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MYCL1, FHIT, SPARC, p16^{INK4} and TP53 genes associated to lung cancer in idiopathic pulmonary fibrosis

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Abstract

Idiopathic pulmonary fibrosis (IPF) is a specific form of chronic interstitial pneumonia limited to the lung and characterized by a fibroproliferative response with only minor signs of inflammation, which almost always causes rapid fibrotic destruction of the lung. In this study, we investigated genomic instability in IPF, using microsatellite DNA analysis, aiming to detect any specific genetic alterations for this disease. We used 40 highly polymorphic microsatellite DNA markers, in multiplex PCR assays, to examine 52 sputum specimens from IPF patients versus correspondent venous blood. Loss of heterozygosity (LOH) was found in 20 (38.5%) patients in at least one locus. These alterations were found on markers previously associated with lung cancer located on 1p34.3, 3p21.32-p21.1, 5q32-q33.1, 9p21 and 17p13.1 where MYCL1, FHIT, SPARC, p16^{Ink4} and TP53 genes have been mapped respectively. These data provide new insights into IPF pathogenesis and a new perspective for its correlation with lung cancer.

Keywords: idiopathic pulmonary fibrosis - microsatellite DNA - loss of heterozygosity

Introduction

Idiopathic pulmonary fibrosis (IPF) represents a specific form of chronic fibrosing interstitial pneumonia limited to the lung. The disease is characterized by a fibroproliferative response with only minor signs of inflammation, and rapid fibrotic destruction of the organ. In early stages of IPF, pulmonary function may be normal or only slightly impaired [1]. The proliferation of fibroblasts and the accumulation of interstitial collagens are the causes of progressive lung fibrosis [2].

It has been estimated that the prevalence of IPF varies from 3 to 6 cases per 100.000 in the general population, with male predominance [3, 4]. The incidence of the disease increases with age and cigarette smoking has been identified as a potential risk factor [1, 4]. Numerous viruses have been implicated in the pathogenesis of IPF, but there is no clear evidence for a viral etiology [5,

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6]. The distribution of this disease does not reveal any distinct geographical, racial or ethnic variation [7]. However, approximately 3% of IPF cases appear to cluster in families, suggesting a genetic susceptibility in some patients [8-10].

A relationship between IPF and lung cancer has been suspected since the late 1960 [11, 12], and 10.4% of IPF mortality was reported due to bronchogenic carcinoma [13]. Whether this results from the effects of scarring and chronic inflammation or from an interaction between cigarette smoking and occupational exposure remains controversial [14]. On the molecular level, previous studies have shown that p53, $p21^{waf1/cip1}$ and apoptosis machinery alterations occurred in hyperplastic bronchial and alveolar epithelial cells of IPF patients [15-18]. Another feature of neoplastic cells, microsatellite DNA alterations was also found in IPF sputum [19] and microdissected lung tissues [20, 21].

In the present study we used multiplex PCR-based microsatellite DNA analysis comparing sputum and venous blood specimens from 52 IPF patients. Forty highly polymorphic markers located on 1p, 1q, 2p, 2q, 3p, 5q, 6p, 7p, 9p, 11q, 14q and 17p were studied in order to identify chromosomal regions that may be altered in IPF patients and thus could be part of the complex genetic basis of the disease and implicated in its etiopathogenesis.

Materials and methods

Specimens

Sputum and venous blood specimens were collected from 52 patients with clinical, radiological (high-resolution computed tomography, HRCT) and histological features consistent with IPF, who were followed at the Department of Pneumology, University Hospital of Heraklion Crete, Greece. The diagnosis of IPF was made by surgical lung biopsy (open lung biopsy or video-assisted thoracoscopic surgery) showing usual interstitial pneumonia (UIP), the presence of persistent bilateral crackles on auscultation, a restrictive ventilatory defect or isolated depression of gas transfer on pulmonary function testing, the presence of bilateral abnormalities with a peripheral distribution and the absence of bilateral patchy infiltrates on HRCT. Patients with environmental exposure to a fibrinogen were excluded. Other exclusion criteria were coexistent chronic disease, lung infection, or malignancy. The median age of the patients was 72 years (range 57 to 82 years); 40 of the patients were male and 12 were female. Thirty-six of the patients were smokers. The smoking history of the patients was 29 ± 21 (mean ± SD) pack-years. The duration of the disease was 3.3 ± 1.4 years. Extra care was taken to ensure that the cell content of IPF patients and normal control subjects remained similar in the morning spontaneously expectorated sputum of the two groups. To ensure that sputum samples were from the lower respiratory tract, they were microscopically examined and considered adequate if squamous epithelial cells were less than 10 per low-power field [22]. Viability measurements were performed as previously described [23]. Mean cell viability was estimated as 48%. Informed consent was obtained from all patients participating in the study, and the study was approved by the medical research ethics committee of University Hospital of Heraklion.

