JOURNAL OF PATHOLOGY, VOL. 180: 129–137 (1996)

A MOLECULAR AND IMMUNOHISTOCHEMICAL STUDY OF THE MDM2 PROTEIN ISOFORMS AND *p53* GENE PRODUCT IN BRONCHOGENIC CARCINOMA

VASSILIS G. GORGOULIS*, VASSILIS ZOUMPOURLIS†, GEORGE Z. RASSIDAKIS*, ANDREAS KARAMERIS‡, ANTONIS N. RASSIDAKIS§, DEMETRIOS A. SPANDIDOS† AND CHRISTOS KITTAS*

**Department of Histology and Embryology, University of Athens, School of Medicine, Athens, Greece; †Institute of Biological Research and Biotechnology, National Hellenic Research Foundation, Athens, Greece; ‡Department of Pathology, 401 Army General Hospital, Athens, Greece; §Department of Pulmonary Medicine, University of Athens, School of Medicine, 'Sotiria' Hospital, Athens, Greece*

SUMMARY

Forty-one bronchogenic carcinomas were investigated for expression of MDM2 protein isoforms and their relationship to p53 protein levels and *p53* gene alterations using molecular and immunohistochemical techniques. The findings were correlated with the pathological features of the carcinomas. MDM2 protein was overexpressed in 26 cases (63 per cent). Western blot analysis with two monoclonal antibodies, 1B10 and IF2, revealed three MDM2 protein isoforms, p90, p57 and p76/74. p90 and p57 are capable of interacting with p53 protein, while p76/74 is not. Various patterns of MDM2 isoforms were seen. Although no correlation between the patterns and pathological features was observed, lymph node metastases were more frequent in the cases with MDM2 overexpression ($P<0{\cdot}005$). In 3 out of 17 specimens of normal lung tissue examined, there was a low level of expression of p90. Molecular analysis revealed that MDM2 overexpression was a consequence of increased transcription rather than *MDM2* gene amplification. p53 protein was overexpressed in 21 cases (51 per cent) and *p53* gene alterations (mutations+allelic deletions) were detected in 23 patients (56 per cent). A high degree of concordance (76 per cent) between *p53* mutations and p53 staining was noticed ($P<10^{-5}$). *p53* gene alterations were significantly associated with lymph node disease ($P<0{\cdot}01$). MDM2 and p53 proteins were simultaneously detected in 21 cases (51 per cent), of which 17 (42 per cent) showed p53 and MDM2 overexpression. The latter group was positively correlated with *p53* mutations ($P<0{\cdot}05$). A strong correlation between MDM2/p53 co-expression and lymph node metastases was observed ($P<0{\cdot}001$). The findings suggest that MDM2 overexpression is a common event in bronchogenic carcinoma. The selective expression of some MDM2 isoforms in neoplastic tissue and not in the surrounding normal areas underscores the pathological role of the various MDM2 products. Finally, the coexistence of MDM2 protein(s) and p53 aberrations (mutations and/or overexpression) in a subset of lung carcinomas may be indicative of a 'gain of function' phenotype, with more aggressive characteristics.

KEY WORDS—**MDM2 protein isoforms; *MDM2* gene amplification; *p53* gene alterations, lung carcinoma**

INTRODUCTION

Bronchogenic carcinoma represents the most frequent fatal malignancy in both sexes and has exceeded breast carcinoma as a cause of death in women.[1] To date, several genetic alterations have been related to lung cancer.[2]

The *p53* oncosuppressor gene is mapped to chromosome 17p13 and encodes a 53 kD nuclear phosphoprotein with a short half-life. It appears to control a cell cycle checkpoint responsible for maintaining the integrity of the genome.[3] Functional loss of p53, mostly via mutations, is the most common genetic lesion in human cancer.[4] Missense mutations may stabilize the protein, prolong its half-life, and permit its immunohistochemical detection.[4] The recent identification of several genes regulated by p53 has increased our understanding of the functions of p53. These genes include *gadd45*, *waf-1/cip-1*, the *Bcl2/bax* duet, and *MDM2*.[3]

The human *MDM2* (murine double minute-2) gene is mapped in chromosome 12q13–14 and encodes a cellular phosphoprotein capable of forming complexes with both wild-type and mutant p53.[5,6] The *MDM2* gene was identified in a tumourigenic derivative of mouse 3T3 cells (3T3DM cell line), where it was amplified and overexpressed.[7] The amino acid sequence of MDM2 is similar to that of transcriptional activators. The amino terminal region of the protein contains a p53 binding domain, suggesting that MDM2 is a transcription factor regulating the p53 protein (Fig. 1a).[8] Thus, on the one hand, MDM2/p53 forms an autoregulatory feedback loop that may be needed to maintain a critical MDM2/p53 ratio within the cell;[9] on the other hand, an excess of MDM2 protein can abrogate the transcriptional activation of wild-type p53, providing an alternative mechanism of p53 inactivation, other than mutation. The *MDM2* gene generates various MDM2 products, of which only a subset complexes with p53 protein.[8] In human cancers, the *MDM2* gene was found to be deregulated in sarcomas,[5,10] glioblastomas,[11] breast carcinomas,[12] leukaemias, and lymphomas.[13,14]

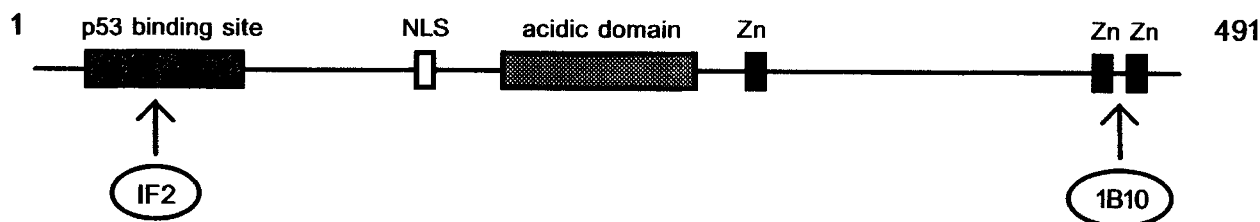
Previously we showed that MDM2 is overexpressed in a significant proportion of lung carcinomas.[15] Wiethege *et al.* have also examined MDM2 expression in human bronchial epithelium.[16] In the present report we investigated in more detail the molecular basis of MDM2 expression and its relationship with p53 protein, in a

Addressee for correspondence: V. G. Gorgoulis, 53 Antaiou str., Lamprini Ano Patisia, Athens, Greece GR-11146.

