EPSTEIN BARR VIRUS AND WILD p53 IN IDIOPATHIC PULMONARY FIBROSIS

She S Lok*	**MRCP,**
James P Stewart#	**PhD,**
Brian G Kelly#	**FIBMS,**
Philip S Hasleton*	**FRCPath,**
Jim J Egan*	**MD FRCP**

*North West Lung Research Centre and Department of Histopathology, Wythenshawe Hospital, Manchester, M23 9LT,UK; #Virus Research Group, Department of Veterinary Pathology. University of Edinburgh, Summerhall, Edinburgh EH9 1QH, UK.

Correspondence to:

She S Lok MRCP
Department of Respiratory Medicine
Queen Elizabeth II Hospital
Howlands
Welwyn Garden City
UK
AL7 4HQ

Tel: +44 1707 5076
Fax: +44 1707 5058
E mail: sslok@aol.com

Keywords: Epstein-Barr Virus, p53, idiopathic pulmonary fibrosis.

ABSTRACT:

Both Epstein Barr virus and p53 have independently been associated with idiopathic pulmonary fibrosis (IPF). This study explores further whether a relationship potentially exists between EBV and p53 in IPF thereby providing a possible mechanism for the role of EBV in the disease progression of IPF.

Lung tissue from open lung biopsies of 14 IPF patients was compared with a control group of 19 patients. EBV status was determined using both immunohistochemistry and PCR while p53 expression was assessed with immunohistochemistry.

Seven of 14 IPF patients expressed p53 compared to 1 of 19 control subjects ($p=0.011$). Eight IPF patients and no controls were positive for EBV ($p<0.01$). Four IPF patients demonstrated both EBV and p53 expression compared with no controls, $p=0.05$.

This study suggests that a relationship between EBV and p53 may exist in patients with IPF.

INTRODUCTION:

Idiopathic pulmonary fibrosis (IPF) occurs following an environmental lung injury in susceptible individuals [1]. The primary injury to the respiratory epithelium results in healing and regeneration [1]. With the loss of cell integrity certain cell cycle control proteins (proto-oncogenes) are expressed to prevent the progression of abnormal and defective cells during repair. These proto-oncogenes result in either a cessation of cellular growth to allow the cell to recover or induce programmed cell death if repair is not possible (apoptosis). One such protein critical to cell cycle regulation is the p53 tumour suppressor protein. During the repair process, the expression of mitogenic factors are suppressed by p53 activity, whose peak action occurs when re-epithelialization is complete [2]. The interference of the normal p53 function may result in unregulated growth factor expression and possibly abnormal epithelial cell repair. A normal intact epithelium itself is important in the control of the repair mechanism [3,4].

Kuwano *et al* have shown that there is over-expression of p53 with evidence of DNA damage in the epithelial layers of lung tissue obtained from patients with IPF [5]. An association between Epstein-Barr virus (EBV) and IPF has also been established implying a potential involvement of viruses in the pathogenesis of IPF [6,7]. The possible mechanisms by which EBV may contribute to the pathogenesis of IPF includes:

A) influencing cytokine balance in lung injury and repair, or
B) influencing proto-oncogenes proteins in the cell cycle involved in cellular growth and control.

Zhang *et al* has previously shown that EBV can modify p53 protein expression [8], therefore we postulated that EBV persistence in pulmonary epithelial cells may modify epithelial cell repair and apoptosis in IPF patients.

The object of this study was to identify whether both p53 expression (wild and mutant) and EBV persistence existed in the epithelial cells of lung tissue from IPF patients compared to controls.

METHODS

Fourteen archival lung biopsies from IPF patients were randomly selected for the study (12 from open lung biopsies (OLB), 2 from lung removed at transplantation). The lung tissue were fixed in buffered formalin and stained with haemotoxylin and eosin. These biopsies were reviewed by a pulmonary histopathologist (PSH) to confirm patterns consistent with the usual interstitial pneumonia (UIP) form of IPF, and to exclude other causes of pulmonary fibrosis including non-specific interstitial pneumonia (NSIP) and desquamative interstitial pneumonia (DIP). Histological features on biopsy examined for were:

1) A variegated picture of fibrosis, inflammation and normal lung.
2) Exclusion of evidence to suggest another pathology (e.g. asbestos bodies, granuloma etc.)
3) A tendency for pleural and sub-pleural based distribution of fibrosis.

Nineteen patients who underwent pneumonectomies or OLB for pulmonary pathology were used as controls (11 OLB, 3 from lung removed at transplantation and 5 from pneumonectomy or lobectomy performed for lung cancer).

