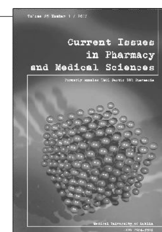
2012 Curr. Issues Pharm. Med. Sci. Vol. 25, No. 2, Pages 159-163

Current Issues in Pharmacy and Medical Sciences

Formerly ANNALES UNIVERSITATIS MARIAE CURIE-SKLODOWSKA, SECTIO DDD, PHARMACIA
on-line: www.umlub.pl/pharmacy



Detection, genotyping and phylogenesis of human papillomavirus (HPV) and Epstein-Barr virus (EBV) in patients with lung cancer

ŁUKASZ PODSIADŁO[1*], SŁAWOMIR MAŃDZIUK[2], MAŁGORZATA POLZ-DACEWICZ[1], AGNIESZKA STEC[1], JAROSŁAW BUCZKOWSKI[2]

[1] Department of Virology, Medical University of Lublin, Poland
[2] Department of Pneumology, Oncology and Allergology, Medical University of Lublin, Poland

ABSTRACT

The lung cancer is one of the most common cancers in humans. It is now believed, that the most common cause of this disease is smoking, nevertheless also research on oncogenic viruses participating in the development of this type of tumor confirms that. Human papillomavirus (HPV) and Epstein-Barr virus (EBV) are the etiologic agents of several types of cancer. The dynamic development of molecular biology techniques and bioinformatics allows for the genotyping of microorganisms in a more accurate and reliable way. In this paper, we carried out the detection and genotyping of HPV and EBV in fragments of fresh tissue obtained from patients with the lung cancer. In addition, typing results are presented graphically in phylogenetic trees.

Keywords: lung cancer, human papillomavirus, Epstein-Barr virus, sequencing, phylogenetic tree

INTRODUCTION

The lung cancer is now one of the worst prognosis and the most common malignancies worldwide, also in Poland. It is worth mentioning, that the lungs are the place where tumors from other body parts often metastasize. Current knowledge indicates that the greatest risk factor for the lung cancer is long-term exposure to inhaled carcinogens, especially tobacco smoke or asbestos. Other important risk factor is for example an exposure to harmful substances such as chromium, silica, radon. In addition, there are reports about the genetic predisposition in causing the lung cancer [2]. Although the etiology of the lung cancer is well recognized, recently we can find papers about oncogenic viruses, as an important factor in the development of this cancer. Human papillomavirus (HPV) belongs primarily to these viruses, but also JCV virus, BKV, SV40 and cytomegalovirus (HCMV) [8, 16, 18, 31].

Human papillomavirus (HPV) belongs to the *Papillomaviridae* family, with more than 100 genotypes [4, 13]. There are some genera in this family where *Alpha*-, *Beta*-and *Gammapapillomavirus* are the most important, which include strains replicate in human epithelial cells [7]. Taxonomy in this case is based primarily on the basis of *L1* gene sequence encoding a larger subunit of the capsid. Here we distinguish taxa, such as 'types', 'subtypes' and 'variants' that are different in the sequence 10%, 2-10% and %, respectively [4].

Corresponding author
* Department of Virology, Medical University of Lublin,
1 Chodzki Str., 20-093 Lublin, Poland
e-mail: lukasz.podsiadlo@op.pl

HPV is responsible for the formation of benign and malignant skin lesions, leading to the development of tumors [7, 26]. The benign lesions include warts or genital warts, caused primarily by the types of low oncogenic potential (6, 11), while the malignant changes include for example cervical cancer, where are involved types of high oncogenic potential (16 and 18) [1, 11, 32].

