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Association of Merkel cell polyomavirus infection with EGFR mutation status in Chinese non-small cell lung cancer patients

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ABSTRACT

Objectives: Female lung cancer patients with no smoking habit and non-mucinous adenocarcinoma have a higher rate of epidermal growth factor receptor (EGFR) gene mutations, which is related to tyrosine kinase inhibitors (TKIs) sensitivity. Unfortunately the cause of EGFR gene mutations is still elusive. In this study, we search for the association between Merkel cell polyomavirus (MCPyV) infection and EGFR gene mutations.

Materials and methods: We studied 189 non-small cell lung cancer (NSCLC) samples for the presence of MCPyV large T (LT) DNA, LT antigen and EGFR hotspot mutations. Clinicopathological parameters of this cohort were also analyzed.

Results: Thirty out of 163 adenocarcinoma and 2 out of 18 squamous cell carcinoma were found to have MCPyV LT DNA by PCR. Immunostaining also showed LT protein expression in most of the DNA positive samples. EGFR mutations were more frequently detected in female ($P = 0.009$) and non-smoking patients ($P = 0.0001$). Furthermore, a significant association between MCPyV infection and EGFR mutations was found ($P = 0.001$).

Conclusion: Our study shows that MCPyV LT DNA is present in a subgroup of NSCLC, which is significantly correlated with EGFR mutations. To the best of our knowledge, this is the first study to find an association between MCPyV infection and EGFR hotspot mutations. These results support the possibility that MCPyV has a partial role in the carcinogenesis of NSCLC in a subgroup of patients.

1. Introduction

Lung cancer is now a leading cause of cancer-related human death globally [1]. The American Cancer Society projected 159,390 deaths from lung cancer in 2009, accounting for about 28% of all cancer deaths [2]. The tobacco epidemic is still growing in Southeast Asia, including China. Lung cancer is becoming a serious human health problem in these regions [3]. Lung cancer patients have different histological types, which need different treatments according to the National Comprehensive Cancer Network (NCCN) guideline. It is already well-known that the EGFR mutation rate of NSCLC in East Asia is significantly higher than in Western countries, allowing for better treatment of the patients in this area through the use of TKIs [4]. The response rate to TKI is around 70% among patients with sensitizing EGFR mutations [5,6]. Female NSCLC patients with no smoking habit and non-mucinous adenocarcinoma have up to 50% EGFR mutation rate, but in male patients with smoking habit it declines to 20% or less. Particularly, for squamous cell carcinoma, the mutation rate is only around 10% [7]. Histological difference could be a reason for the variable mutation rates, but why the other different clinical groups have different molecular events is still unknown [8]. Tobacco was proven to be a mutagen in lung cancer [9]. Because it has been observed that EGFR mutation frequencies decrease with increasing tobacco usage [10,11], tobacco is clearly not a direct inducer of EGFR mutations.

Merkel cell polyomavirus (MCPyV), discovered in 2008, is clonally integrated in 80% of Merkel cell carcinoma (MCC) [12]. MCPyV is a common skin flora and initiates cancer in susceptible hosts only after it acquires a precise set of mutations that render it replication incompetently. The cause, diagnosis and therapy for the intractable MCC have been changed due to the molecular discovery of MCPyV. MCPyV infection has been investigated in both small cell lung cancer (SCLC), which has histological similarity with MCC and in NSCLC [13–15]. It was firstly reported in 2009 that MCPyV infected 4.7% of NSCLC in an 86-patient cohort [14]. Recently its infection rate was shown to be 9.1% and 17.8% in two different cohorts [16,17]. These studies support the possibility that MCPyV is partly associated with the pathogenesis of NSCLC in a subset of patients, but the underlying tumorigenic functions of MCPyV in NSCLC are still elusive.

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It is interesting that the v-raf murine sarcoma viral oncogene homolog B1 (BRAF) gene expression was found to be higher in MCPyV positive samples than negative ones [17]. Since BRAF is a downstream target of EGFR pathway, activated EGFR could increase BRAF expression, suggesting a potential association between MCPyV infection and EGFR activated mutations. Furthermore, MCPyV itself undergoes specific mutations during MCC tumorigenesis, so we hypothesize that MCPyV might be one inducer of EGFR mutations in NSCLC. In this study, we screened MCPyV infection and the EGFR mutations of a 189 NSCLC cohort. A significant correlation was demonstrated between virus infection and EGFR mutations.

2. Materials and methods

2.1. Patients and samples

NSCLCs were retrospectively sampled from the tissue blocks in the pathology department, Sir Run Run Shaw Hospital, Medical School, Zhejiang University, China. The protocol was approved by the Ethics Committee of Bio-medicine Research in the hospital. All the patients gave informed consent for this study. We investigated a total of 198 NSCLC samples. Diagnosis of NSCLC was established histologically and immunohistologically according to World Health Organization (WHO) criteria [18].

2.2. Antibodies

Antibodies were purchased mainly from Dako, NeoMarkers and Santa Cruz. They are anti-TTF1 (Dako M357501, Glostrup, Denmark), anti-CK7 (Dako M701801, Glostrup, Denmark), anti-CK19 (Dako M088801, Glostrup, Denmark), anti-CK-H (Dako M063001, Glostrup, Denmark), anti-CK-L (NeoMarkers MS-341-PO, MA, USA), anti-synaptophysin (Dako M077601, Glostrup, Denmark), anti-Ki-67 (Dako M724001, Glostrup, Denmark) and anti-MCPyV LT (Santa Cruz sc-136172, TX, USA).