OLB were performed either by a VATS procedure or by open thoracotomy with the site of operation directed by high-resolution computer tomography (HRCT). In those control lung specimens where the primary diagnosis was cancer, lung tissue away from the malignant lesion was used for the study. The lung tissues were examined for the presence of p53 (both mutant and wild-type) using immunohistochemisitry, while EBV was detected by both PCR and immunohistochemisitry. The lung specimens were coded and assessed blind for the presence of p53 and EBV by the histopathologist (PSH).

Immunohistochemistry for p53: Using paraffin preserved lung tissue 5µm sections were cut. These were dried overnight in an incubator at 37°C and then at 56°C for 30 minutes. The sections were dewaxed using xylene and dehydrated by bringing to absolute alcohol. Staining of the p53 employed a streptavidin peroxidase technique (duet, DAKO) according to the manufacturers' instructions using DAB (3,3'-diaminobenzidine tetrahydrochloride) as substrate, which visualises the peroxidase tracer. Two primary p53 antibodies were used. NCL-p53-1801 (Novo Castra Laboratories Ltd, Newcastle UK) an index mouse antibody in 1:40 dilution, which recognises both wild and mutant p53. MCA909 (Serotec Ltd, Oxford UK), an index mouse antibody in 1:40 dilution which recognises mutant p53 only. Samples staining positive for the NCL-p53-1801 and negative to MCA909 were considered positive for wild p53. Negative controls were sections deprived of the primary antibody and the positive controls were sections known to contain p53. Only nuclear staining of the cell by the antibody was considered positive for p53.

Immunohistochemistry for EBV was performed as described in a previous study [7]: In each case, 5µm sections were analysed for the presence of the EBV productive cycle by using monoclonal antibodies specific for the EBV structural antigen gp350/220 and VCA. Sections were first incubated at 4°C overnight in a solution of 20% (w/v) bovine serum albumin to block non-specific binding of antibodies. Staining for EBV lytic cycle antigens and cellular antigen was performed using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA, USA) according to the manufacturers protocol. Primary antisera were mouse monoclonal reagents including; anti-EBV membrane antigen gp340/220; anti-EBV VCA (MAB 817; Chemicon International Temecula, California USA); anti-epithelial membrane antigen

EMA (M613; dakopatts) anti-CD20, a pan B cell antibody (M755; Dakopatts). Purified anti-gp 340/220 was used at a concentration of 10ug/ml and anti-VCA was used at a 1:400 dilution. To assess the specificities of the reagents used, reaction against an EBV positive B-cell line B95-8 and EBV-negative B cell line BL41 were used as control. Antigens expressed during the latent phase of EBV life cycle (LMP) were not studied.

PCR for EBV was performed as previously described [7]: DNA was extracted from 5μm sections that were serially adjacent to those analysed by IHC using Qiamp tissue kits (Qiagen). Control DNA was extracted from the B lymphocyte cell lines AM [8] (EBV-positive) and BL41 [9] (EBV-negative). Pilot studies were performed to analyse the ability of extracted DNA to be amplified by PCR using primers specific for the human p53 gene [10]. This indicated that amplifiable DNA was best obtained if proteinase K digestion of the sample was extended from 12h to 5 days. Amplifiable DNA (100ng) was subjected to nested PCR analysis using oligonucleotide primers specific for EBV and recombinant *Taq* DNA polymerase (Gibco/BRL). The first round of PCR used the primers 5'-GGTCCCGTAGTGACAACTATGCTG and 5'-GAGTGCACCACAGCCAACTCCATG at an annealing temperature of 60°C for 40 cycles as described by Lees *et al* [11]. The second (nested) round of PCR used the primers 5'-GGCTTTGGGTTCCATTGTGTGC and 5'-TGTACAGAACCAAAGAGGTGGC at an annealing temperature of 60°C for 25 cycles. PCR products were electrophoresed through 2% agarose gels and DNA visualised by ethidium bromide staining. Molecular weight determinations were made using the 1Kb ladder (Gibco/BRL). Positive signal for EBV yielded a 284bp product. Limiting dilution of a known amount of cloned target DNA showed that the

PCR was sensitive to one copy of EBV DNA. Sections were randomised, analysed by PCR blind and the results decoded subsequently.

STATISTICS

The demographic data was compared using a non-parametric statistical analysis (Mann-Whitney U test). EBV and p53 staining in the IPF and control patients were compared using the Chi-squared test with Yates' correction. To determine if there was a statistical relationship between EBV and p53 positivity crosstabs was used with kappa correction. The SPSS package was used for the statistical calculations.