Epstein-Barr virus belongs to the *Herpesviridae* family, *Lymphocryptovirus* genus and is also known as human herpesvirus 4 (HHV-4). This is one of the most common viruses in humans [3]. This virus has the ability to replicate and enter a latency phase in B cells, which can lead to a development of various types of cancer [9]. So far, two types of EBV (EBV-1 and EBV-2) have been distinguished, with the significant differences in the *EBNA-2*, *EBNA-3A*, *EBNA-3B* and *EBNA-3C* genes sequences [14, 25, 29]. Type 1 is found primarily in Europe, North and South America and Asia, while type 2 is dominant mainly in Africa [25]. EBNA-2 protein plays a key role in the initiation of carcinogenesis by disrupting mitotic checkpoints and causing chromosomal instability [24]. EBV can cause infectious mononucleosis, and participates in the development of cancers such as Burkitt's and Hodgkin's lymphoma, gastric and nasopharyngeal carcinoma (NPC) [9, 25, 29].

The dynamic development of molecular biology techniques and bioinformatics has led to a revolution in our knowledge of biological evolution and the relationships between living organisms. Now we have laboratory techniques and computer software that allow us to reconstruct the evolution process in a more reliable way. For this purpose, we use the phylogenetic trees construction methods, which

can be divided into two basic groups: distance-based methods and character-based methods. The first group is characterized by a fast rate of tree building, whereas in the second group we have more reliably phylogeny reconstruction [19]. Distance-based methods include UPGMA and neighbor joining method (NJ) [5, 15, 19, 27]. Character-based methods, on the other hand include the maximum parsimony (MP), maximum likelihood (ML) and Bayesian inference method [17, 20, 22]. The maximum likelihood method has a high value of the credibility logarithm. A number of computer programs analyzing different models of molecular evolution of DNA with their parameters. In order to choose the appropriate model of molecular evolution, the most often used method is hLRTs (hierarchical Likelihood-Ratio Tests) and AIC (Akaike Information Criterion) [17, 20].

The aim of this study was the detection and genotyping of HPV and EBV in fragments of fresh tissue obtained from patients with the lung cancer.

MATERIALS AND METHODS

Biologic material

The material consisted of 33 fresh tumor tissue fragments taken from patients with the lung cancer (Table 1.) hospitalized in the Department of Pulmonary Diseases of Autonomous Public University Hospital No. 4 in Lublin in 2011. The analyzed samples were taken during the bronchoscopy procedure.

Table 1. Patients characteristics

		n (%)
Age	Median (years)	64
Gender	Male	29 (88)
	Female	4 (12)
Histological type	Small cell lung carcinoma (SCLC)	7 (21)
	Non-small cell lung carcinoma (NSCLC)	26 (79)

Sequence numbers of reference strains

In the construction of phylogenetic trees were used *L1* gene sequences of HPV reference strains taken from the public database GenBank (http://www.ncbi.nlm.nih.gov/genbank/). Selected strains of viruses belong to the genus *Alphapapillomavirus* and *Betapapillomavirus*. Sequence numbers are summarized in Table 2.

Table 2. Sequence numbers of reference strains

Strain	Number
Alphapapillomavirus	
HPV 10	X74465.1
HPV 16	K02718.1
HPV 18	X05015.1
HPV 31	J04353.1
HPV 33	M12732.1
Betapapillomavirus	
HPV 5	M17463.1
HPV 9	X74464.1
HPV 49	X74480.1
HPV120	GQ845442.1

Isolation of DNA

Fragments of fresh tissue obtained from patients with the lung cancer were cut and then subjected to isolation of genetic material using the QIAamp DNA Mini Kit (QIAGEN) according to manufacturer's instructions.

Polymerase Chain Reaction

Amplification of the HPV *L1* gene

For the genotyping of HPV, amplification was carried out on the gene encoding the L1 protein, a component of the capsid. The primers used in the study are summarized in Table 3. Concentrations of the PCR reaction components was prepared in accordance with Winder et al. [30] Amplification was performed under the following conditions: initial denaturation 95°C 5 min., followed by 40 cycles: 95°C 1 min., 55°C 1 min., 72°C 1 min.; final extension: 72°C for 10 min. The reaction mixture was subjected to electrophoresis on 3% agarose gel, and then the products were purified by using Gel-Out kit (A&A Biotechnology) for further analysis.

