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The presence of Merkel cell polyomavirus is associated with deregulated expression of *BRAF* and *Bcl-2* genes in non-small cell lung cancer

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Polyomaviruses such as BK virus (BKV), JC virus (JCV) and Merkel cell polyomavirus (MCPyV) are typically nononcogenic, although they have been detected in a variety of human neoplasms. The aim of our study was to determine the frequency of the most common polyomaviruses MCPyV, BKV and JCV as well as the gene expression profile of genes involved in oncogenesis including *K-ras, BRAF, RKIP, Bax, Bcl-2, p53* and *RB1* in a cohort of non-small cell lung cancer (NSCLC) patients. Real-time and nested polymerase chain reaction (PCR) were used to assess the presence of polyomaviruses DNA in tissue biopsies from 110 patients with primary NSCLC and 14 tissue specimens from macroscopically healthy sites of their lung. Real-time PCR was also used to determine the mRNA expression of *K-ras, BRAF, RKIP, Bax, Bcl-2, p53* and *RB1* in selected samples. Results showed that ten NSCLC specimens were positive for the presence of MCPyV DNA (10/110, 9.1%), whereas no control sample was tested positive for the virus. The MCPyV-positive samples were predominantly obtained from male smokers (9/10). BKV and JCV DNA were not detected either in lung tissues biopsies or the control specimens. Interestingly, gene expression analysis revealed increased mRNA and protein expression of *BRAF* gene in association with *BRAF* phosphorylation in the MCPyV-positive samples, whereas *Bcl-2* gene expression was downregulated in the same type of samples. The detected MCPyV prevalence in NSCLC in combination with the deregulated expression of *BRAF* and *Bcl-2* genes suggests that these events are likely to contribute to the pathogenesis of NSCLC.

In 2011, lung cancer worldwide constituted 13% of all malignant tumors and accounted for 18% of cancer deaths.[1] The main risk factors that have been implicated thus far include cigarette smoking, environmental pollution, radiation and exposure to asbestos,[2] although numerous lung cancer cases have failed to be attributed to these factors. It has been postulated that the gene–environment interaction is also involved in carcinogenesis, where environmental factors cause genomic instability either directly or *via* an inflammatory process.[3]

Human polyomaviruses (HPyVs) are double-stranded DNA viruses encapsulated in an icosahedral capsid, with no lipoprotein envelope. JC (JCV), BK (BKV), Karolinska Institute (KIV), Washington University (WUV), Merkel cell (MCPyV) viruses and the newly discovered skin-infecting HPyV6, HPyV7 and trichodysplasia spinulosa-associated polyomavirus have been detected in humans.[4] Similar studies with the newly discovered HPyV are lacking. A number of cases in which HPyV exhibited a higher prevalence compared to the corresponding normal tissue have recently been identified.[5] However, a direct causative role of HPyV in cancer remains controversial.

Merkel cell polyomavirus (MCPyV) was first discovered in Merkel cell carcinoma (MCC) of the skin, a highly aggressive tumor from neuroendocrine cells.[6] Since the discovery of MCPyV as a major etiopathological factor of MCC, additional associations have been sought between MCPyV and other human neoplasia, including lung carcinomas. In small cell lung cancer (SCLC), this virus has been correlated with hypermethylation of the tumor suppressor gene RASSF1A,[7] whereas in non-small cell lung cancer (NSCLC) MCPyV has been detected either alone[8] or in association with human papillomavirus (HPV) in 33% of the patients.[9]

MCPyV expresses the large T antigen (LTA) that may be truncated in cancer cells, resulting in elimination of the viral helicase function and replication, while the ability of the

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What's new?

An estimated one in five cancer cases worldwide are caused by infection, with the majority being caused by tumor viruses. The present study aimed to establish an association between human polyomavirus infection and non-small cell lung cancer (NSCLC). The data implicate Merkel cell polyomavirus (MCPyV) in 9.1% of the cases in a cohort of 110 NSCLC patients. The expression profile of genes involved in oncogenesis also point to the deregulation of *BRAF* and *Bcl-2* in MCPyV-positive NSCLC cells. These findings suggest the involvement of MCPyV in non-small cell lung cancer through deregulation of *BRAF* and *Bcl-2*.

truncated LTA to bind critical cell cycle-regulating proteins may be preserved leading to cell proliferation and enhanced transformation potential.[10] LTA expression by MCPyV is crucial for inhibiting important responses to UVR-induced DNA damage, suggesting that progressive MCV-mediated abrogation of genomic stability may be involved in Merkel cell carcinogenesis.[11] Furthermore, the LTA may target and interact with cellular proteins such as the retinoblastoma protein, as an intact retinoblastoma protein-binding site in MCPyV LTA is required for promoting growth of MCC cells.[12]