CCC 0022–3417/96/100129–09
© 1996 by John Wiley & Sons, Ltd.

Received 12 September 1995
Accepted 3 April 1996

1a



NLS : Nuclear Localization Signal domain
Zn : Zinc finger domains

1b : Reactivity of MDM2 isoforms detected in our series by antibodies 1B10 and IF2

	p90	*p76/74*	*p57*
1B10	+	+	-
IF2	+	-	+

Fig. 1—(a) Reactivity of 1B10 and IF2 antibodies with the full length MDM2 protein epitopes. (b) Reactivity of MDM2 isoforms detected in our series by antibodies 1B10 and IF2

series of 41 bronchogenic carcinomas. The aims of our study were the following: to examine the immunolocalization of MDM2 and the expression of its isoforms; to look into the mechanism of MDM2 overexpression using Northern blotting and differential polymerase chain reaction (D-PCR) analysis; to study the concordance between p53 protein abnormality, detected by immunohistochemistry and immunoblotting, and *p53* gene alterations, detected by the single-strand conformation polymorphism (SSCP) technique and Southern blotting; and, finally, to correlate MDM2 expression and isoform patterns with the p53 protein status, gene alterations, and pathological features of the carcinomas.

MATERIALS AND METHODS

Tissue samples

Forty-one lung carcinomas were taken shortly after surgery. Two samples of each tumour were obtained. One sample of each tumour was snap-frozen in liquid nitrogen and stored at − 70°C; the other was formalin-fixed and paraffin-embedded (FFPE). In addition, adjacent normal tissue was included from each specimen examined. The patients had not undergone any chemo- or radiotherapy prior to surgical resection, thus avoiding up- and down-regulation of p53 and MDM2 proteins, respectively, due to DNA damage. Tumours were classified following the World Health Organization criteria (Table I) and the TNM staging system.[1] Seventeen tumours were stage I (T1–2, N0, M0), 15 stage II (T1–2, N1, M0), and nine stage IIIa (eight T1–2, N2, M0 and one T3, N1, M0).

Antibodies

Immunohistochemical and immunoblotting analyses of p53 and MDM2 were carried out with monoclonal antibodies (MAbs) DO7 (Dako, Denmark), DO1 (kindly provided by Dr D. P. Lane, Dundee, U.K.), 1B10 (Novocastra Laboratories, U.K.), and IF2 (Oncogene Science, NY, U.S.A.) (Table II, Fig. 1a).

MDM2 *and* p53 *immunohistochemistry (IHC)*

Immunohistochemical analyses was performed on tissue sections using the streptavidin–biotin–peroxidase methodology (Dako, Denmark). p53 and MDM2 proteins were unmasked with the heat-mediated antigen

Table I—Summary of immunohistochemical and molecular findings in relation to pathological features

Patient No.	Dx	Grade	LN	p53 IHC	p53 WB	p53 SSCP	p53 LOH	MDM2 IHC (1B10)	MDM2 WB (1B10)	MDM2 WB (1B10)	MDM2 WB (IF2)	MDM2 WB (IF2)
									74/76	90	57	90
1	Sq	WD	–	–	–	–	NI	–	–	LL	–	LL
2	Sq	WD	–	+	LL	–	NI	++	–	×	×	×
3	Sq	MD	–	++	×	NI	ND	–	–	–	–	–
4	Sq	MD	+	+++	×	Exon 5	No	–	–	–	–	–
5	Sq	MD	+	–	–	–	ND	++	×	×	×	
6	Sq	WD	–	+	LL	–	Yes	+++	×	×	×	×
7	Sq	MD	+	+++	×	Exon 5	Yes	+++	×	×	×	×
8	Sq	MD	+	+++	×	Exon 7	Yes	+++	–	×	×	×
9	Sq	MD	–	+++	×	Exon 8	NI	++	–	×	–	×
10	Sq	PD	+	+++	×	Exon 8	Yes	+++	–	×	–	×
11	Sq	PD	+	++	×	Exon 7	Yes	+++	×	×	×	×
12	Sq	PD	+	+++	×	Exon 6	NI	++	–	×	×	×
13	Sq	MD	–	–	–	–	NI	–	–	–	–	–
14	Sq	MD	–	–	LL	–	No	–	–	–	–	–
15	Sq	MD	–	–	–	–	ND	–	–	LL	–	LL
16	Sq	PD	+	+++	×	Exon 7	NI	++	×	–	–	–
17	Ad	MD	–	–	–	–	ND	–	–	–	–	–
18	Ad	PD	+	+++	×	Exon 5	Yes	+++	×	×	–	×
19	Ad	MD	+	+++	×	Exon 8	No	+++	–	×	×	×
20	Ad	MD	–	+++	×	Exon 7	NI	–	–	–	–	–
21	Ad	MD	+	–	–	–	ND	+++	×	LL	–	LL
22	Ad	MD	+	+	LL	–	NI	+++	×	–	–	–
23	Ad	MD	–	+++	×	Exon 7	NI	++	–	×	–	×
24	Ad	MD	+	–	–	Exon 5	Yes	++	×	–	–	–
25	Ad	PD	–	+++	×	Exon 8	Yes	–	–	–	–	–
26	Ad	PD	+	+++	×	–	No	+++	×	×	×	×
27	Ad	PD	+	+++	×	–	No	+++	×	×	×	×
28	Ad	WD	–	–	–	–	No	–	–	–	–	–
29	Ad	WD	–	–	–	–	NI	++	×	–	–	–
30	Ad	MD	–	–	–	NI	ND	+	–	LL	–	LL
31	Ad	MD	+	–	LL	–	No	–	–	–	–	–
32	SCLC		–	–	–	–	No	–	–	–	–	–
33	SCLC		+	–	–	–	Yes	–	–	–	–	–
34	SCLC		+	–	–	–	Yes	++	–	×	×	×
35	SCLC		+	+	LL	Exon 8	Yes	++	–	×	×	×
36	SCLC		+	+++	×	Exon 7	NI	++	×	×	–	×
37	SCLC		+	+++	×	Exon 7	Yes	+++	×	LL	–	LL
38	SCLC		+	++	×	Exon 7	NI	+++	×	×	×	×
39	SCLC		+	+++	×	Exon 6	Yes	+++	×	–	–	–
40	SCLC		+	+++	×	Exon 8	NI	+++	×	×	×	×
41	SCLC		–	–	–	–	NI	–	–	–	–	–