Amplification of the EBV *EBNA-2* gene

In the case of EBV, nested PCR was carried out on the *EBNA-2* gene, encoding a latent protein involved in carcinogenesis. Primers are listed in Table 3. Concentrations of the PCR reaction components were prepared in accordance with Peh et al. [25]. Activation of HotStarTaq polymerase was carried out at 95°C for 15 min. The first reaction in an amount of 35 cycles was performed under the following conditions: 94°C 1 min., 55°C 1 min., 72°C 2 min. with a final extension at 72°C for 5 min. Nested PCR reaction was performed in 30 cycles with an annealing temperature of 60°C. PCR products were separated on 3% agarose gel and purified using Gel-Out kit for further analysis. The products have different length depending on the virus type: 368 bp (EBV-1) and 473 bp (EBV-2).

Table 3. Primers used in the PCR

Primers	5'>3' sequence	Product
HPV		
MY09	CGT CCM ARR GGA WAC TGA TC	450 bp
MY11	GCM CAG GGW CAT AAY AAT GG	
EBV		
Out primers		
A1	TTT CAC CAA TAC ATG ACC C	
A2	TGG CAA AGT GCT GAG AGC AA	
In primers		
B1	CAA TAC ATG AAC CRG AGT CC	368 bp (type 1)
B2	AAG TGC TGA GAG CAA GGC MC	473 bp (type 2)

Genotyping of HPV and EBV

Genotyping of HPV and EBV was based on the *L1* and *EBNA-2* gene fragments sequences, respectively. Purified PCR products were sent to the Genomed Warsaw Company for sequencing. The results were analyzed using the computer programs: Chromas Lite 2.0.0.0, ClustalX 2.1.0.0, GeneDoc 2.7.0.0 and BioEdit 7.0.9.1. Typing was performed using the BLAST algorithm (Basic Local Alignment Search Tool; http://blast.ncbi.nlm.nih. gov/Blast.cgi).

Phylogenesis of HPV and EBV

The *L1* and *EBNA*-2 gene sequences were used in order to construct phylogenetic trees. Phylogeny was based on the maximum likelihood method (ML). This method requires the use of computer programs such as PAUP 4.0, ModelTest

3.7, PhyML 2.4.4 and MEGA 4.1. In order to choose the appropriate model of molecular evolution hLRTs (hierarchical Likelihood-Ratio Tests) and AIC (Akaike Information Criterion) tests were used. The reference strains sequences used in the study was taken from the public database GenBank (http://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

The molecular biology methods have allowed for the detection of human papillomavirus and Epstein-Barr virus in specimens from 33 patients with the lung cancer. HPV was detected in one sample (3%) and EBV was detected in 8 samples (24%).

The genotyping and construction of phylogenetic trees of tested viruses was based on the genes sequences. In the case of HPV we detected 120 type (5B, Fig. 3.), whereas in the case of EBV in all eight positive samples we detected type 1. In order to show the sequences alignment and percentage similarity were used the computer programs: GeneDoc 2.7.0.0 and BioEdit 7.0.9.1, respectively.

The phylogenetic trees were constructed using the maximum likelihood method (ML), which is shown in Figure 3. (HPV) and 4. (EBV). Type 120 belongs to the *Betapapillomavirus* genus, hence the reference strains sequences received from GenBank database (http://www.ncbi.nlm.nih.gov/genbank/) also belong to this genus. In addition, for rooting the tree, viruses sequences of the genus *Alphapapillomavirus* were also downloaded.

