 does not recognise recombinant human p53 protein in western blots. The N terminal truncation of p63α, designated ΔNp63α, generated as a consequence of alternate promoter usage,²¹ and other potential splice variants of p63 that retain the C terminus, would be recognised by SC1.²² p63 protein expression, as judged both by western blotting and immunohistochemistry, has been reported to be very widespread in many human tissues.²² Importantly, in our studies, no staining was found with preimmune serum

Figure 1 p63 (SC1) immunostaining in non-neoplastic intestinal type Barrett's mucosa. There is nuclear staining best seen in the proliferative zone in the lower aspect of the crypt. There is also occasional positivity in nuclei in the surface epithelium.

even when used at higher concentrations than employed for the SC1 serum. This finding was also observed in normal rodent tissues (unpublished results), and is in stark contrast to the expression pattern of p53 which is rarely detectable by immunohistochemical methods in normal unstressed tissues and cells, even when sensitive amplification systems are employed.²⁷

The results of the immunohistochemical assessment by the two histopathological observers are summarised in table 1. Two general observations can be made concerning the localisation and distribution of p63 in oesophageal tissues. SC1 immunoreactivity was usually found to be nuclear and in particular in the nuclei of epithelial cells (figs 1, 2), although occasionally cytoplasmic immunoreactivity was also observed. In non-neoplastic mucosa, if observed at all, SC1 immunoreactivity was restricted to the proliferative basal layer of the oesophageal stratified squamous epithelium and to nuclei of the cells in the immediate parabasal layer. In those cases of non-neoplastic Barrett's mucosa that expressed SC1, expression was particularly seen in intestinalised mucosa. Nuclear staining was predominantly in the proliferative zone but was also occasionally observed in the surface epithelium (fig 1).

In neoplastic epithelium, while the staining remained generally nuclear, it demonstrated an increased intensity and wider distribution. This distribution pattern broadly paralleled the site(s) of proliferative activity in this epithelium. No staining was seen in the smooth muscle of the muscularis propria nor in connective

Figure 2 p63 (SC1) immunostaining in an adenocarcinoma arising in Barrett's oesophagus. There is nuclear staining in the infiltrating adenocarcinoma (below) while the overlying non-neoplastic Barrett's mucosa is negative. In this case there was a very similar staining pattern, in the carcinoma, for p53.

tissue fibroblasts or endothelium. This proliferation associated distribution of epithelial p63 nuclear immunoreactivity is consistent with previous studies in human^{22 23} and rodent (data unpublished) tissues. Occasionally, staining of suprabasal keratinocytes has also been observed but this is inconsistent and in studies of oral squamous epithelium such staining has been found to be non-specific.²⁴ Consequently, we interpret this pattern with some caution. Demonstration of some cytoplasmic staining in the glandular epithelium of CLO and also in normal columnar epithelium of oesophageal glands is of interest and initially somewhat surprising. However, cytoplasmic expression of p53 is now well documented^{28 29} and it may be that p63 (and other p53 homologues) can also on occasion exist in cytoplasmic pools.

The extent of nuclear p63 expression was observed to increase with the severity of neoplastic change in CLO (table 1). Thus in low grade dysplasia, only two of 10 cases showed strong and widespread nuclear staining while two showed focal nuclear staining, more marked in the crypts rather than on the surface, in a manner analogous to that seen in non-neoplastic intestinal type CLO. In high grade dysplasia, strong nuclear staining was prevalent and more widely distributed (table 1). A similar distribution of staining was seen in invasive adenocarcinoma (fig 2) although there was no evidence of a relationship between p63 expression and the grade of carcinoma.

Comparing p53 and p63 staining, the neoplastic CLO cases with strong widespread staining for p63 paralleled the profile of p53

immunoreactivity in those same cases (table 1). Significantly, SC1 serum does not recognise p53, even in stringent biochemical assays, and the epitope recognised by DO-1 (in the N terminus of human p53) is not present in p63 (or p73).²² Most of the non-neoplastic CLO mucosa was negative for p63, as it was for p53 (table 1), although some cases with p63 show restricted nuclear staining in the proliferative zone of the epithelium with some expression in surface epithelium (fig 1). Interestingly, this was specifically observed in intestinal type CLO mucosa, in patients with and without evidence of neoplasia elsewhere in the CLO segment. This pattern of staining was not observed in any cases with p53 (table 1). An important technical issue, pertaining to the p63 staining in normal tissues in these studies, is that no staining was detected with preimmune serum even when used at higher concentrations than employed for the SC1 serum.