Dx=diagnosis; LN=lymph node metastases; IHC=immunohistochemistry; WB=Western blot; SSCP=single strand conformation polymorphism; LOH=loss of heterozygosity; Sq=squamous cell lung carcinoma; Ad=adenocarcinoma; SCLC=small cell lung carcinoma; WD=well differentiated; MD=moderately differentiated; PD=poorly differentiated; LL=low levels; NI=non-informative; ND=not done; × =elevated levels.

retrieval (HMAR) method, as previously described.[15] Visualization was carried out with diaminobenzidine as chromogen. Laryngeal carcinomas expressing p53 and a leukaemic cell line, K562, overexpressing MDM2[13] were used as positive controls. Mouse IgG1 MAb of unrelated specificity and the IgG fraction of normal rabbit serum were used as negative controls. The p53, MDM2 positive cases were classified using the following semi-quantitative method: 0=negative; (+)=<20 per cent of cells positive (mild expression); (++)=20–50 per cent of cells positive (moderate expression); and (+++)=>50 per cent of cells positive (intense expression).

Simultaneous extraction of nuclear proteins and DNA

The samples were homogenized in a hypotonic buffer (25 mM Tris–HCl, pH 7·5; 5 mM KCl; 0·5 mM $MgCl_2$; 0·5 mM DTT; 0·5 mM PMSF) at 5–10 mg/ml. The nuclei were pelleted at 2500 rpm for 10 min at 4°C, washed 3 times with an isotonic buffer (25 mM Tris–HCl, pH 7·5; 5 mM KCl; 0·5 mM $MgCl_2$; 0·2 M sucrose; 0·5 mM DTT; 1 mM PMSF), and resuspended in nuclear extraction buffer (25 mM Tris–HCl, pH 7·5; 1 mM EDTA; 0·1 per cent NP-40; 0·5 mM DTT; 0·5 mM PMSF). Nuclear extracts were clarified after centrifugation at 25 000 rpm

Table II—Characteristics of the antibodies used in the present study

MAb	Class	Epitope
DO-7	IgG2b	Residues 1–45 of p53
DO-1	IgG2a	Residues 21–25 of p53
1B10	IgM	–COOH terminal portion of MDM2
IF2	IgG2b	$-NH_2$ terminal portion of MDM2

for 60 min at 4°C. Supernatant containing the extracts was stored at − 70°C. DNA was extracted from the final pellet, which contained a mixture of nucleic acids and nuclear debris, following standard protocols.[17]

RNA extraction–MDM2 Northern blotting

RNA was extracted from the specimens using the Rnazol B reagent (BioGenesis) and Northern blot hybridization was performed with a 585-base pair *MDM2* ^{32}P-dCTP-labelled probe spanning nucleotides 650–1214 of the published cDNA sequence, as previously described.[15] The levels of *MDM2* mRNA were scored by eye on a relative basis from 0 (negative) to + + + (strong). The scoring was standardized by including samples of K562 mRNA in all gels.

D-PCR for MDM2 gene amplification

D-PCR was carried out as previously described.[18] As target and reference sequences, we used a 230 bp and a 150 bp fragment of the *MDM2*[5] and interferon gamma (*IFN-γ*) genes,[18] respectively. The primer sequences for the 230 bp *MDM2* gene fragment were 5′-TGAGTGAGAACAGGTGTCACC-3′ (sense) and 5′-TTCTAGATGAGGTAGATG-3′ (antisense). A leiomyosarcoma carrying an eight-fold *MDM2* gene amplification was used as a positive control.

MDM2 *and* p53 *Western blot analysis*–MDM2 *immunoprecipitation*

(A) Twenty micrograms of nuclear proteins was electrophoresed on 10 per cent polyacrylamide-SDS gel and transferred to nitrocellulose membrane.[17] Blots were blocked for 2 h in 5 per cent non-fat dry milk/PBST (PBST=PBS, 0·1 per cent Tween) at room temperature. Subsequently, the membranes were incubated overnight with 1B10 and IF2 antibodies (diluted 1:500) and DO-1 (diluted 1:200) at 4°C. For detection, the biotinylated rabbit anti-mouse immunoglobulin (1:1000) (Dako, Denmark) and streptavidin–biotin–peroxidase complex (Dako, Denmark) were applied. The MDM2, p53 levels were analysed using an enhanced chemiluminescence system (Amersham Corp., Arlington Heights, IL, U.S.A.). Protein from a leiomyosarcoma overexpressing MDM2 and normal lung tissue with undetectable levels of MDM2 protein were used as positive and negative controls, respectively. Protein from the HT29 colon cancer cell line, which overexpresses p53, was used as a positive control (American Type Culture Collection). Band sizes were determined by comparison with migration of broad range protein ladder (Biolabs, MA, U.S.A.). Protein levels of MDM2 and p53 were scored by eye on a relative basis as follows: 0, negative; +, low levels; + + and + + +, elevated levels (×).