RESULTS

The demographics of the patients studied were closely matched for age and sex (table 1). The primary diagnoses of the control patients are shown in table 2.

Staining of lung tissue for p53 and EBV are summarised in tables 2,3 and 4. Lung tissue from IPF patients had more frequent staining for p53 (7 of 14 IPF patients compared to 1 of 19 controls, $p=0.011$). Six IPF patients were positive for wild p53 and only 1 of the control patients, $p=0.029$. One IPF patients was positive for mutant p53 compared with no controls, $p=0.88$). Ten IPF patients stained positive for EBV compared with 4 controls. Increased specificity for EBV was observed by 8 IPF patients who were positive by immunohistochemistry and PCR compared to no controls ($p<0.01$). Only IPF patients were positive for both EBV and p53 (4 of 14) compared to no controls (0 of 19), $p=0.05$. There was no statistical correlation between EBV and p53 staining.

DISCUSSION:

This study suggests that a potential relationship exist between p53 and EBV in IPF patients providing a possible mechanism by which EBV may contribute to epithelial cell injury. Both wild p53 staining and EBV positivity were localised to the epithelial cell layer of IPF patients. However a statistical correlation between p53 staining and EBV was not established.

This data shows that wild p53 expression occurs more frequently than the mutant p53 in IPF patients, which is consistent with the findings of Kuwano and colleagues. In their study, Kuwano *et al* established a relationship between p53 and IPF [5]. They studied 14 IPF patients and 17 controls (normal lungs and pulmonary emphysema) and found that p53 was detected in the hyperplastic epithelial layer of IPF lung tissue but not controls. This correlated with evidence of cell death in this same region of IPF patients, as detected by the TUNEL (TdT-mediated dUTP-biotin nick end labelling) method. They postulated that the up-regulation of p53 in the face of chronic DNA damage may increase the potential in mutation of p53 leading to increased tumorigenesis in IPF. However, they did not differentiate between wild and mutant p53, as this study has done. Kuwano inferred that p53 staining was wild type because of the presence of p21 (a downstream protein important in the function of p53 and only produced by wild p53).

The presence of p53 in fibrotic lung may therefore reflect: 1) an excess of injured cells or 2) a normal response to control the growth of damaged cells! The up-regulation of p53 seen in the data may reflect a response to injury. Cells with damaged DNA are halted at the G_1 checkpoint and prevented from entering the S phase of cell replication by the p53 checkpoint protein [12]. Cell division is also

halted at the G_2 checkpoint and prevented from entering interphase hence preventing proliferation of abnormal cells. Cells either undergo DNA repair or programmed death (apoptosis) [12].

EBV can interfere with the function of p53. This activity can be achieved by the action of BZLF-1 (also called ZEBRA (BamH1 Z EBV replication activator), Zta or EB1) an AP-1 transcriptional *trans*-activator protein important in EBV replication, as shown by Zhang *et al* [14]. The role of the BZLF-1 protein is to facilitate the propagation of EBV in the host by interfering with the regulation of the cell cycle. Such an action has led to the proposed tumourigenicity effect of EBV [14], although in EBV-related malignancies such as Burkitt's lymphoma there is no relationship between EBV and p53 [14]. In addition Szekely *et al* have suggested that the EBV leader protein expressed during latency, EBNA-5 (EBV nuclear antigen 5) can also interact with p53 [15]. In this study they found that the EBNA-5 protein binds with p53 resulting in an immunoprecpitate. However, it is not known whether p53 function is affected by this particular interaction. Gan *et al* has shown that ZEBRA does not affect mutant p53 and functions only in the presence of wild p53 [16]. This study supports the role of a potential interaction between p53 and EBV in IPF patients. Therefore it is possible that an interaction between EBV proteins and p53 may play a role in the disease progression.

We have not identified p53 staining in the fibroblasts. The abnormal degree of fibrosis implies overactivity of collagen production from fibroblasts, and in patients with Li-Fraumeni syndrome, examination of fibroblasts show abnormal p53 with resultant prolongation and even immortalisation of fibroblasts [17]. However, the role of p53 on the actions of inflammatory cells and fibroblasts can be indirect through its effects

on the epithelium. We believe epithelial cells are critically important effector cells in IPF [3,4,18].