Table 4. Percentage similarity of *L1* gene sequences

Seq-	5B	HPV120	HPV5	HPV9	HPV49	HPV10	HPV16	HPV18	HPV31	HPV33
5B	ID	0,98	0.648	0.734	0.685	0.559	0.604	0.591	0.594	0.647
HPV120	0.98	ID	0.65	0.741	0.685	0.564	0.609	0.591	0.606	0.66
HPV5	0.648	0.65	ID	0.689	0.684	0.567	0.556	0.56	0.55	0.597
HPV9	0.734	0.741	0.689	ID	0.698	0.586	0.582	0.613	0.606	0.633
HPV49	0.685	0.685	0.684	0.698	ID	0.587	0.58	0.585	0.595	0.607
HPV10	0.559	0.564	0.567	0.586	0.587	ID	0.65	0.715	0.679	0.657
HPV16	0.604	0.609	0.556	0.582	0.58	0.65	ID	0.665	0.744	0.736
HPV18	0.591	0.591	0.56	0.613	0.585	0.715	0.665	ID	0.66	0.697
HPV31	0.594	0.606	0.55	0.606	0.595	0.679	0.744	0.66	ID	0.782
HPV33	0.647	0.66	0.597	0.633	0.607	0.657	0.736	0.697	0.782	ID

Table 5. Percentage similarity of *EBNA-2* gene sequences

Seq-	4	5	10	15	16	23	24	33
4	ID	0.994	0.985	0.973	0.988	0.97	0.952	0.985
5	0.994	ID	0.979	0.967	0.988	0.97	0.952	0.985
10	0.985	0.979	ID	0.964	0.985	0.955	0.952	0.976
15	0.973	0.967	0.964	ID	0.967	0.979	0.967	0.958
16	0.988	0.988	0.985	0.967	ID	0.961	0.949	0.982
23	0.97	0.97	0.955	0.979	0.961	ID	0.982	0.964
24	0.952	0.952	0.952	0.967	0.949	0.982	ID	0.949
33	0.985	0.985	0.976	0.958	0.982	0.964	0.949	ID

EBV phylogenetic tree does not include reference strains because of their large differences in the sequence of *EBNA-2* gene. The genotyping in this case was conducted only using the BLAST algorithm (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