(B) Nuclear extracts from cases 2, 5, 6, 16, and 35 were immunoprecipitated using protein A-Sepharose (Sigma), following standard protocols.[17] Following immunoprecipitation, immunoprecipitates were separated on 10 per cent polyacrylamide gels and immunoblotted.[17]

Nested PCR/SSCP and Southern blot analysis for p53 *gene alterations*

(A) Nested PCR and SSCP analysis was performed on matched normal and tumour DNA as previously described.[19] Briefly, we first determined the optimal conditions for amplification of the 2·9 kb *p53* gene fragment, which contains exons 4–9, and then we amplified individual exons 4–8 with nested PCR. Next we analysed with the SSCP technique exons 4–8, as most previous studies have shown that the majority of *p53* mutations in lung carcinomas are found in this region.[4]

(B) For Southern blot analysis, 10 μg of DNA was digested with *PstI* (Boehringer Mannheim Biochemica, Mannheim, Germany). The resulting fragments were subjected to electrophoresis in 0·8 per cent agarose gels, transferred to nylon membranes (Hybond-N, Amersham), and baked for 3 h at 80°C. The membranes were prehybridized and then hybridized to random primer ^{32}P-dCTP-labelled pYNZ22 probe, which locates chromosomal region 17p13.3. This probe detects restriction fragment length polymorphisms on *PstI* and *BamHI* digested DNA. After hybridization, the membranes were washed under stringent conditions (0·2 × SSC at 65°C for 20 min) and autoradiographed.

Statistical analysis

All statistical correlations were based on the chi-square (χ^2) test with Yates' correction. An additional two-tailed Fisher's exact test was used only when the number of samples in any cell of a given statistical table was 5 or fewer.

RESULTS

MDM2 protein, mRNA, and gene analysis

MDM2 ***protein immunolocalization***—Twenty-seven carcinomas (66 per cent) showed a positive signal for MDM2 protein (any number of tumour cells positive). Moderate or intense MDM2 reactivity was observed in 26 cases (63 per cent) (Table I). Twenty samples were positive with both antibodies, while seven (cases 16, 18, 20, 21, 29, 37, and 39) reacted only with 1B10. MDM2 protein distribution was exclusively nuclear, and finely granular (Figs 2 and 3). Its expression was found not only in dysplastic or neoplastic regions, but also in normal bronchial cells. The dysplastic and normal areas were positive with both antibodies.

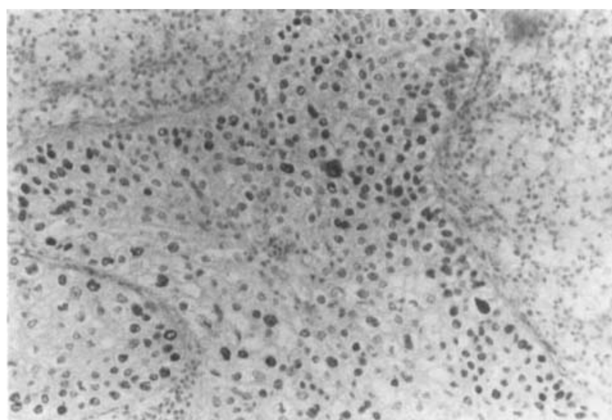


Fig. 2—Intense MDM2 immunoreactivity in a moderately differentiated squamous cell lung carcinoma with the monoclonal antibody 1B10. Streptavidin–biotin–peroxidase technique with haematoxylin counterstain (× 125)

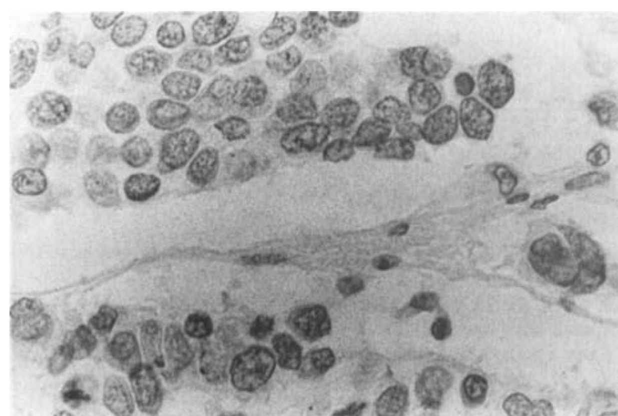


Fig. 3—Finely granular nuclear MDM2 staining in a moderately differentiated lung adenocarcinoma, with the monoclonal antibody 1B10. Streptavidin–biotin–peroxidase technique with haematoxylin counterstain (× 500)

MDM2 ***protein isoform expression***—The immunohistochemical findings were confirmed with Western blot analysis, which also showed three MDM2 protein isoforms of approximately 90 kD (p90), 76/74 kD (p76/74), and 57 kD (p57) (Table I, Figs 4A and 4B). The reactivity of these MDM2 proteins to the antibodies that we used is presented in Fig. 1b. Immunoprecipitation, with the same antibodies, was performed in five selected specimens (cases 5, 8, 9, 16, and 35) and verified the specificity of the bands shown on Western blot. MDM2 isoforms p90, p76/74, and p57 were overexpressed in 19 (46 per cent), 17 (41 per cent), and 14 (34 per cent) carcinomas respectively, while p90 displayed low levels in five cases (Table I). Various MDM2 protein isoform patterns were observed and are shown in Table III. No correlation between isoform patterns, histological subtypes, and metastatic disease was seen. We also examined 17 cases of normal lung tissue and found a low level of expression of the p90 isoform in three samples, two of which were MDM2 immunohistochemically positive (+).

MDM2 ***mRNA and DNA analysis***—Northern hybridization demonstrated a 5·5 kb transcript, corresponding in size to that reported previously[5] (Fig. 5A). Increased levels of *MDM2* mRNA (as compared with the K562 cell line) were absolutely correlated with MDM2 protein(s) overexpression. *MDM2* transcript was not detectable in six out of seven normal lung tissue samples examined, while one showed a weak 5·5 kb band. D-PCR analysis did not detect any *MDM2* gene amplification in our series (Fig. 5B).

p53 protein and gene analysis

p53 ***protein expression***—Twenty-five carcinomas (61 per cent) showed immunoreactivity for p53 protein (any number of tumour cells positive). Moderate or intense p53 staining was observed in 21 cases (51 per cent) (Table I). p53 staining was exclusively nuclear (Fig. 6). Reactivity was confined to dysplastic and neoplastic areas. Immunohistochemistry was confirmed with immunoblotting. Of note, four cases with mild and two with negative p53 immunoexpression demonstrated low levels of p53 protein (Table I, Fig. 4C).

p53 gene analysis—SSCP was used to detect point mutations and insertions or deletions of a short nucleotide sequence, while loss of heterozygosity (LOH) affecting chromosome band 17p13 was determined using the 1·7 kb BamHI fragment from pYNZ22 on Southern blots. SSCP revealed tumour-specific mobility shifts in 20 out of 41 cases (49 per cent). Mutations were observed in exons 5, 6, 7, and 8 in four, two, eight, and six cases, respectively (Table I, Figs 7A–7D). From 21 examined informative specimens, LOH of chromosome 17p13 was observed in 13 cases. In ten of the 13 cases (77 percent), LOH in the *p53* region was accompanied by *p53* mutations, while in the remaining three, no detectable abnormalities of the other *p53* allele were seen (Table I, Fig. 8). Thus, in total, *p53* gene alterations (*p53* mutations and/or LOH) were detected in 23 out of 41 cases (56 per cent).