Epithelial cells appear to have a role in normal fibroblast function, as a result of cell to cell interaction. An intact, normally functioning epithelium may be responsible for controlling the release and actions of cytokines present during epithelial repair [18]. Our data showed that p53 and EBV were both found in the epithelial layer. The consequence is a potential viral mediated inhibition of p53 action (through the expression of ZEBRA) on the repair of cells with damaged DNA [14]. The failure of repair to the epithelium could therefore affect the cytokine balance. The potential role of EBV on the actions of p53 is comparable to the effects of human papillomavirus on p53 [19]. Papilloma virus produces proteins that bind with and degrades the p53 protein.

EBV in the course of replication can also interfere with cytokine balance. Klein *et al* in a study of EBV infected cell lines found that there was increased expression of IL-8, IL-10, TNF-α and TNF-β by these infected cells [20]. The cytokine imbalance induced by EBV together with the impairment of cytokine regulation through the effect on the epithelial layer may initiate the events leading to the development of fibrosis.

This study demonstrates an association between p53 and EBV in the epithelial cells of lung tissue of IPF patents supporting a hypothesis of a viral mediated dysregulation of epithelial cell repair. However, further in vitro studies would be required to determine the precise relationship.

Table1: Demographics of patients in the study.

	IPF	Controls	p value
Patients	14	19	
Sex (females/%)	4 (28)	10 (55)	0.38
Age	59	53	0.11

Table 2: p53 and EBV staining of IPF patients

PATIENT	W&M p53	M p53	W p53	EBV PCR	EBV IHC	PCR & IHC
1	+	-	+	+	+	+
2	-	-	-	+	+	+
3	-	-	-	+	+	+
4	-	-	-	-	+	-
5	-	-	-	-	-	-
6	+	-	+	-	-	-
7	+	-	+	-	+	-
8	+	+	-	-	-	-
9	-	-	-	+	+	+
10	+	-	+	+	+	+
11	-	-	-	+	+	+
12	+	-	+	+	+	+
13	-	-	-	-	-	-
14	+	-	-	+	+	+

W&M p53 = staining for wild and mutant p53, M p53 = mutant p53 present
W p53 = wild p53 present, EBV PCR = staining for EBV DNA
EBV IHC = staining for EBV VCA and gp 340/220

Table 3: p53 and EBV staining of controls

PATIENT	W&M p53	M p53	W p53	EBV PCR	EBV IHC	PCR & IHC	DIAGNOSIS
15	-	-	-	-	+	-	lung ca
16	-	-	-	-	+	-	wegener
17	-	-	-	-	-	-	normal
18	-	-	-	-	-	-	sarcoid
19	-	-	-	-	-	-	sarcoid
20	-	-	-	-	-	-	sarcoid
21	-	-	-	-	-	-	bronchiectasis
22	-	-	-	-	-	-	normal
23	-	-	-	-	-	-	sarcoid
24	-	-	-	-	-	-	lung ca
25	+	-	+	-	-	-	EAA
26	-	-	-	-	+	-	lung ca
27	-	-	-	-	-	-	sarcoid
28	-	-	-	-	-	-	normal
29	-	-	-	+	-	-	sarcoid
30	-	-	-	-	-	-	sarcoid
31	-	-	-	-	-	-	CF
32	-	-	-	-	-	-	lung ca
33	-	-	-	-	-	-	sarcoid

Lung Ca = lung carcinoma (non-small cell), normal = no abnormalities found
Sarcoid = sarcoidosis, EAA = extrinsic allergic alveolitis, CF = Cystic fibrosis.

Figure 4: Comparison of IPF with controls

	IPF (n=14)	Controls (n=19)	p value
p53 (W & M) positive	7	1	0.011
Wild p53 positive	6	1	0.029
Mutant p53 positive	1	0	0.88
EBV positive (PCR &IHC)	8	0	<0.01
EBV and p53 positive	4	0	0.05

LEGENDS:

Table 1: Demographic data of the 33 patients in the study

Table 2: Results of p53 and EBV staining in IPF patients

Table 3: Results of the p53 and EBV staining in the control population.