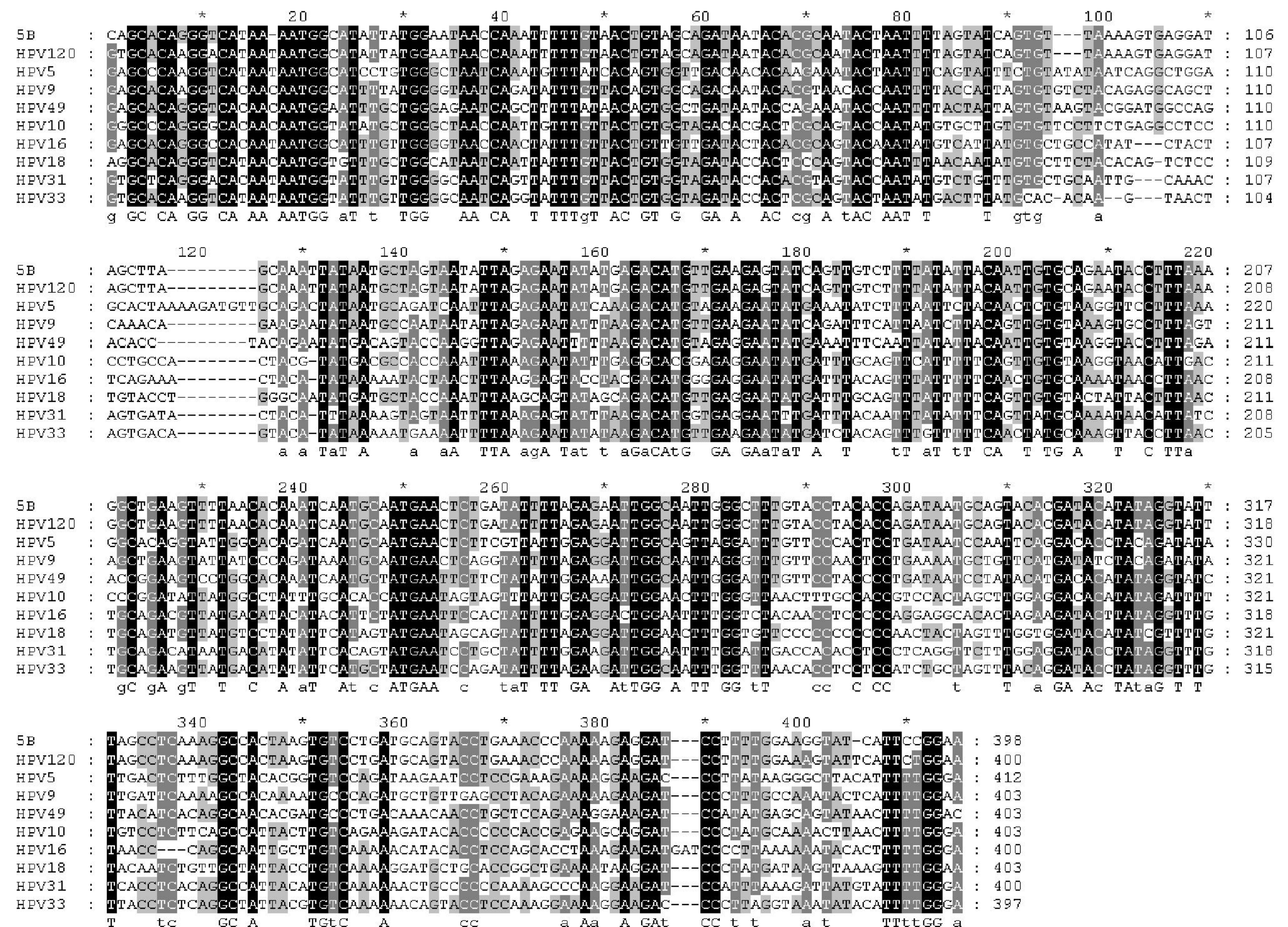


Fig. 1. Alignment of HPV *L1* gene sequences

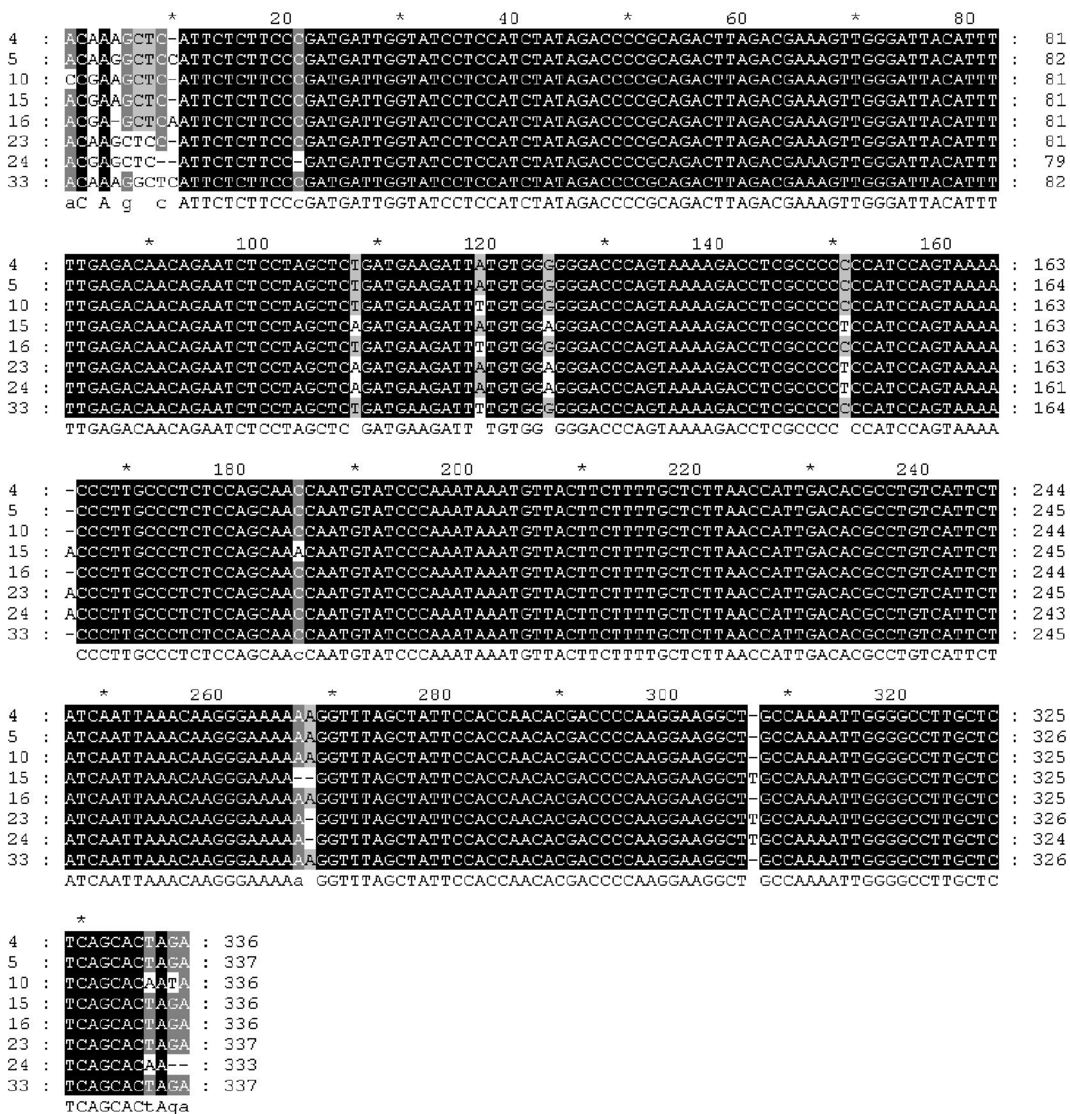


Fig. 2. Alignment of EBV *EBNA-2* gene sequences

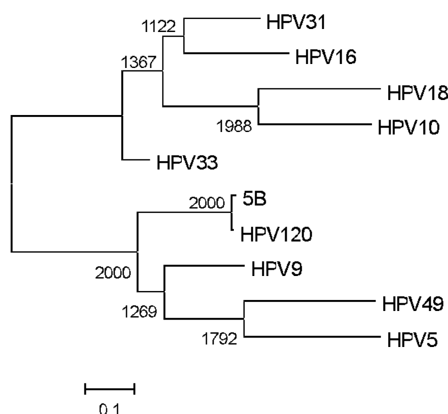


Fig. 3. Phylogenetic tree of HPV constructed based on the maximum likelihood method (ML)

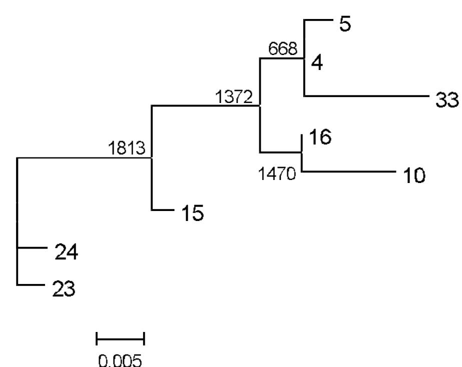


Fig. 4. Phylogenetic tree of EBV constructed based on the maximum likelihood method (ML)

DISCUSSION OF THE RESULTS

The lung cancer is one of the leading causes of death in Western countries. Recently, we can observe an increase in interest in the role of oncogenic viruses in the development of various types of cancer, which of course is reflected in the high amount of literature available on this subject. The most commonly studied viruses are primarily HPV, SV40, JCV, BKV, EBV, and HCMV [16]. Currently, HPV is considered the most important virus involved in the formation of tumors, and has a particular affinity for the squamous epithelium covering the skin and mucous membranes. Among this virus family there are the types of a low oncogenic potential (e.g. 6, 11, 42), a medium oncogenic potential (e.g. 31, 33, 35) and a high oncogenic potential (e.g. 16, 18) [4, 11, 32]. Syrjänen [28] first presented the hypothesis of the HPV contribution in the development of the lung cancer.