Relationship between MDM2 *protein expression,* p53 *protein levels, p53 gene alterations, and pathological features (detailed data are listed in Table IV)*

The cases with MDM2 immunoreactivity were more frequently correlated with lymph node metastases ($P<0{\cdot}005$), while those with moderate or intense p53 staining were associated with nodal disease ($P=0{\cdot}08$). On the other hand, a strong correlation was observed between *p53* mutations, *p53* gene alteration, and lymph node metastases ($P<0{\cdot}05$ and $P<0{\cdot}01$, respectively, Table IV). In addition, a highly significant statistical correlation was found between *p53* mutation and p53 staining—19 out of 25 (75 per cent) vs. 1 out of 16 (6 per cent), $P<10^{-5}$.

The two proteins, MDM2 and p53, were simultaneously detected with IHC and/or immunoblotting in 21 cases (51 per cent), of which 17 (42 per cent) showed moderate or intense p53 and MDM2 staining. The latter group was strongly associated with *p53* mutations—15

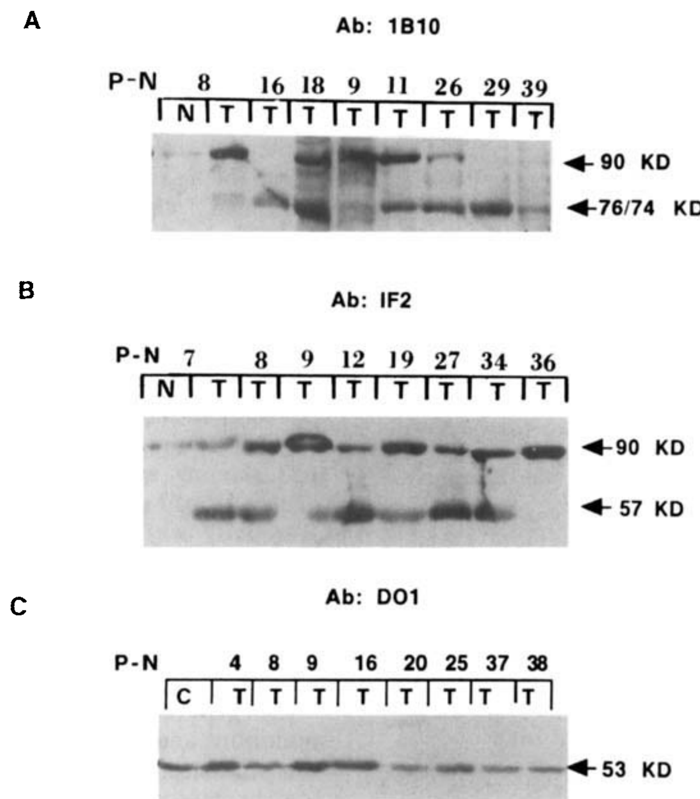


Fig. 4—Representative Western blots showing expression of MDM2 isoform patterns and p53 protein in lung carcinomas. (A) Overexpression of isoforms p90 and p76/74 in neoplastic cases 8, 16, 18, 9, 11, 26, 29, and 39 with the monoclonal antibody 1B10 (lanes 2–9), compared with low levels of p90 in adjacent normal lung tissue from case 8 (lane 1). (B) Overexpression of isoforms p90 and p57 in neoplastic cases 7, 8, 9, 12, 19, 27, 34, and 36 with the monoclonal antibody IF2 (lanes 2–9), compared with low levels of p90 in adjacent normal lung tissue from case 7 (lane 1). (C) Overexpression of p53 in neoplastic cases 4, 8, 9, 16, 20, 25, 37, and 38 with the monoclonal antibody DO1 (lanes 2–9), compared with the cell line HT-29, which overexpresses p53 (lane 1). T=tumour; N=normal; P-N=patient number

out of 20 (75 per cent) vs. 2 out of 21 (9 per cent), $P<10^{-4}$. Moreover, the carcinomas with both proteins detected (any number of cells positive) were statistically correlated with *p53* gene alterations—17 out of 23 (74 per cent) vs. 6 out of 18 (32 per cent), $P<0{\cdot}05$.

Table III—MDM2 protein isoform patterns in the present series of bronchogenic carcinoma

p57	p76/74	p90*	No. of cases
+	−	−	0 (0%)
−	−	+	3 (11%)
−	+	−	7 (27%)
+	+	−	0 (0%)
−	+	+	2 (8%)
+	−	+	6 (23%)
+	+	+	8 (31%)
Total			26 (100%)

*The five cases which displayed low levels of p90 are not included in the table.

Finally, the relationship between the latter groups and lymph node metastases was highly significant ($P<0{\cdot}005$ and $P<0{\cdot}001$, respectively) (Table IV).

DISCUSSION

To the best of our knowledge, this is the first report which deals with the concept of MDM2 protein isoforms in lung cancer. We have examined their expression and relationship to p53 protein levels, gene alterations, and pathological features in a series of 41 surgically resected bronchogenic carcinomas.