DNA extraction

DNA was isolated from peripheral white blood cells and sputum cells using the IsoQuick Nucleic Acid Extraction kit (ORCA; Research, Inc., Bothell, WA), according to the manufacturer's instructions.

PCR parameters

Forty microsatellite DNA markers were amplified in 11 panels of 3 and 4-plex reactions (Table 1). We introduced 100ng of genomic DNA in a PCR reaction mixture containing 1X PCR buffer, 400 μM dNTPs, 2.66 mM $MgCl_2$ and 0.35U Taq DNA polymerase (Life Technologies Ltd., Gaithersburg, Scotland, United Kingdom). To optimize the reactions, different concentrations of each marker primer set were used (Table 1). Amplification parameters were: initial denaturation for 3 min; 30 cycles at 94ºC for 30 sec, 55ºC for 30 sec and 72ºC for 30 sec; final extension step at 72ºC for 10 min. The PCR assays were done in a PTC-100 programmable thermal controller (MJ Research, Inc., Watertown, MA, USA).

Microsatellite alteration analysis

The PCR products were analyzed by using 10% polyacrylamide gel electrophoresis (29:1 ratio of acrylamide to bisacrylamide) and silver staining. Gels were sealed in a plastic transparent bag and scanned on an Agfa SnapScan 1212u (Agfa-Gevaert N.V., Mortsel, Belgium). Integrated density (ID) was calculated as *(Mean OD - Background OD)*pixels*, where OD stands for optical density. OD of each band was used as quantitative parameter and was calculated by digital imaging using the Adobe Photoshop 6.0 software

Table 1. Microsatellite DNA markers studied.

Marker	Cytogenetic location	Heterozygosity	Allele size range	Optimal multiplex PCR primer concentration (nM)	Panel*
D1S416	1q24	0.82	146-162	130	I
D1S186	1p34.3	0.84	82-106	100	G
D1S116	1p31-p21	0.65	89-101	100	F
APOA2	1q21-q23	0.70	131-145	150	E
D1S104	1q2-q23	0.76	152-168	150	G
D1S180	1q42	0.90	163-189	150	H
ACTN2	1q42-q43	0.50	105	120	H
D2S119	2p22.3-p16.1	0.80	221	175	A
D2S123	2p22.3-p16.1	0.76	196	140	A
D2S177	2p22.3-p16.1	0.85	276	225	A
D2S147	2p22.3-p16.1	0.73	111	80	A
D2S2182	2p22.3-p16.1	0.78	234	180	C
D2S288	2p22.3-p16.1	0.62	276-284	250	C
D2S2291	2p22.3-p16.1	0.76	245	320	D
D2S138	2q24.1-q32.1	0.67	115	100	B
D2S164	2q24.1-q32.1	0.83	195	175	B
D2S103	2q24.1-q32.1	0.82	109-125	120	D
D2S311	2q24.1-q32.1	0.81	185-207	150	D
D2S105	2q24.1-q32.1	0.69	107-125	130	E
D3S1611	3p21.32-p21.1	0.66	258	260	B
D3S1612	3p21.32-p21.1	0.69	100	80	C
D3S1478	3p21.32-p21.1	0.98	109-152	130	C
D3S647	3p21.32-p21.1	0.73	100	90	D
D3S1260	3p21.32-p21.1	0.66	268	230	E
D3S1561	3p21.32-p21.1	0.65	226	180	E
D5S207	5q31.3-q33.3	0.68	135-143	130	K
D5S376	5q32-q33.1	0.72	117-129	100	I
D6S2225	6p21.3	0.38	152	140	J
D6S1002	6p21.3-p22.1	0.32	240-244	200	K
D6S429	6p23-p25	0.74	222-238	200	I
D6S259	6p23-p25	0.73	267-285	260	K
D6S263	6pter, 6p23-p25	0.82	90-114	80	J
D7S519	7p15-q22	0.81	256-268	230	F
D9S161	9p21	0.78	119-135	120	F
D9S270	9p21	0.71	87-101	100	H
D11S1876	11q21-q22.3	1.00	123-135	100	J
D14S72	14pter, 14q11.1-q11.2	0.83	257-271	200	J
D14S258	14q23-q24.3	0.80	170-182	160	I
D14S292	14qter, 14q32.1-q32.3	0.74	110-118	100	G
TP53	17p13.1	0.90	103-135	130	K