Discussion

An extensive understanding of the biochemical properties of p53 has now developed and it is clear that regulation of the p53 pathway involves many levels of control.^{6 7 30} These include regulation of p53 protein stability, modulation of post-translational modifications such as phosphorylation and acetylation, a range of protein-protein interactions, and manipulations in the subcellular localisation of the p53 protein. The cellular insults that can activate the p53 pathway are diverse. They include DNA damage, induced by a wide range of physical and chemical agents, from heat shock and alterations in pO₂ tension through to altered cell-substrate and cell anchorage interactions, growth factor and cytokine modulated events, and the effects of activated oncogenes via an ARF dependent pathway. The downstream consequences of p53 activation remain incompletely understood although most data are consistent with the view that p53 is a transcription factor capable of activating (and in some cases repressing) the activity of target genes via binding to canonical p53 responsive elements. The physiological activities so elicited include (but are certainly not restricted to) apoptosis and growth arrest. The p53 pathway is carefully regulated in a cell type and tissue type specific manner that remains poorly understood.^{27 31}

The discovery of p53 homologues and the recognition that both p73²⁰ and p63^{21 22} can exist in multiple forms has greatly complicated an already complex field. All members of the p53 family are transcription factors with very similar DNA binding domains capable of binding to p53 consensus sequences and trans-activating downstream target genes. However, there is increasing evidence that there may be a differential ability to activate (or repress) p53 responsive elements.^{32 33} Furthermore, there appear to be transdominant negative forms of p63 which as a consequence of alternate promoter usage give rise to N terminal truncated forms of p63 devoid of the transcriptional activation domain.²¹ Additional alternate

splice forms, some with long C terminal extensions, may form a range of hetero-oligomers with novel properties, both in terms of transcriptional activation of different promoters as well as in protein-protein interactions, post-translational modifications, and related regulatory mechanisms.²⁰⁻³⁴ Finally, competition between various species and isoforms at promoters may profoundly alter the functional properties of the p53 pathway. Supporting this notion is the idea that the previously reported differentiation related activities of p53³⁵⁻³⁷ may in fact be a consequence of p53 perturbing the differentiation and developmental roles of p63 and p73 rather than the presence of specific p53 differentiation regulating properties. This may be manifest by mutant p53 overexpression altering the properties of p63 and p73 in tumours. It is notable that, in this study, an absolute concordance of overexpression of p63 and p53 in more advanced neoplasia, high grade dysplasia and invasive adenocarcinoma in CLO, was observed. These close interactions are being increasingly recognised in other families of transcription factors, such as the helix-loop-helix and Id family³⁸⁻³⁹ where the stoichiometry modifications and subcellular localisation of members profoundly alter the expression of target genes.

In the light of these findings, the observation that p63 is widely expressed in human tissues²² and is demonstrable in the proliferative compartments of both the normal squamous mucosa and the metaplastic glandular epithelium of the oesophagus raises important questions. It may be that p63 isoforms have important biological roles in epithelia.⁴⁰⁻⁴¹ However, it must be emphasised that in this study, p63 expression, in non-neoplastic Barrett's mucosa, was also seen in surface epithelium so that there is certainly not absolute concordance between p63 expression and proliferation. Also, there is no clearcut relationship in this study and in one other²² between p63 isoform expression and apoptosis.

Evidence for the important role of p63 in biological systems comes from the dramatic phenotype of p63 null mice⁴²⁻⁴³ and the observation that mutations in p63 can account for autosomal dominant ectodermal defects.⁴⁴ Our previous observation that it is the ΔN form of p63, devoid of the transcriptional activation domain, that is the predominant form of p63 in the proliferative compartments of tissues may suggest that this represents an endogenous dominant negative regulator of p53 activity, an endogenous "anti-p53 protein". Alternatively, the exact concordance of p53 and p63 expression in more advanced forms of neoplasia in CLO, high grade dysplasia and invasive adenocarcinoma, suggests a strong positive relationship in the protein expression of the two genes in such neoplasia. To understand the function of the p53 pathway and its critical role of carcinogenesis will require the detailed definition of all components of the p53 pathway in human cells and their complex interplay. Thus while there is often a good correlation between p53 expression and the neoplastic phenotype, the relative failure of studies of p53 to equate

with clinically useful parameters in malignant CLO¹⁰⁻¹⁸ may be due to the incomplete nature of our definition of the various components of the p53 pathway (including p63).

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