Detection of MDM2 was assessed with the MAbs 1B10 and IF2 raised against the C- and N-terminal portion of the protein, respectively. We used these particular antibodies in order to avoid false-negative results due to the production of MDM2 proteins with a truncated $-NH_2$ or $-COOH$ terminal region.[8] Twenty carcinomas reacted with both antibodies, while seven reacted only with 1B10. This discrepancy is most likely due to the sole overexpression of the p76/74 isoform in these seven cases, as shown on Western blot. The p76/74

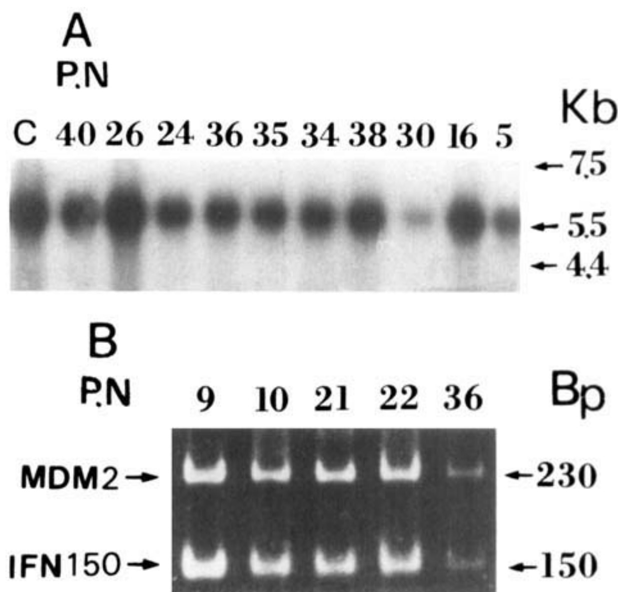


Fig. 5—(A) Representative Northern blot showing overexpression of *MDM2* mRNA in samples 40, 26, 24, 36, 35, 34, 38, 30, 16, and 5 (lanes 2–11). C=K562 cell line (positive control) (lane 1). (B) Representative D-PCR analysis with no indication of *MDM2* gene amplification in samples 9, 10, 21, 22, and 36 (lanes 1–5). IFN150=150 bp fragment of the reference gene interferon-gamma; P-N=patient number

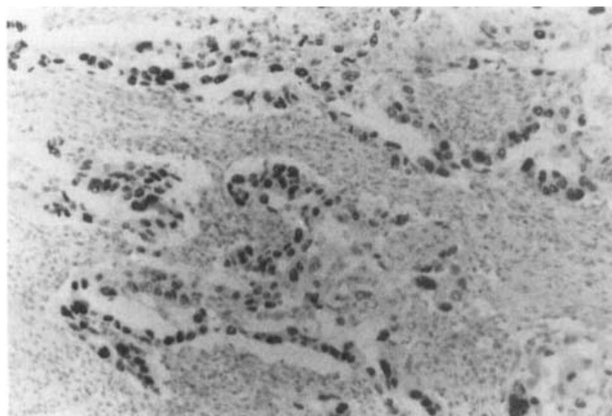


Fig. 6—Moderately differentiated lung adenocarcinoma with intense p53 immunoreactivity with the monoclonal antibody DO7. Streptavidin–biotin–peroxidase technique with haematoxylin counterstain (× 250)

isoform has lost the $-NH_2$ terminal region and thus is not detectable by IF2 MAb[8] (Figs 1a and 1b). MDM2 immunoreactivity was nuclear and was found not only in neoplastic areas, but also in normal bronchial epithelium. Immunoblotting analysis in 17 normal lung tissue specimens revealed a low level of expression of the p90 isoform in three samples. Bronchial epithelium is not the only normal tissue expressing MDM2 protein.[20] MDM2 expression in normal tissues probably reflects an appropriate response to certain external or internal cellular stimuli.[21]

On the other hand, the selective overexpression of some MDM2 isoforms in neoplastic areas compared with the surrounding normal tissue underscores the pathological relevance of the various MDM2 products. Immunoblotting analysis revealed the overexpression of three MDM2-related proteins of molecular weight 90 (p90), 76/74 (p76/74), and 57 (p57) kD. p90 and p57 can form complexes with p53 because they possess the p53 binding domain of the full length molecule, while p76/74 has lost it. In addition, the –COOH terminal portion in p57 is missing (Fig. 1b).

Several MDM2 isoform patterns were observed (Table III). They had no specific correlation with the pathological features of the carcinomas. Most likely these proteins arise from alternatively spliced mRNAs,[22] proteolytic processing, or post-translational modification. Although our study revealed a single *MDM2* transcript, the possibility of the MDM2 isoforms arising from alternatively spliced mRNAs cannot be excluded, since Zauberman *et al.* showed the small difference in the size of the various *MDM2* transcripts (71–276 bp).[22] Such lengths cannot be separated by Northern blotting. Furthermore, Barak *et al.* demonstrated that each *MDM2* transcript, under certain circumstances, has various translational profiles.[23]

The role of these isoforms in tumourigenesis remains obscure. Possibly, overexpressed p90 and p57 either overcome p53 suppression or gain novel oncogenic properties by forming complexes with mutant p53,[6] while p76/74 contributes, via an unknown mechanism, to tumour development.

There are several studies in cell lines[8] and human malignancies[24,25] which have identified several MDM2 isoforms ranging from 44 to 125 kD. In one of these reports, breast carcinomas overexpressing MDM2 were associated with aggressive pathological features.[25] The latter is in agreement with our finding which showed a significant association between elevated levels of MDM2 and lymph node disease ($P<0{\cdot}005$).

MDM2 gene analysis revealed that MDM2 overexpression was due to increased mRNA expression rather than gene amplification. Unlike in sarcomas,[5,10] *MDM2* gene amplification seems to be a rare event in certain types of cancer.[26,27]

In order to estimate more accurately the incidence of p53 overexpression and gene alterations, we carried out a comprehensive analysis using various techniques. p53 was overexpressed in 21 cases (51 per cent). This figure is consistent with those presented in most studies.[2] The protein was also detected in preneoplastic areas, supporting previous reports which suggest its early involvement in lung carcinogenesis.[2,4] As we expected, a high degree of concordance (76 per cent) between SSCP and immunopositivity was observed. *p53* mutations were not found in the remaining 24 per cent, implying either that they exist outside the examined region or that other cellular factors (e.g., MDM2) stabilize p53, permitting its detection.[4]

Seventy-seven per cent of the cases with LOH near the *p53* locus were accompanied by *p53* mutations in the remaining allele. This finding is in accordance with the 'two-hit' hypothesis of oncosuppressor gene action, proposed by Knudson.[29] Two informative tumours with *p53* mutations retained the wild-type allele, implying functional dominance of the mutant over the wild-type form.[3]