Lung carcinomas harbor multiple mutations in genes involved in cell growth, proliferation or apoptosis. Such mutations may include the Ras/raf pathway whose defect results in uncontrolled cell growth *via* MAPK/ERK regulation, while the *p53* pathway is situated at the crossroads of a network of signaling cascades, essential for cell growth regulation and apoptosis induced by genotoxic and nongenotoxic stresses. Mutations of the *ras* genes, including the *K-ras* oncogene, in lung cancer are entirely limited to NSCLC (predominantly adenocarcinoma),[13] whereas in a recent study[14] it has been suggested that the mutant *BRAF* is a driving "event" in lung adenocarcinoma. Deficiency of the inhibitor *RKIP* is positively correlated with carcinogenesis as well as invasion and metastasis of lung squamous cell carcinoma,[15] while the total level of *RKIP* has no predictive value for lung cancer patient outcome.[16] Genotoxic stress results in the activation of the "guardian of the genome" gene *p53*. In turn, *p53* reduces RB1 phosphorylation and induces a repair-or-apoptosis cell cycle stop through *Bax/Bcl-2* and other *p53*-induced gene regulation processes.[17] *Bax* is an apoptosis-inducing gene, whereas *Bcl-2* is an antiapoptotic gene and the heterodimer *Bax/Bcl-2* induces a neutralization of *Bax* and a loss of apoptosis.[18] Mutation of *p53* is very frequent in lung cancer and occurs in 50% of NSCLCs and 75% of SCLCs.[19]

A viral etiology of lung tumors is attractive because the virus can be regarded as a main oncogenic event or as an important cofactor as in human mesotheliomas (Simian Virus 40 of the polyoma family plus asbestosis). In this view and considering the fact that the polyoma family has already been linked with human malignancies, we aimed to examine the MCPyV, BKV and JCV frequency as well as the expression patterns of *K-ras, BRAF, RKIP, Bax, Bcl-2, p53* and *RB1* genes in the same series of NSCLC specimens.

Material and Methods

Patients

The lung tissue group of patients comprised of 110 consecutive patients with NSCLC from the Department of Thoracic Medicine, University Hospital of Heraklion, Crete, Greece. The patients included in our study were classified according to the criteria of WHO (1997). The tissue control group consisted of 14 samples from macroscopically healthy sites of the lung derived from NSCLC patients ongoing lobectomy and were verified histologically.

Ethics statement

The Ethics Committee of the University Hospital of Heraklion, Crete approved the protocol and all patients and control subjects provided informed consent in written form.

Biological samples and processing

One hundred and ten lung cancer tissue specimens were obtained from paraffin-embedded blocks from the Laboratories of Pathology, University Hospital of Heraklion, Crete. All the samples were obtained from surgical resections. Among the 110 patients, 16 underwent further surgical resection of the lesion, from which fresh lung tissue specimens were obtained and stored at −80°C until further processing.

DNA extraction and PCR amplification

To extract DNA, lung cancer tissue biopsy samples from paraffin-embedded blocks were processed using the QIAamp DNA FFPE Tissue Kit (Qiagen, Germany), according to the manufacturer's instructions. Genomic DNA from the lung cancer tissue samples from controls and fresh lung cancer tissue biopsies was extracted after homogenization and subsequent treatment using proteinase K, followed by phenol extraction and ethanol precipitation according to the standard procedures. All the DNA samples were quantified spectrophotometrically and normalized aliquots were produced for each sample.

Real-time quantitative polymerase chain reaction (PCR) reactions for the BKV and JCV assays were performed using commercial kits. For the detection of BKV DNA, the BKV real-time Q-PCR Alert AmpliMIX kit (Cat. No. RTS175 BKV) was applied according to the manufacturer's instructions (Nanogen Advanced Diagnostics S.p.A., Italy), whereas for the detection of JCV DNA the JCV/BKV oligomix Alert

nested PCR kit (Cat. No. BAN075) was used, according to the manufacturer's instructions (Nanogen Advanced Diagnostics S.p.A.). The presence of MCPyV DNA was tested using real-time quantitative PCR protocols, using the MCV138 set of primers that targets the LTA region, as previously described.[20] The integrity and quality of the extracted DNA were confirmed after the successful amplification of the beta2-microglobulin gene in all samples.