* Panel of multiplex reaction.

(Adobe Systems Inc., U.S.A.). Loss of heterozygosity (LOH) was scored in a heterozygous case when the ratio

$$\frac{ID\ \ allele1\ \ (sputum\ \ DNA)\ /\ ID\ \ allele2\ \ (sputum\ \ DNA)}{ID\ \ allele1\ \ (blood\ \ DNA)\ /\ ID\ \ allele2\ \ (blood\ \ DNA)}$$

was calculated >1.49 or <0.58, with a 99.5% confidence interval, as determined from independent reproducibility experiments. Microsatellite instability (MIN) was scored when a novel generated microsatellite allele was observed in sputum-extracted DNA compared to the correspondent venous blood-extracted DNA. The analysis in LOH or MIN positive cases was repeated three times and the results were reproducible. Representative examples of LOH are shown in Fig. 1 and MIN in Fig. 2.

Statistical analysis

Data analysis was done with SPSS statistical software (SPSS Inc., Chicago, IL, USA). Results are expressed as mean ± SD or median (range). Differences in the mean values of quantitative measurements were tested with the Student's t or the Mann–Whitney U test. The chi-square test was used for comparison of percentages. Analysis of covariance (logistic regression) was used when appropriate. A p value of 0.05 was considered statistically significant.

Results

We assayed 52 sputum/venous blood DNA pairs from idiopathic pulmonary fibrosis patients with a total of 40 microsatellite markers. The incidence of imbalance for each marker ranged from 0% to 10.0%, while the degree of heterozygosity was from 0.32 to 1.00 (Table 1). Twenty specimens from IPF patients exhibited LOH (38.5%) in at least one of the examined loci. Although, MIN was observed in 10 cases (19.2%), none of them fulfilled the criterion of the co-finding of at least 5 markers to be characterized as replication error-