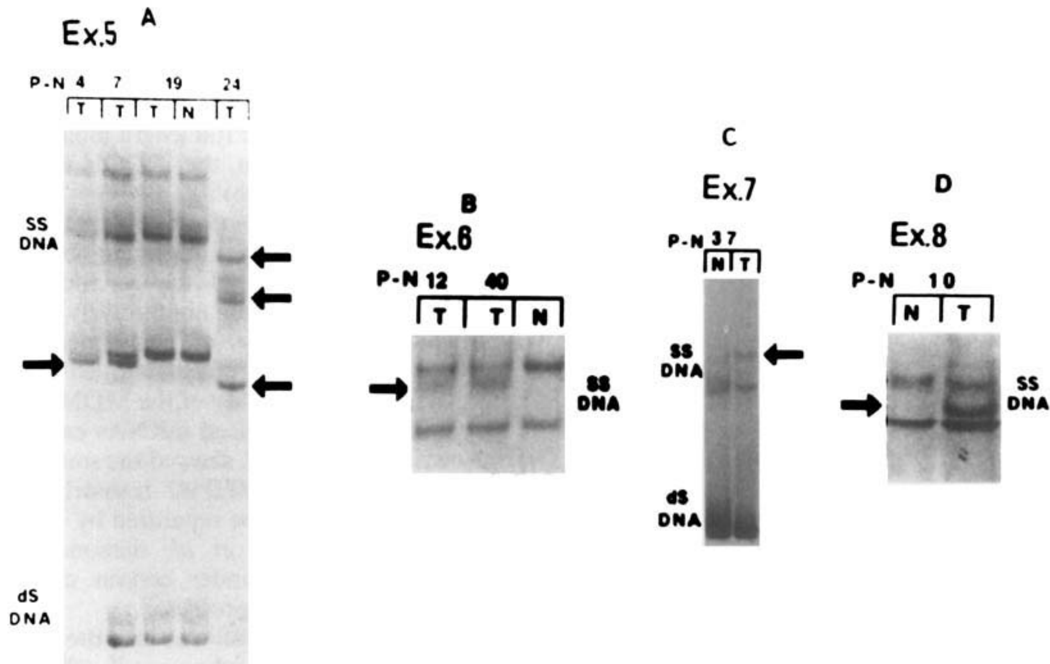


Fig. 7—Representative SSCP analysis of exon 5 (A), exon 6 (B), exon 7 (C), and exon 8 (D). Mobility shifts indicating point mutations, insertions, or deletions of a short nucleotide sequence are presented as aberrant bands (arrows), compared with normal tissue samples. T=tumour; N=normal. The numbers indicate patient numbers (P-N)

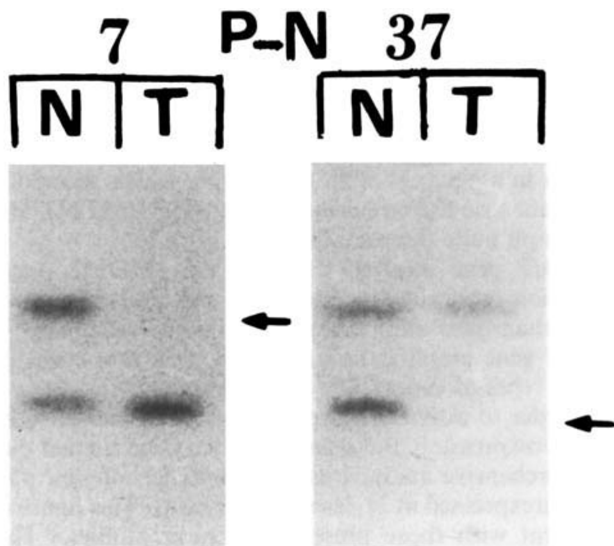


Fig. 8—Representative Southern blot analysis showing loss of heterozygosity in cases 7 and 37, with disappearance of the longer and shorter allele, respectively (arrows). T=tumour; N=normal. The numbers indicate patient numbers (P-N)

We observed a strong association between *p53* gene alteration and lymph node metastases ($P=0{\cdot}01$), but not between p53 overexpression and metastatic disease ($P=0{\cdot}08$). The prognostic significance of p53 alterations in lung cancer remains controversial[2] and other factors are likely to modulate the final outcome.

Our most interesting finding was the highly significant relationship between the cases which co-expressed MDM2 and p53, and lymph node metastases ($P<9\times10^{-4}$). Interestingly, 86 per cent of these patients possessed an alteration at least in one of the two *p53* alleles. This is similar to the findings of Cordon-Cardo *et al.* in sarcomas.[10] It seems that in a subset of bronchogenic carcinomas, the MDM2 protein co-exists with the mutant p53. This may be indicative of mutant p53–MDM2 protein complex activity,[6] or may reflect a 'gain of function' phenotype. The latter hypothesis is based on evidence which demonstrates that p53 mutants acquire novel 'oncogenic' properties.[3,30]

Table IV—Relationship of MDM2 and p53 status with lymph node metastases

	LN (+) No. (24)	LN (−) No. (17)	*P*
MDM2			
IHC (+)-(+++)	21 (91%)	6 (35%)	<0·005
p53			
IHC (+)-(+++)	17 (71%)	8 (47%)	0·1
IHC (++) or (+++)	15 (63%)	6 (35%)	0·08
Mutations	15 (63%)	5 (29%)	<0·05
Gene alterations	18 (75%)	5 (29%)	<0·01
MDM2–p53 co-expression			
IHC (+)-(+++)	18 (75%)	3 (18%)	<0·001
IHC (++) or (+++)	15 (65%)	2 (12%)	<0·005

(+)-(+++): any number of tumour cells positive.
(++) or (+++): moderate or intense expression.

In conclusion, our findings show that *MDM2* gene products are overexpressed in a subset of bronchogenic carcinomas. It appears that they may play an important role in tumourigenicity and/or progression in this type of neoplasia. Further studies are needed in order to elucidate the molecular nature of the p53–MDM2 relationship, as well as its possible clinical value.