Particular care was taken and all manipulations were performed inside a PCR hood to avoid potential contamination. Each PCR reaction contained two negative controls. The positive controls for the BKV and JCV reactions were obtained from Nanogen Advanced Diagnostics S.p.A. (Cat. No. CTR075), while DNA from a MCC patient served as the positive control. All real-time PCRs were carried out in an Mx3000P real-time PCR system while additionally, PCR end products were analyzed on 2% agarose gel, stained with ethidium bromide and the bands were visualized under a UV transilluminator (260 nm). These viral PCR products were then subjected to direct sequencing analysis to verify the initial amplification results.

Gene expression

Sixteen fresh tissue specimens from NSCLC patients and 14 controls were processed using the TRIzol reagent (Invitrogen, Carlsbad, CA) protocol for total RNA extraction according to the manufacturer's instructions. The extracted RNA was subsequently treated with DNase to avoid DNA contamination. RNA concentration and purity were evaluated by a spectrophotometer. Aliquots of RNA were stored at −80°C until use. cDNA from each sample was derived by reverse transcription of 1 μg of total RNA using the the SuperScript® First-Strand Synthesis System (Invitrogen) and random hexamers. To remove the RNA template, cDNA was incubated with *Escherichia coli* RNaseH (Invitrogen) and stored at −20°C until use.

Transcript levels of *K-ras, BRAF, RKIP, Bax, Bcl-2, p53* and *RB1* were determined using the Mx3000P real-time PCR system (Stratagene, La Jolla, CA) and SYBR-Green I Master Mix (Stratagene) according to the manufacturer's instructions, as previously described.[21] The primers were designed to span at least one intron in order to avoid amplification of contaminating genomic DNA. GAPDH was used as an internal control to normalize mRNA expression levels. To verify the results of the melt-curve analysis, PCR products were analyzed by electrophoresis on 2% agarose gels, stained with ethidium bromide and visualized under a UV light transilluminator. Primer sequences and annealing temperatures for all the genes analyzed as well as for GAPDH are shown in Table 1.

Table 1. Primer sequences used for quantitative real-time RT-PCR

Gene	Primer pair sequence (5′-3′)	Annealing temperature (°C)
GAPDH	FOR: GGAAGGTGAAGGTCGGAGTCA	60
	REV: GTCATTGATGGCAACAATATCCACT	
BRAF	FOR: AGAAAGCACTGATGATGAGAGG	58
	REV: GGAAATATCAGTGTCCCAACCA	
RKIP	FOR: AGACCCACCAGCATTTCGTG	58
	REV: GCTGATGTCATTGCCCTTCA-3	
p53	FOR: GTGAGCGCTTCGAGATGTTC	60
	REV: ATGGCGGGAGGTAGACTGAC	
K-ras	FOR: ACTGAATATAAACTTGTGGTAGTTGGACCT	60
	REV: TCAAAGAATGGTCCTGGACC	
Bcl-2	FOR: GAAACCCCTAGTGCCATCAA	55
	REV: GGGACGTCAGGTCACTGAAT	
Bax	FOR: TTCTGACGGCAACTTCAACTGG	61
	REV: TTGGTGCACAGGGCCTGTAATC	
RB1	FOR: CTTGCATGGCTCTCAGATTCAC	60
	REV: AGAGGACAAGCAGATTCAAGGTG	

Protein extraction and Western blotting

Fresh tissue samples were homogenized in RIPA lysis buffer (Tris-buffered saline pH 7.4, NP-40Na-deoxycholate, NaCl, EDTA, PMSF, Protease Inhibitor Cocktail, Phosphatase inhibitors, Na-Vanadat and NaF) to extract the total protein. Proteins were quantified using the Bradford Protein Quantitation protocol. After a 12% SDS–PAGE gel was cast, 40 mg of total protein was loaded into each lane, and the electrophoresis separation was performed. Proteins in the gel were then transferred to a PVDF membrane in a semidry blotting system. The PVDF membrane was blocked with TBST (10 mM Tris–HCl, pH 7.5, 150 mM NaCl and 0.1% Tween-20) solution containing 5% skimmed milk at room temperature for 1 hr. Primary antibodies were then added at the appropriate dilution [phosphor-BRAF (Ser445), BRAF and Bcl-2 1/1,000; beta-actin 1/2,000) with overnight incubation at 4°C. All primary antibodies were purchased from Cell Signaling Technology, MA. Subsequently, the HRP-conjugated goat anti-rabbit IgG polyclonal antibodies (1:2,000 dilution) were added, with incubation at room temperature for 1 hr. After TBST washing, autoradiography was conducted with enhanced chemiluminescence reagents. The results were analyzed with the QuantityOne software.