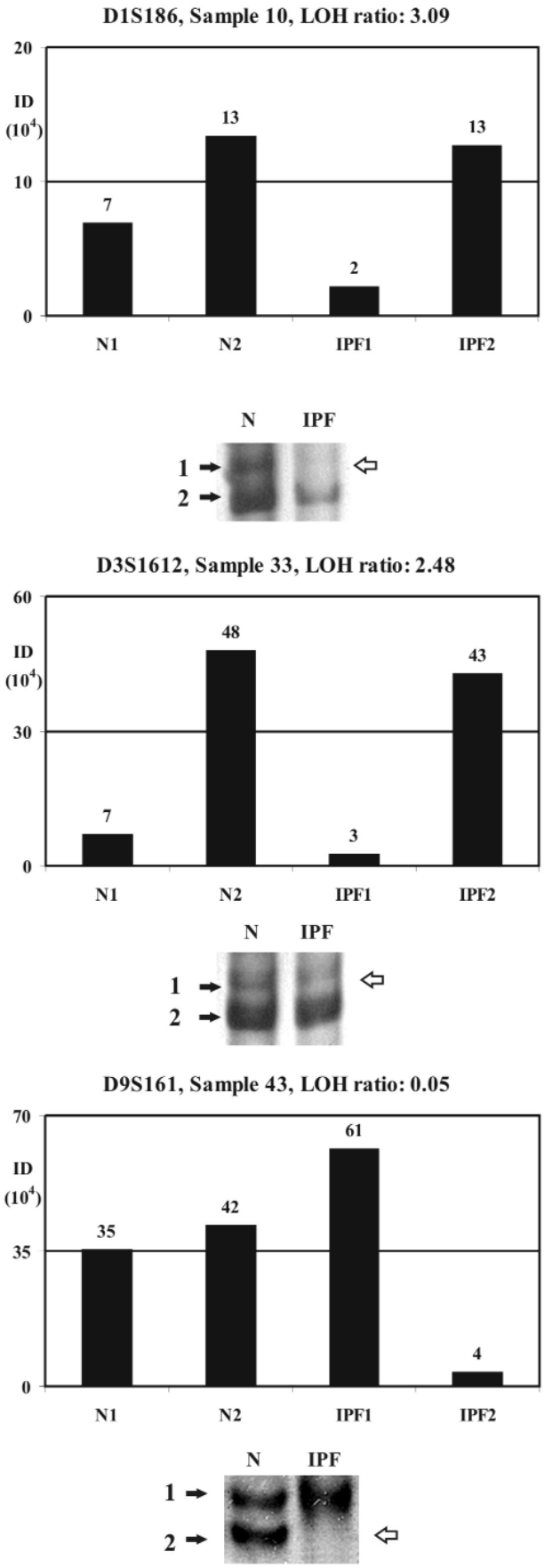

Fig. 1 Characteristic examples of LOH positive cases. ID, integrated density; N, normal DNA; IPF, idiopathic pulmonary fibrosis DNA. Black arrows and numbers indicate each allelic band. White arrows indicate the affected allele.

Table 2 Genotypes of LOH and MIN positive IPF cases.