REFERENCES

1. Tumors of the Lower Respiratory Tract. 3rd edn. AFIP, 1995.
2. Kalemkarian PG. Biology of lung cancer. *Curr Opin Oncol* 1994; **6:** 147–155.
3. Hainaut P. The tumor suppressor protein p53: a receptor to genotoxic stress that controls cell growth and survival. *Curr Opin Oncol* 1995; **7:** 76–82.
4. Greenblatt MS, Bennett WP, Hollstein M, Harris CC. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. *Cancer Res* 1994; **54:** 4855–4878.
5. Oliner GD, Kinzler KW, Meltzer PS, George DL, Vogelstein B. Amplification of a gene encoding a p53-associated protein in human sarcomas. *Nature* 1992; **358:** 80–83.
6. Momand J, Zambetti GP, Olson DC, George D, Levine AJ. The MDM2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* 1992; **69:** 1237–1245.
7. Cahilly-Snyder L, Yang-Feng T, Francke U, George DL. Molecular analysis and chromosomal mapping of amplified genes isolated from a transformed mouse 3T3 cell line. *Somatic Cell Mol Genet* 1987; **13:** 235–244.
8. Olson D, Marechal V, Momand J, Chen J, Romochi C, Levine AJ. Identification and characterization of multiple mdm2 proteins and mdm2 p53 protein complexes. *Oncogene* 1993; **8:** 2353–2360.
9. Wu X, Bayle JH, Olson D, Levine AJ. The p53–MDM2 autoregulatory feedback loop. *Genes Dev* 1993; **7:** 1126–1132.
10. Cordon-Cardo C, Latres E, Drobnjak M, *et al.* Molecular abnormalities of mdm2 and p53 genes in adult soft tissue sarcomas. *Cancer Res* 1994; **54:** 794–799.
11. Reifenberger G, Liu L, Ichimura K, Schmidt E, Collins V. Amplification and overexpression of MDM2 gene in a subset of human malignant gliomas without p53 mutations. *Cancer Res* 1993; **53:** 2736–2739.
12. Marchetti A, Buttitta F, Girlando S, *et al.* mdm2 gene alterations and mdm2 protein expression in breast carcinomas. *J Pathol* 1995; **175:** 31–38.
13. Buesco-Ramos CE, Yang Y, deLeon E, McCown P, Stass SA, Albitar M. The human MDM2 oncogene is overexpressed in leukemias. *Blood* 1993; **82:** 2617–2623.
14. Watanabe T, Hotta T, Ichikawa A, *et al.* The MDM2 oncogene overexpression in chronic lymphocytic leukemia and low-grade lymphoma of B-cell origin. *Blood* 1994; **84:** 3158–3165.
15. Gorgoulis V, Rassidakis G, Karameris A, Papastamatiou H, Trigidou R, Veslemes M, Rassidakis A, Kittas C. Immunohistochemical and molecular evaluation of the *MDM2* gene product in bronchogenic carcinoma. *Mod Pathol* 1996; **9:** 544–554.
16. Wiethege T, Voss B, Muller KM. Detection of MDM2-proto-oncogene in paraffin embedded human bronchial epithelium. *J Cancer Res Clin Oncol* 1994; **124:** 252–255.
17. Sambrook J, Fritsh EF, Maniatis T. Molecular Cloning. A Laboratory Manual. Plainview, NY: Cold Spring Harbor Laboratory, 1989.
18. Gorgoulis V, Sfikakis PP, Karameris A, *et al.* Molecular and immunohistochemical study of class I growth factor receptors in squamous cell lung carcinomas. *Pathol Res Pract* 1995; **191:** 973–981.
19. Gorgoulis V, Zoumpourlis V, Rassidakis G, *et al.* Molecular analysis of p53 gene in laryngeal premalignant and malignant lesions: p53 protein immunohistochemical expression is positively related to proliferating cell nuclear antigen labelling index. *Virchows Arch* 1995; **426:** 339–344.
20. Martinez JC, Mateo M, Sanchez-Beato M, *et al.* MDM2 expression in lymphoid cells and reactive and neoplastic lymphoid tissue. Comparative study with p53 expression. *J Pathol* 1995; **177:** 27–34.
21. Price BD, Park SJ. DNA damage increases the levels of MDM2 messenger RNA in wt p53 human cells. *Cancer Res* 1994; **54:** 896–899.
22. Zauberman A, Flusberg D, Haupt Y, Barak Y, Oren M. A functional p53-responsive intronic promoter is contained within the human mdm2 gene. *Nucleic Acids Res* 1995; **23:** 2584–2592.
23. Barak Y, Gottlieb E, Juven-Gershon T, Oren M. Regulation of mdm2 expression by p53: alternative promoters produce transcripts with non-identical translation potential. *Genes Dev* 1994; **8:** 1739–1749.
24. Bueso-Ramos CE, Manshouri T, Haidar M, Huh YO, Keating MJ, Albitar M. Multiple patterns of MDM2 deregulation in human leukemias: implications in leukemogenesis and prognosis. *Leukemia Lymphoma* 1995; **17:** 13–18.
25. Bueso-Ramos CE, Manshouri T, Yang Y, Sneige N, Albitar M. MDM2 deregulation in breast carcinomas and sarcomas (Abstract). *Mod Pathol* 1994; **7:** 13.
26. Waber PG, Chen J, Nisen DP. Infrequency of MDM2 gene amplification in pediatric solid tumors and lack of association with p53 mutations in adult squamous cell carcinomas. *Cancer Res* 1993; **53:** 6028.
27. Esteve A, Lehman T, Jiang W, *et al.* Correlation of p53 mutations with epidermal growth factor receptor overexpression and absence of MDM2 amplification in human esophageal carcinomas. *Mol Carcinog* 1993; **8:** 306–311.
28. Kessis TD, Slebos RJ, Han SM, *et al.* p53 gene mutations and MDM2 gene amplification are uncommon in primary carcinomas of the uterine cervix. *Am J Pathol* 1993; **143:** 1398–1405.
29. Knudson AG. Mutation and cancer: statistical study of retinoblastoma. *Proc Natl Acad Sci USA* 1971; **68:** 820–823.
30. Dittmer D, Parti S, Zambetti G, *et al.* Gain of function mutations in p53. *Nature Genet* 1993; **4:** 42–46.