In the literature, the detection of HPV and EBV DNA in patients with the lung cancer ranges from 0% to 100%. The

results depend mainly on the laboratory techniques used, matched primers for PCR, as well as the multiplicity of tested samples [16]. The most commonly used method is PCR, nested PCR and *in situ* hybridization.

In this paper, in order to detect the genetic material of HPV and EBV in fragments of fresh tissue obtained from patients with the lung cancer we used the method of sequencing. Analysis was carried out on the *L1* and *EBNA-2* genes, which are used for genotyping of HPV and EBV, respectively. From 33 studied samples, one was HPV positive (3%). In other works the level of this virus detection was: 12.8% (78 analyzed samples) [16], 0% (110, 122 samples) [8, 23], 39.4% (109 samples) [31] and 36.9% (217 samples) [21]. In the case of EBV eight patients were positive (24%). Interestingly, in the other four works this virus was not detected despite of the large number of tested samples (110, 122, 130, 23 samples) [8, 10, 12, 23]. In these works, the authors used the method of PCR and *in situ* hybridization, suggesting a connection between the level of detection and a laboratory method used.

In the case of HPV we diagnosed genotype 120. This type has a tropism for a wide range of multi-layered epithelium in humans. HPV120 was found in the mouth, hair, eyebrows, channel of the penis, vulva and anal area [6]. The detection of EBV type 1 in our specimens is not surprising because it is the dominant genotype in this geographical region [14, 25].

CONCLUSIONS

The results suggest no effect of HPV on the development of the lung cancer. In this study HPV type 120 was detected, which had not yet been diagnosed in the lung cancer tissue. It might consider the relationship between EBV and the lung cancer, which requires further research.