Statistical analysis

The Kolmogorov–Smirnov test was used to determine whether the expression data obtained followed a normal distribution pattern. The mRNA expression of all the genes was compared between the groups of normal and pathological samples, as well as between groups with different histological features, using parametric procedures (Student's *t*-test and one-way ANOVA).

Probability values (p-values) <0.05 were considered statistically significant. Statistical calculations were performed using SPSS 11.5 software (SPSS, Chicago, IL).

Table 2. The clinicopathological characteristics of the subjects

Characteristics	Control group	NSCLC patients	*p* value
Number	14	110	
Age[1]	56.33 ± 5.47	62.92 ± 1.12	NS
Sex (male/female)[2]	12/2	101/9	NS
Nonsmokers[2]	9	12	0.0011
Smokers[2]	2	96	0.0007
Ex-smokers[2]	3	2	NS
FEV1[1]	86.78 ± 8.71	84.12 ± 3.53	NS
FVC[1]	93.22 ± 7.50	89.89 ± 4.11	0.0274
FEV1/FVC[1]	73.33 ± 4.29	74.46±1.80	NS
DLCO[1]	74.43 ± 11.37	83.77 ± 9.15	0.00386
KCO[1]	83.15 ± 10.89	91.36 ± 14.19	NS

Values are expressed as means ± SEM (standard error of the mean).
[1] *t*-test; $P < 0.05$ is considered statistically significant.
[2] χ^2 test; $P < 0.05$ is considered statistically significant.
Abbreviations: DL_{CO}: diffusing capacity for carbon monoxide; FEV1: forced expiratory volume in 1 sec; FVC: forced vital capacity; KCO: DLCO per unit lung volume; NS: not significant.

Table 3. Histological types of biopsies of NSCLC patients

NSLC subtype	All	Male	Female
Number	110	101	9
Adenocarcinoma	58	52	6
Squamous	39	38	1
Adenosquamous	4	4	–
Large cell undifferentiated	3	2	1
Bronchioalveolar	3	2	1
Undifferentiated	2	2	–
Giant cell	1	1	–

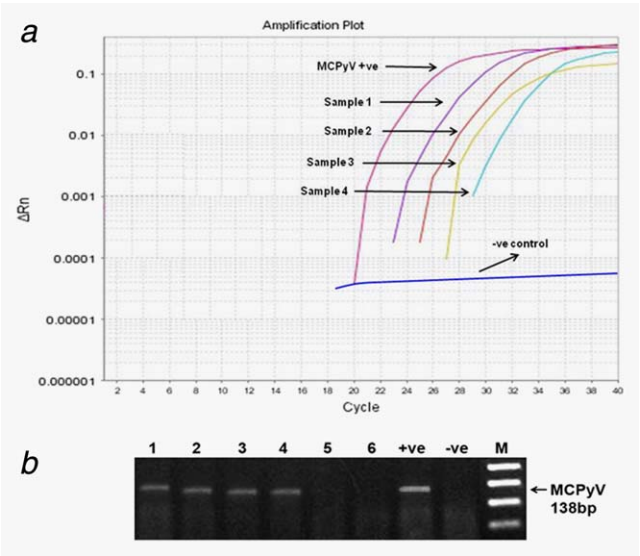

Figure 1. Representative examples of MCPyV as detected by (*a*) real-time PCR and (*b*) on agarose gel. +ve: positive control; −ve: negative control; M: 50-bp DNA ladder. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Results

Polyomavirus detection

The NSCLC tissue study sample included 101 male and nine female patients with NSCLC. Demographics and pulmonary function tests of patients are shown in Table 2. Biopsy results and malignant subtypes of NSCLC of all patients are shown in Table 3. Briefly, the mean age of patients was 62.92 ± 1.12 years, while the majority of the NSCLC group consisted of active smokers (87.3%).

The lung tissue biopsies from the NSCLC patients and the control population tested negative for the presence of BKV and JCV DNA.

MCPyV DNA was detected in ten of 110 (9.1%) specimens from the lung cancer group, whereas MCPyV genomes were not detected in any of the control samples (Fig. 1). The vast majority of the MCPyV-positive samples were obtained from male patients (9/10), while the mean age was 63.78 ± 11.25 years (Table 4). The histological types consisted mainly of adenocarcinomas and squamous cell carcinomas. The two predominant histological MCPyV-positive types showed similarities in mean age of the patients.