2.3. Immunohistochemistry

4 μm paraffin sections were de-paraffinised through xylene and graded ethanol series. Endogenous peroxidase was quenched by 0.3% H_2O_2 in methanol. Sections were microwaved (10 mM citrate buffer, pH 6.0, 4–5 min, 600 W) and incubated for 30 min at room temperature either with anti-TTF1 (1:1000), anti-CK7 (1:1200), anti-CK19 (1:300), anti-CK-H (1:300), anti-CK-L (1:200), anti-synaptophysin (1:100), anti-Ki-67 (1:100), or anti-MCPyV LT (1:30). Further steps were performed with the Polink-2 HRP Broad Spectrum for DAB Bulk kit (GBI Labs D22–110, WA, USA) and REAL™ EnVision™ Detection System (Dako K500711, Glostrup, Denmark) following manufacturers' instructions. Sections were counterstained with Mayer's hemalum solution (Merck 1092490500, NJ, USA).

2.4. DNA isolation

Five 4 μm tissue sections from paraffin-embedded tumor blocks were macro-dissected according to their consecutive H&E stained slides to enrich over 70% abundance of tumor cells in each sample. Adjacent normal controls were collected directly without macro-dissection. DNA was isolated using a QIAamp FFPE DNA extraction kit (Qiagen 56404, Germany). Concentrations of DNA were determined by SmartSpec Plus Spectrophotometer (Bio-Rad Life Science, CA, USA).

Table 1
Sequences of primers.

Primer name	Primer sequence (5′–3′)
MCPyV-f	ACTTGGGAAAGTTTTGACTGGTGGCAA
MCPyV-r	GGGCCTCGTCAACCTAGATGGGAAAG
Globin-f	AGAAGAGCCAAGGACAGGTACG
Globin-r	ATAGGCAGAGAGAGTCAGTGCC
MCPyV-seq	GGGAAAGTTTTGACTGGTGGC
Globin-seq	GAAGAGCCAAGGACAGG
Exon 18-f	actgtaaaacgacggccagtGCTGAGGTGACCCTTGTCTCTGTGT
Exon 18-r	accaggaaacagctatgaccATACAGCTTGCAAGGACTCTGGGCT
Exon 19-f	actgtaaaacgacggccagtCAGCATGTGGCACCATCTCACAAT
Exon 19-r	accaggaaacagctatgaccAGACATGAGAAAAGGTGGGCCTGAG
Exon 20-f	actgtaaaacgacggccagtGAAGCCACACTGACGTGCCTCTC
Exon 20-r	accaggaaacagctatgaccGCTCCTTATCTCCCCTCCCCGTAT
Exon 21-f	actgtaaaacgacggccagtATCTGTCCCTCACAGCAGGGTCTTC
Exon 21-r	accaggaaacagctatgaccGCAGCCTGGTCCCTGGTGTC
Seq-f	tgtaaaacgacggccagt
Seq-r	caggaaacagctatgacc

2.5. MCPyV identification

The four-step virus DNA detection procedure including PCR mixture preparation, tissue DNA isolation, PCR amplification and PCR product analysis was carried out in 4 separated rooms of a gene diagnostic laboratory to avoid contamination from different samples and previous PCR products, which was approved by the Chinese National Center of Clinical Laboratories.

A duplex PCR was performed with the MCPyV specific primers and β-globin primers (Table 1), using 200 ng of the genomic DNA as template for a 10-min initial denaturation step, 40 cycles at 95 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a 5-min final extension step at 72 °C in 50 μl containing 200 μmol dNTPs, 20 pmol of MCPyV-f and MCPyV-r primer, 5 pmol of Globin-f and Globin-r primer, and 1 unit of FastStart Taq DNA Polymerase (Roche 12032929001, Switzerland). The housekeeping gene β-globin was utilized as an internal control for DNA integrity. 10 μl of PCR product was analyzed in a 1% Tris-borate EDTA agarose gel. Visible PCR product bands close to 200 bp and 400 bp length bands were purified by QIAquick Gel Extraction Kit (Qiagen, 28704, Germany) respectively, and then sequenced directly. The sequencing was conducted by repeating 25 cycles of 10-s, 5-s and 4-min treatments at 96 °C, 50 °C, and 60 °C, respectively, with 20 ng of separated PCR product in a 20 μl mixed solution, including 8 μl of Big Dye Terminator v3.1 (Life Technologies, CA, USA) and 2 pmol of MCPyV-seq or Globin-seq primer respectively, by using an ABI PRISM 3130 DNA Analyzer (Applied Biosystem, Tokyo, Japan). The nucleotide sequences were aligned by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Samples with MCPyV-specific PCR amplification were classified as MCPyV-positive samples.

A sensitivity analysis of the duplex PCR was also performed. A recombinant plasmid pMD-18T containing the PCR product by MCPyV-f and MCPyV-r primers was quantified using SmartSpec Plus Spectrophotometer. The plasmid copy number was calculated by its molecular weight and Avogadro's constant. Serial 2-fold dilutions of the plasmid representing 125–1 plasmid molecules/μl were made and 4 μl of each dilution was added into the each PCR reaction with 200 ng MCPyV LT DNA negative genomic DNA.

2.6. EGFR mutation analysis

EGFR mutation analysis was also carried on in the same laboratory. 4 pairs of primers, covering the hotspot mutation regions of EGFR exon 18–21 were utilized for four separate PCR amplifications (Table 1). 200 ng of the genomic DNA was used as template for a 10-min initial denaturation step, and then 40 cycles at 95 °C for 30 s, 61 °C for 30 s, 72 °C for 30 s with a 5-min final extension step at 72 °C