Table 4: Comparison of the p53 and EBV staining between IPF patients and controls

REFERENCES:

1. Crystal RG, Bitterman PB, Renard SI, Hance AJ, Keogh BA. Interstitial disease of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract (first of two parts). *N Engl J Med 1984; 310: 154-166.*

2. Antoniades HN, Galanopoulos T, Neville-Golden J, Kitritsy CP, Lynch SE. p53 expression during normal tissue regeneration in response to acute cutaneous injury in swine. *J Clin Invest 1994; 93: 2206-2214.*

3. Terzaghi M, Nettesheim P, Williams ML. Re-population of denuded tracheal grafts with normal, pre-neoplastic and neoplastic epithelial cell populations. *Cancer Res 1978; 38: 4546-4553.*

4. Adamson IYR, Young L, Bowden DH. Relationship of alveolar epithelial injury and repair to the induction of pulmonary fibrosis. *Am J Pathol 1988; 130: 377-383.*

5. Kuwano K, Kunitake R, Kawasaki M, Nomoto Y, Hagimoto N, Nakanishi Y, Hara N. $p21^{Waf1/Cip1/Sdi11}$ and p53 expression in assocation with DNA strand breaks in idiopathic pulmonary fibrosis. *Am J Respir Crit Care Med 1996; 154: 477-483.*

6. Egan JJ, Stewart JP, Hasleton PS, Arrand JP, Carroll KB, Woodcock AA. Epstein-Barr Virus replication within pulmonary epithelial cells in cryptogenic fibrosing alveolitis. *Thorax 1995; 50: 1234-1239.*

7. Stewart JP, Egan JJ, Ross AJ, Kelly BG, Lok SS, Hasleton PS, Woodcock AA. The detection of Epstein-Barr virus DNA in lung tissue from idiopathic pulmonary fibrosis patients. *Am J Respir Crit Care Med 1999; 159: 1336-1341.*

8. Wrightam MN, Stewart JP, Janjua NJ, Pepper S, Sample C, Rooney CM and Arrand JR. Antigenic and sequence variation in the C-terminal unique domain of the Epstein-Barr virus nuclear antigen EBNA-1. *Virology 1995; 208: 724-732.*

9. Lenoir GM, Vuillaume M, Bonnardel C. The use of lymphomatous and lymphoblastoid cell lines in the study of Burkitt's lymphoma. *In* GM lenoir, G O'Conner and CLM Olweny editors. Burkitt's lymphoma, a human cancer model. IARC publication, Lyon. 309-318.

10. Gaidano G, Ballerini P, Gong JZ, Inghirami G, Neri A, Newcomb EW, Magrath IT, Knowles DM, Dalla-Favera R. p53 mutations in human lymphoid malignacies: association with Burkitt's lymphoma and chronic lymphocytic leukaemia. *Proc. Natl. Acad. Sci.1991; 88: 5413-5417.*

11. Lees JF, Goodeve AC, Arrand JE, Ghosh AK, Jones PH, Arrand JR. Detection of EBV DNA in post-nasal space biopsy tissue from asymptomatic EBV-seropostive individuals. *J Med Virol 1992; 37: 30-38.*

12. Ko LJ, Prives C. p53: puzzle and paradigm. *Genes Dev 1996; 10: 1054-1072*

13. Turner-Warwick M, Lebowitz M, Burrows B, Johnson A. Cryptogenic fibrosing alveolitis and lung cancer. *Thorax 1980; 35: 349-355.*

14. Zhang Q, Gutsch D, Kenney S. Functional and physical interaction between p53 and BZLF1: implications for Epstein Barr virus latency. *Mol. Cell. Biol. 1994: 14; 1939-1948.*

15. Szekely L, Selivanova G, Magnusson KP, Klein G, Wiman KG. EBNA-5, an Epstein Barr irus encoded nuclear antigen, binds to the retinoblastoma and p53 proteins. *Proc. Natl. Acad. Sci. 1993; 90: 5455-5499.*

16. Gan Y J, Shirley P, Zeng Y, Sixbey JW. Human rorpharyngeal lesions with a defective Epstein Barr Virus that disrupts viral latency. *J Infect Dis 1993; 168: 1349-55.*

17. Bischolff FZ, Yim SO, Pathak et al. Spontaneous abnormalities in normal fibroblasts from patients with Li-Fraumeni cancer syndrome: aneuploidy and immortilization. *Cancer Res 1990; 50: 7979-7984.*

18. Polunovsky VA, Chen B, Henke C, Snover D, Wendt C, Ingbar DH, Bitterman PB. Role of mesenchymal cell death in lung remodelling after injury. *J Clin Invest 1993; 92: 388-397.*

19. Scheffner M. The E6 oncoprotein encoded by human papilloma virus type 16 and 18 promotes degradation of p53. *Cell 1990; 63: 1129-1136.*

20. Klein SC, Kube D, Abts H, Diehl V, Tesch H. Promotion of IL8, IL10, TNFα and TNFβ production by EBV infection. *Leukaemia Research 1996; 20: 633-636.*