Among the MCPyV-positive samples only one had a no smoking history, and although one patient was an ex-smoker, the mean pack year (p.y.) smoking history was 51 p.y. In both the adenocarcinoma and squamous cell carcinoma MCPyV-positive group, we observed a smoking history of ∼39 pack years, although in the squamous cell carcinoma MCPyV-positive group one patient had no smoking history. No difference was identified in clinical parameters between virus-positive and virus-negative patients in adenocarcinoma or squamous cell carcinoma histological NSCLC subtypes.

Gene expression

Transcript levels of *K-ras, BRAF, RKIP*, as well as of *p53* and *RB1* were not statistically significantly altered between NSCLC specimens and the control population (Table 5). In contrast, *Bcl-2* expression was significantly downregulated in the lung cancer samples compared to the controls (mean ± SD: 1.19 ± 1.39 and 1.23 ± 1.49, respectively, ratio of the means 0.97, $p = 0.047$). Of note, considerably lower levels of *Bax* expression were also found in the NSCLC specimens compared to the controls (mean ± SD: 1.15 ± 0.23 and 9.91 ± 0.16, respectively, ratio of the means 0.12, $p = 0.038$). The *Bax/Bcl2* ratio was 0.97 for the lung cancer group and 8.06 for the controls.

We subsequently determined whether the MCPyV infection affected the expression levels of the abovementioned cellular genes. Six MCPyV-positive samples and ten MCPyV-negative specimens were used in the gene expression analysis. Among NSCLC specimens, *BRAF* was upregulated in MCPyV-positive tissue samples compared to MCPyV-negative samples

Table 4. Clinicopathological characteristics of Merkel cell polyomavirus-positive NSCLC patients

Lung cancer tissue group							
Patient no.	Sex	Age (years)	Histological type	Smoking status Y/N (pack-years)	FEV1 (%)	FVC (%)	FEV1/FVC
27	M	47	Undifferentiated large cell lung carcinoma	Y (65)	91.8	113.2	64.77
44	M	66	Adenocarcinoma	Y (40)	100.8	121	65.9
57	M	73	Adenocarcinoma	Ex-smoker (10)	92.1	95.5	63.97
62	M	67	Squamous cell carcinoma	N	87.2	83.8	76.61
63	M	73	Adenocarcinoma	Y (80)	88.5	80	85.25
67	M	65	Bronchoalveolar carcinoma	Y (55)	68.9	89.3	61.14
71	M	74	Adenocarcinoma	Y (43)	101.6	83.7	96.03
78	M	85	Squamous cell carcinoma	Y (85)	63.6	89.3	51.83
80	M	54	Squamous cell carcinoma	Y (30)	90.3	91.8	73.56
88	F	55	Adenocarcinoma	Y (25)	79.1	91.9	72.67
Mean		63.78 ± 11.25		51 ± 29	86.39 ± 12.47	93.95 ± 13.16	71.17 ± 12.69

Values are expressed as means ± SEM (standard error of the mean).
Abbreviations: FEV1: forced expiratory volume in 1 sec; FVC: forced vital capacity; NSCLC: non-small cell lung cancer; SCLC: small cell lung cancer.

Table 5. Expression profile of cellular genes in lung tissue samples of NSCLC patients and controls in relation to the presence or absence of MCPyV

Mean ± SD	Lung cancer tissue	Healthy lung tissue	Ratio[1] of means	*p*1 value	McPyV-positive samples	McPyV-negative samples	Ratio[2] of means	*p*2 value
K-ras	1.21 ± 0.20	1.08 ± 0.16	1.12	NS	1.24 ± 0.39	1.21 ± 0.21	1.03	NS
BRAF	1.16 ± 0.20	1.08 ± 0.23	1.07	NS	1.29 ± 0.10	1.12 ± 0.21	1.15	$p = 0.0411$
RKIP	1.11 ± 0.19	1.04±0.19	1.07	NS	1.17 ± 0.18	1.09 ± 0.19	1.07	NS
p53	1.21 ± 0.22	1.13 ± 0.15	1.07	NS	1.29 ± 0.23	1.21 ± 0.23	1.07	NS
RB1	1.37±0.31	1.34 ± 0.12	1.02	NS	1.29 ± 0.54	1.42 ± 0.32	0.91	NS
Bcl-2	1.19 ± 1.39	1.23 ± 1.49	0.97	$p = 0.047$	1.12 ± 0.14	1.26 ± 0.12	0.89	$p = 0.050$
Bax	1.15 ± 0.23	9.91 ± 0.16	0.12	$p = 0.038$	1.24 ± 0.25	1.11 ± 0.21	1.12	NS

*p*1: *p* value lung cancer tissue *vs*. healthy lung tissue; *p*2: *p* value MCPyV-positive samples *vs*. MCPyV-negative samples.
[1]Ratio of the means lung cancer tissue/healthy lung tissue.
[2]Ratio of the means MCPyV-positive samples/MCPyV-negative samples.