REFERENCES

1. Aguayo F. et al.: Human papillomavirus-16 presence and physical status in lung carcinomas from Asia. *Infectious Agents and Cancer*, 5, 20, 2010.
2. Alberg AJ, Samet JM: Epidemiology of Lung Cancer. *Chest*, 123, 2003.
3. Alipov G et al.: Epstein-Barr virus-associated gastric carcinoma in Kazakhstan. *World J. Gastroenterol.*, 11, 1, 2005.
4. Bernard H.U. et al.: Classification of papillomaviruses (PVs) based on 189 PV types and proposal of taxonomic amendments. *Virology*, 401, 2010.
5. Bos D.H., Posada D.: Using models of nucleotide evolution to build phylogenetic trees. *Dev. Comp. Immunol.*, 29, 2005.
6. Bottalico D. et al.: Characterization of human papillomavirus type 120 (HPV120): a novel Betapapillomavirus with tropism for multiple anatomic niches. *J. Gen. Virol.*, 5, 2012.
7. Bravo I.G., de Sanjose S., Gottschling M.: The clinical importance of understanding the evolution of papillomaviruses. *Trends Microbiol.*, 18, 10, 2010.
8. Brouchet L. et al.: Detection of oncogenic virus genomes and gene products in lung carcinoma. *Brit. J. Cancer*, 92, 2005.
9. Chen M.R.: Epstein–Barr virus, the immune system, and associated diseases. *Frontiers in Microbiology*, 2, 5, 2011.
10. Chu P.G. et al.: Epstein–Barr virus plays no role in the tumorigenesis of small-cell carcinoma of the lung. *Mod. Pathol.*, 17, 2004.
11. Coissard C.J. et al.: Prevalence of human papillomaviruses in lung carcinomas: a study of 218 cases. *Modern Pathol.*, 18, 2005.
12. Conway E.J. et al.: Absence of evidence for an etiologic role for Epstein-Barr virus in neoplasms of the lung and pleura. *Mod. Pathol.*, 9, 5, 1996.
13. Cornut G. et al.: Polymorphism of the Capsid L1 Gene of Human Papillomavirus Types 31, 33, and 35. *J. Med. Virol.*, 82, 2010.
14. Correa R.M. et al.: Epstein Barr Virus Genotypes and LMP-1 Variants in HIV-Infected Patients. *J. Med. Virol.*, 79, 2007.
15. Criscuolo A., Gascuel O.: Fast NJ-like algorithms to deal with incomplete distance matrices. *BMC Bioinformatics*, 9, 2008.
16. Giuliani L. et al.: Detection of oncogenic viruses (SV40, BKV, JCV, HCMV, HPV) and p53 codon 72 polymorphism in lung carcinoma. *Lung Cancer*, 57, 2007.
17. Guindon S., Gascuel O.A.: Simple, Fast, and Accurate Algorithm to Estimate Large Phylogenies by Maximum Likelihood. *Syst. Biol.*, 52, 2003.
18. Haley S.A. et al.: Unique susceptibility of a human lung carcinoid tumor cell line to infection with BK virus. *Virus Res.*, 149, 2010.
19. Higgs P.G., Attwood T.K. (2004). Bioinformatics and Molecular Evolution. New Jersey: Wiley-Blackwell.
20. Holder M., Lewis P.O.: Phylogeny Estimation: Traditional and Bayesian Approaches. *Nat. Rev. Genet.*, 4, 2003.
21. Hsu N.Y. et al.: Association between expression of human papillomavirus 16/18 E6 oncoprotein and survival in patients with stage I non-small cell lung cancer. *Oncol. Rep.*, 21, 2009.
22. Kolaczkowski B., Thornton JW: Performance of maximum parsimony and likelihood phylogenetics when evolution is heterogeneous. *Nature,* 431, 2004.
23. Lim W.T. et al.: Assessment of human papillomavirus and Epstein-Barr virus in lung adenocarcinoma. *Oncol. Rep.*, 21, 2009.
24. Pan S.H. et al.: Epstein-Barr virus nuclear antigen 2 disrupts mitotic checkpoint and causes chromosomal instability. *Carcinogenesis*, 30, 2, 2009.
25. Peh S.C. et al.: Epstein-Barr virus (EBV) Subtypes and Variants in Malignant Tissue from Malaysian Patients. *J. Clin. Exp. Hematopathol.*, 43, 2, 2003.
26. Picconi M.A. et al.: Human Papillomavirus Type-16 Variants in Quechua Aboriginals from Argentina. *J. Med. Virol.*, 69, 2003.
27. Saitou N., Nei M.: The Neighbor-joining Method: A New Method for Reconstructing Phylogenetic Trees. *Mol. Biol. Evol.*, 4, 1987.
28. Syrjänen K.J.: Condylomatous changes in neoplastic bronchial epithelium. Report of a case. *Respiration*, 38, 5, 1979.
29. Tiwawech D. et al.: Association between EBNA2 and LMP1 subtypes of Epstein-Barr virus and nasopharyngeal carcinoma in Thais. *J. Clin. Virol.*, 42, 2008.
30. Winder D.M. et al.: Sensitive HPV detection in oropharyngeal cancers. *BMC Cancer*, 9, 440, 2009.
31. Yu Y. et al.: Correlation of HPV-16/18 infection of human papillomavirus with lung squamous cell carcinomas in Western China. *Oncol. Rep.*, 21, 2009.
32. Zafer E. et al.: Detection and Typing of Human Papillomavirus in Non-Small Cell Lung Cancer. *Respiration*, 71, 2004.