Marker	1	5	8	10	11	12	13	16	18	20	23	24	25	27	29	30	31	33	35	38	40	41	43	47	49	51	LOH (%)	MIN (%)
D1S416	H	H	H	H	H	H	NI	H	H	NI	H	NI	H	H	H	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D1S186	H	H	H	LOH	H	H	H	H	H	H	H	H	H	NI	LOH	H	NI	H	H	H	NI	H	H	H	H	H	4,3	0,0
D1S116	NI	NI	H	H	H	H	H	H	NI	H	H	NI	H	H	H	NI	H	H	NI	H	H	NI	NI	H	H	H	0,0	0,0
APOA2	H	H	NI	H	NI	H	NI	H	H	H	H	H	NI	NI	H	H	H	H	NI	H	H	H	H	H	H	MIN	0,0	1,9
D1S104	H	H	MIN	H	H	H	H	NI	H	NI	H	NI	H	H	H	NI	NI	H	H	H	NI	H	H	H	H	H	0,0	1,9
D1S180	H	H	H	H	H	H	H	LOH	H	H	H	H	H	H	H	LOH	H	H	H	H	H	H	NI	H	H	H	4,0	0,0
ACTN2	H	NI	NI	H	NI	H	H	NI	NI	H	H	NI	NI	NI	H	H	NI	H	H	H	H	H	H	NI	H	NI	0,0	0,0
D2S119	NI	H	H	NI	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	NI	H	H	H	H	H	0,0	0,0
D2S123	H	NI	H	H	H	NI	H	NI	H	H	H	NI	H	MIN	H	H	H	NI	H	H	H	H	H	H	H	NI	0,0	1,9
D2S177	H	H	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	NI	H	H	NI	H	H	H	H	0,0	0,0
D2S147	H	H	H	H	H	H	H	H	NI	NI	H	H	H	H	H	NI	H	H	H	H	H	H	NI	NI	H	H	0,0	0,0
D2S2182	NI	MIN	H	H	H	H	NI	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	H	H	H	NI	H	0,0	1,9
D2S288	H	H	NI	NI	H	H	NI	H	NI	H	H	H	NI	H	NI	NI	H	H	H	NI	H	NI	NI	H	H	H	0,0	0,0
D2S2291	H	H	H	H	NI	H	H	H	NI	H	H	NI	H	NI	H	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D2S138	H	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	NI	H	NI	NI	NI	NI	H	H	H	H	NI	0,0	0,0
D2S164	H	H	H	NI	H	H	H	H	H	H	H	NI	MIN	NI	H	H	H	H	H	H	H	H	H	H	H	H	0,0	1,9
D2S103	H	H	H	H	H	H	H	NI	H	H	NI	H	NI	H	H	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D2S311	H	NI	NI	H	H	H	NI	H	H	H	H	H	H	H	NI	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D2S105	H	H	H	H	NI	NI	H	NI	H	H	H	NI	H	H	H	NI	NI	H	H	H	H	H	H	NI	H	H	0,0	0,0
D3S1611	NI	NI	H	H	NI	H	H	H	MIN	H	H	H	NI	H	H	H	H	H	NI	NI	H	NI	H	H	H	H	0,0	1,9
D3S1612	LOH	LOH	NI	H	H	H	NI	H	H	H	H	NI	H	H	H	H	H	LOH	NI	H	H	LOH	H	NI	NI	H	10,0	0,0
D3S1478	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D3S647	H	H	NI	H	H	NI	H	H	H	LOH	H	H	H	H	NI	H	H	H	H	H	H	H	H	NI	H	NI	4,8	0,0
D3S1260	H	NI	H	H	H	H	LOH	NI	H	H	H	LOH	H	H	H	NI	H	H	LOH	NI	NI	NI	NI	LOH	H	H	10,5	0,0
D3S1561	H	H	H	H	H	H	H	H	H	H	NI	H	NI	NI	H	NI	NI	H	NI	H	H	H	H	H	NI	H	0,0	0,0
D5S207	H	H	NI	H	H	NI	H	H	NI	H	H	H	NI	H	NI	H	H	H	H	MIN	NI	H	H	H	H	NI	0,0	1,9
D5S376	NI	NI	H	H	H	LOH	NI	H	H	H	NI	NI	H	H	H	H	H	H	H	LOH	H	H	NI	NI	H	H	5,3	0,0
D6S2225	H	NI	NI	H	H	NI	NI	H	NI	NI	NI	NI	NI	NI	H	NI	H	H	NI	H	NI	H	H	NI	H	NI	0,0	0,0
D6S1002	H	H	NI	NI	NI	NI	NI	NI	NI	NI	NI	NI	H	NI	NI	H	NI	NI	NI	H	NI	H	H	H	NI	H	0,0	0,0
D6S429	NI	H	H	NI	H	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	NI	NI	NI	H	H	H	H	0,0	0,0
D6S259	H	NI	NI	H	H	H	H	H	H	NI	H	NI	H	H	H	H	H	H	NI	H	H	H	H	NI	H	H	0,0	0,0
D6S263	H	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	NI	H	H	NI	H	H	H	NI	H	H	H	0,0	0,0
D7S519	H	H	H	H	MIN	H	NI	H	NI	H	H	H	H	H	NI	H	H	NI	H	H	NI	H	H	H	H	H	0,0	1,9
D9S161	H	H	H	NI	LOH	H	H	NI	H	H	MIN	NI	H	H	H	H	H	NI	H	H	NI	H	LOH	H	H	H	4,8	1,9
D9S270	H	H	NI	H	H	NI	H	H	NI	H	H	H	H	H	NI	H	H	H	H	H	H	NI	H	H	H	NI	0,0	0,0
D11S1876	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	0,0	0,0
D14S72	H	H	H	H	H	H	H	H	H	NI	H	H	H	NI	H	H	H	H	NI	H	H	H	H	H	H	H	0,0	0,0
D14S258	H	H	H	H	H	H	H	H	H	H	H	H	NI	H	NI	H	H	H	H	H	H	H	NI	NI	H	H	0,0	0,0
D14S292	NI	H	H	H	H	H	H	H	H	H	NI	H	H	H	H	NI	MIN	H	H	NI	H	H	NI	H	H	NI	0,0	1,9
TP53	H	NI	H	H	LOH	H	H	H	H	H	H	H	LOH	H	H	H	H	H	H	H	LOH	H	H	H	LOH	H	8,0	0,0

positive (RER+) phenotype [24]. Analytical presentation of the genotypes in microsatellite alteration positive cases is provided in Table 2.