(mean ± SD: 1.29 ± 0.10 and 1.12 ± 0.21, respectively, ratio of the means 0.97, $p = 0.0411$) (Table 5). Unlike *BRAF*, *Bcl-2* gene was markedly downregulated in the MCPyV-positive specimens (mean ± SD: 1.12 ± 0.14 and 1.26 ± 0.12, respectively, ratio of the means 0.89, $p = 0.050$).

The aforementioned differences observed in the gene expression depending on the MCPyV status were additionally examined at the protein level. Western blot analysis confirmed the increased expression of *BRAF* in the MCPyV-infected samples as well as the downregulation of *Bcl-2* in the uninfected specimens (Fig. 2). Moreover, we investigated the activation status of BRAF in a proportion of the samples. For that purpose, a specific phospho-BRAF (Ser445) antibody that detects endogenous levels of B-RAF when phosphorylated at serine 445 was used. Of note, the phosphorylation of BRAF at this particular residue was significantly increased in the MCPyV-infected samples compared to the basal levels of phosphorylation in the MCPyV noninfected specimens (Fig. 2*a*).

No statistically significant association was observed between the expression patterns and any of the clinical parameters.

Discussion

An estimated one in five cancer cases worldwide are caused by infection, with the majority being caused by tumor viruses.[22,23] Most of the viruses are capable of expressing viral

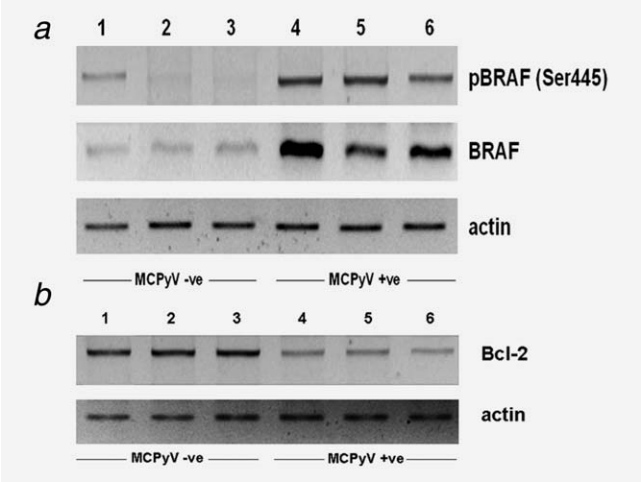

Figure 2. Western blot analysis of phospho-BRAF (Ser445), BRAF and Bcl-2 in MCPyV-infected and noninfected NSCLC specimens. (*a*) Compared to MCPyV noninfected cells (lanes 1–3), both activation of BRAF at Ser445 and overexpression of BRAF protein were evident in MCPyV-infected tissues (lanes 4–6). (*b*) Bcl-2 protein levels were significantly decreased in MCPyV-positive samples (lanes 4–6) compared to noninfected tissue specimens. Actin was used as a stable endogenous control. −ve: negative samples; +ve: positive samples.

genes, inducing alterations in cell growth, proliferation and apoptosis of the infected cells. Our study aimed to establish an association between HPyV infection and NSCLC. Moreover, we investigated the expression of oncogenes, tumor suppressor genes and apoptosis-related genes in MCPyV-infected NSCLC samples, in an effort to evaluate possible mechanisms of oncogenesis in this group of patients. Our major finding is the 9.1% prevalence of MCPyV in the NSCLC group. Moreover, we found an increased mRNA expression of *BRAF* and decreased *Bcl-2* transcription level in the MCPyV-positive NSCLC samples. These findings suggest the involvement of MCPyV in this type of lung cancer through deregulation of the above genes.

The MCPyV-positive samples were predominantly obtained from male smokers, and included mainly adenocarcinomas (50%) followed by squamous cell carcinomas (30%). These findings are noteworthy because a marked increase in the incidence of lung adenocarcinoma has been observed surpassing that of squamous cell carcinoma. The "gene–environment" interaction that is hypothesized to promote this change[24] may be a widely spread infectious agent, capable of interacting with environmental insults causing a susceptibility to genetic mutation and ultimately oncogenesis.

The detected frequency of MCPyV-positive samples (9.1%) in NSCLC is identified in the prevalence rates of two previous studies' identified prevalence rates; a MCPyV and HPV costudy[9] and a recent study of MCPyV DNA presence in NSCLC from Chile.[8] The HPV and MCPyV costudy suggested the presence of MCPyV in 16.7% of 30 NSCLC patients, mainly from squamous cell carcinomas of smoker patients. Interestingly, these findings came from both malignant and adjacent benign tissue. Researchers also detected two benign MCPyV-positive samples but not their corresponding malignant ones. Our study screened 110 NSCLC patients, including a control group that proved to be negative for MCPyV DNA. The discordance among these three studies may be a result of differences in the population of patients studied as well as the difference in the control group tested. Notably, we have also shown a positive trend in smoking status, because the majority of MCPyV-positive samples were obtained from smokers. The differences among these three studies may also be attributed to sample composition variations. The study from Chile used only adenocarcinomas and squamous cell carcinomas at a 1:1 ratio, whereas our study included all NSCLC histological types and the ratio between them reflects their trend in a different geographical area. Moreover, the authors from Chile have stated that their poor percentage is potentially attributed to low sample quality. It is of note that a quantitative study[25] has proven that the incidence of MCPyV DNA in lung cancer tissue is twice as common as in normal lung tissue, although in our study no normal tissue was found to be MCPyV-positive. It is noted that the absence of MCPyV in the control group of our study may be a result of the small control sample size.

It is known that the presence of DNA in this virus is not a sufficient condition to support its involvement in tumorigenesis. Several DNA viruses may not be strong promoters of neoplastic transformation. Instead, the long-term presence of these viruses and their associated proteins in the cell may contribute to an environment that is beneficial to the virus, playing a critical role in the transition from neoplastic precursor toward malignancy. Thus, our study further explored the gene expression of a variety of genes involved in fundamental cellular processes to investigate a possible association between the virus and oncogenesis. The major finding in our study was the downregulation of the *Bcl-2* gene in NSCLC. Moreover, an increased expression of the *BRAF* gene was observed in the MCPyV-positive cases. Research has shown that ablation of *BRAF* had no significant impact on NSCLC tumor development.[26] Moreover, *BRAF* mutations undergo careful observation as they are thought to be excellent therapeutic targets, with *BRAF* exon 15 mutation in NSCLC patients without other gene mutations being sufficient to identify candidates for treatment, in NSCLC male and patients who are heavy smokers.[27] An incidence of 3% of *BRAF* mutations in lung adenocarcinomas was detected, indicating that all patients with a *BRAF* mutation were current or former smokers.[14] The regulatory proteins of HPyV interfere with the normal activity of cell cycle regulatory proteins such as p53, pRb, cyclins, cyclin-dependent kinases (CDK) and CDK inhibitors, inducing telomerase activity, DNA instability, perturbed protein degradation and preventing apoptosis.[28] Two of the principal signaling pathways that are stimulated by the polyoma middle T antigen (MT) are the mitogen-activated protein kinase (MAPK) *via* the Ras/raf pathway[29] and the phosphatidylinositol 3-kinase (PI3K)

cascades. *BRAF* upregulation that leads to a downstream upregulation of the MAPK/ERK pathway and ultimately to uncontrolled cell proliferation may be a direct result of polyoma protein transformation of the cell, thus contributing to carcinogenesis.