LOH was most frequently observed in D3S1260 (10.5%) followed by D3S1612 (10.0%) and TP53 (8.0%). Homozygote genotype of a sample in a microsatellite DNA marker was considered to be non-informative in LOH analysis. The chromosomal arms exhibiting LOH were 1p34.3 (4.3%), 3p21.32-p21.1 (15.4%), 5q32-

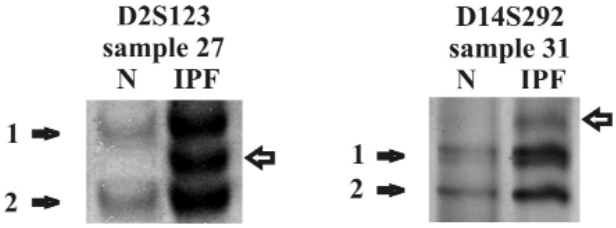

Fig. 2 Characteristic examples of MIN cases. N, normal DNA; IPF, idiopathic pulmonary fibrosis DNA. Black arrows and numbers indicate each allelic band. White arrows indicate the novel generated allele.

q33.1 (5.3%), 9p21 (4.8%) and 17p13.1 (8.0%) where MYCL1, FHIT, SPARC, $p16^{Ink4}$ and TP53 genes have been mapped respectively (http://www.ncbi.nlm.nih.gov/LocusLink/).

Subgroups of IPF patients, positive and negative for LOH, were compared. No statistically significant difference was found between subgroups in relation to age, sex, stage, duration of illness and smoking habit.

Discussion

We employed microsatellite DNA analysis, with highly polymorphic markers, to identify candidate genetic loci for IPF development. LOH incidence was 38.5% in IPF patients, with at least one locus affected. LOH positive cases were observed in microsatellite DNA markers located in MYCL1, FHIT, SPARC, $p16^{Ink4}$ and TP53 genes at 4.3, 15.4, 5.3, 4.8 and 8.0% respectively. Previous reports of LOH in IPF showed incidences between 39 and 52% [19, 21]. Such variations could be due to different kind of specimens, sputum [19] where the contamination of healthy cells is greater instead of microdissected lung tissues [21] from patients and to the exclusion versus inclusion of malignancies.

MYCL1 is a member of a family of genes encoding short-lived nuclear regulatory proteins that act as transcription factors and regulate cell proliferation and differentiation [25]. Amplification and rearrangements of this locus have been described in lung cancer [26] as well as LOH of this chromosomal region [27].

Abnormalities of fragile histidine triad, FHIT gene, at expression [28] or microsatellite level have been reported to occur frequently in multiple tumor types including non-small cell lung cancer (NSCLC) [29] and IPF [21]. Uematsu *et al*. found a LOH incidence of 17% in FHIT gene, which was confirmed by FISH analysis, in IPF patients without cancer [21], while we report 15.4% for the same locus.

Expressed during many stages of development, the matricellular protein SPARC (secreted protein acidic and rich in cysteine) gene is restricted in adults primarily to tissues that undergo consistent turnover or to sites of injury and disease [30]. The capacity of SPARC to bind to several resident proteins of the ECM, to modulate growth factor efficacy, to affect the expression of matrix metalloproteinases, and to alter cell shape as a counter adhesive factor, supports the idea that SPARC acts to regulate cell interaction with the extracellular milieu during development and in response to injury [30]. Recent studies suggest that idiopathic pulmonary fibrosis (IPF) is associated more closely with abnormal wound healing than with inflammation [31]. Thus the connection between SPARC and IPF may reflect this feature.

The association between IPF and cancer [11-14] leads to the assumption that LOH on $p16^{Ink4}$ and p53, as determined in this study, could be implicated in disease pathogenesis by loss of a functional allele. Alterations in p53 expression profiles have been detected on protein level [15], while mutations of the gene have also been found [17]. Noteworthy, p53 alterations do not attribute only to a carcinogenesis process but also to an acute inflammatory one.

MIN was found in 19.2% of cases in our set of samples. However, none of them had a replication error-positive (RER+) phenotype, probably because of minor alterations of DNA mismatch repair system (MMR) activity [24].

In conclusion, our data suggest that IPF exhibits alterations at the molecular level towards tumor as well as wound healing progression. Genes involved in distinct cellular pathways such as cell cycle, apoptosis or inflammation responses could account for the pathogenesis of this disease.

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