Our third major finding, the downregulation of the *Bcl-2* gene in the MCPyV-positive lung cancer specimens, has been correlated in previous studies with a more aggressive behavior of NSCLC.[30] The second principal signaling pathway that is stimulated by the polyoma MT is the phosphatidylinositol 3-kinase (*PI3K*) cascade, which results, *via Akt*, in the phosphorylation of the *Bcl-2*-associated death agonist (*BAD*), which in turn restores the antiapoptotic effects of the *BAD*-binding protein *BCL-X_L*.[29] Although the main aim of our study was not to explore the *PI3K/Akt* pathway, we investigated the deregulation of apoptosis by the expression of two genes situated downstream the *PI3K* cascade: *Bax* and *Bcl-2*. A ratio <1 of *Bax/Bcl-2* indicates hyperexpression of *Bcl-2* and loss of *Bax* when compared to normal bronchial epithelium and has been shown to increase with the severity of the preneoplastic lesions, from low grade to high grade.[18] In our study, we have shown a statistically significant downregulation of *Bax* expression in lung cancer samples, whose role consists of accelerating programmed cell death by binding to, and antagonizing, the apoptosis repressor *Bcl-2*. The total of the lung cancer tissue samples had a *Bax/Bcl-2* ratio < 1, in accordance with the studies implicating such ratios with neoplasias.[18] Interestingly, the MCPyV-positive samples had lower *Bcl-2 l*evels compared to MCPyV-negative samples, leading toward a loss of apoptosis-repressor activity, which may contribute to aberrant proliferation. We hypothesize that the susceptibility of Bcl-2 deregulation may be a contributing factor to MCPyV infection. A recent study has shown that the Bcl-2-associated athanogene, *Bag3*, has the ability to interact with the JCV LTA and downregulate the level of LTA expression in primitive neuroectodermal tumor cells, suggesting that *Bag3* affects the expression of LTA at the post-transcriptional level, most likely by destabilizing the protein.[31] The findings of a recent study suggest that *Bcl-2* positivity indicates a better clinical stage at the time of diagnosis with less metastasis and a longer survival rate in MCC.[32] In our study, all the MCPyV-positive samples tested positive for *Bcl-2* expression, and while data from survival rates cannot be correlated at this time with *Bcl-2* expression, we may hypothesize that MCPyV infection in lung cancer cells may alter the expression pattern but not repress the *Bcl-2* expression. Further studies may provide information on lung cancer survival rates in correlation with *Bcl-2* expression.

LTA of MCPyV contains a supposed *p53*-binding site that may be lost when the virus undergoes a truncating mutation in tissue.[10] Recently, it was shown in MCCs that higher viral abundance tended to be associated with lower *p53* expression and longer survival,[33] whereas in another study *p53* expression in tumor was associated with unfavorable MCC-specific survival.[34] In addition, a recent study aiming to improve the sensitivity of detection of MCPyV in MCC revealed that although all MCC may contain MCPyV viral DNA, tumors may be selected for the presence of inactivating p53 mutations when the viral T antigens are not expressed. Furthermore, in the case that MCPyVpositive MCC tumors disrupt p53 signaling, then the above selection is accomplished by a different mechanism that does not involve the C terminus of the LTA.[35] In our study, although a trend for *p53* upregulation in the MCPyV-positive sample was observed, *p53* was not significantly affected by the MCPyV in the NSCLC samples, suggesting that among different human tumors, MCPyV may induce different oncogenic pathways.

Regarding the *RB1* expression, we found that the presence of MCPyV did not correlate with the abundance in the *RB1* transcripts in NSCLC samples. This finding is in agreement with the comparable expression and phosphorylation of the retinoblastoma protein in MCPyV-positive and -negative MCC cells.[36]

Recent studies have shown that although MCPyV is possibly an oncogenic polyomavirus in humans and is potentially causally related to the development of MCC, it is not to the morphological similar to SCLC.[37]

Studies have shown that presence of MCPyV DNA is higher in respiratory specimens from asymptomatic patients following lung transplantation compared to KIV and WUV,[38] whereas in a study on acute respiratory tract infection more adults than children were positive for respiratory MCPyV in contrast to KIV and WUV.[39] It seems likely that presence of MCPyV in the lung parenchyma may play a critical role in the progression of pulmonary diseases in adults, and because inflammation has been postulated to be important in lung carcinogenesis,[40] viral infections may be a key component of the process.

There was no detection of BKV or JCV DNA in any of the NSCLC samples, in accordance with the lack of evidence toward an etiopathological role of these polyomaviruses and lung carcinomas. Although HPyVs have a global presence and high seropositivity worldwide, none of our control population samples was tested positive for MCPyV, BKV or JCV DNA.

The connection between lung cancer and infectious agents such as viruses may lead to a better understanding of the incidence of the disease in nonsmokers. Although smoking accounts for the majority of lung cancer cases, the fact that only a minority of smokers develop lung cancer in their lifetimes makes this disease an important model for assessing gene–environment interactions. Our study contributes to the field of the pathogenesis of lung carcinogenesis by providing data implicating MCPyV infection in a notable 10% of NSCLC patients, while suggesting that the deregulation of BRAF and Bcl-2 in MCPyV-infected lung cancer cells may be one of the critical steps involved in the contribution to carcinogenesis *via* MCPyV cell transformation.

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