

Short Communication

Human papillomavirus detection in paraffin-embedded colorectal cancer tissues

Elisabetta Tanzi,¹ Silvia Bianchi,¹ Elena R. Frati,¹ Daniela Amicizia,² Marianna Martinelli,¹ Nicola L. Bragazzi,² Maria Pia Brisigotti,³ Daniela Colzani,¹ Ester Fasoli,¹ Gianguglielmo Zehender,⁴ Donatella Panatto² and Roberto Gasparini²

Correspondence

Elisabetta Tanzi
elisabetta.tanzi@unimi.it

¹Department of Biomedical Sciences for Health, University of Milan, Milan, Italy

²Department of Health Sciences, University of Genoa, Genoa, Italy

³Pathological Anatomy and Histology Unit, IRCCS AOU San Martino-IST, Genoa, Italy

⁴Department of Biomedical and Clinical Sciences 'L. Sacco', University of Milan, Milan, Italy

Human papillomavirus (HPV) has a well-recognized aetiological role in the development of cervical cancer and other anogenital tumours. Recently, an association between colorectal cancer and HPV infection has been suggested, although this is still controversial. This study aimed at detecting and characterizing HPV infection in 57 paired biopsies from colorectal cancers and adjacent intact tissues using a degenerate PCR approach. All amplified fragments were genotyped by means of sequencing. Overall, HPV prevalence was 12.3%. In particular, 15.8% of tumour tissues and 8.8% of non-cancerous tissue samples were HPV DNA-positive. Of these samples, 85.7% were genotyped successfully, with 41.7% of sequences identifying four genotypes of the HR (high oncogenic risk) clade Group 1; the remaining 58.3% of HPV-genotyped specimens had an unclassified β -HPV. Examining additional cases and analysing whole genomes will help to outline the significance of these findings.

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The aetiological role of human papillomavirus (HPV) in cervical and anogenital cancers is well recognized. Recent clinical and laboratory evidence has highlighted the association between HPV infection and the development of other malignancies, such as head and neck cancer, and skin tumours (HPV Information Centre, 2014; IARC, 2011). Papillomaviruses are small epitheliotropic viruses that infect all vertebrates, including humans. HPVs are grouped into five genera, *Alphapapillomavirus*, *Betapapillomavirus*, *Gammapapillomavirus*, *Mupapillomavirus* and *Nupapillomavirus*, with *Alphapapillomavirus* and *Betapapillomavirus* being associated primarily with benign and malignant lesions of the mucosal and cutaneous epithelia. The International Agency for Research on Cancer (IARC) classification includes 25 alphapapillomavirus genotypes in the HR (high oncogenic risk) clade due to their association with malignancy; however, insufficient evidence is available to exclude other genotypes from this risk (IARC, 2011).

Colorectal cancer is one of the most common tumours in the world (Jemal *et al.*, 2011), but its pathogenesis remains

poorly understood. Colorectal carcinogenesis is a multistep process that involves genetic instability, DNA repair defects, aberrant DNA methylation and mutational inactivation of tumour suppressor genes. Specifically, inactivation of the tumour suppressor protein p53 has been implicated in carcinogenesis (Kanthan *et al.*, 2012). As the HPV protein E6 efficiently inactivates p53, HPV has the potential to contribute to colorectal cancer development (Moody & Laimins, 2010). However, the association between HPV infection and colorectal cancer is still controversial (Damin *et al.*, 2013; Gornick *et al.*, 2010; Lorenzon *et al.*, 2011); several studies report a prevalence of HPV infection ranging from 0 to 84.2% (Damin *et al.*, 2013).

Here, we report the presence of HPV and the unexpected evidence of an unclassified β -HPV in paraffin-embedded colorectal cancer tissues collected for a wider ongoing study aimed at evaluating the role of HPV infection in the etiopathogenesis of colorectal cancer.

Between March 2011 and September 2012, 57 patients (26 women, 31 men; mean age 72 years; range 34–91 years) with colorectal adenocarcinoma histologically diagnosed at the IRCCS AOU San Martino-IST, Genoa, Italy, were biopsied. A total of 114 paraffin-embedded specimens, including 57 colorectal tumours and 57 paired samples of

The GenBank/EMBL/DDBJ accession numbers for the L1 sequences of the unclassified β -HPV are KJ680282–KJ680288.

One supplementary table is available with the online Supplementary Material.

adjacent intact tissue (non-cancerous colorectal tissue from an area located 15 cm proximally to the tumour) were analysed to detect HPV DNA. Specimens were collected and prepared in accordance with good laboratory practice in order to avoid the possibility of HPV contamination.

Ethics approval was obtained from the Ethics Committee of IRCCS San Martino Hospital, Genoa, Italy (19/2011).

Slices (10 µm thick) from the paraffin-embedded tissues were used to detect HPV DNA. Samples were pretreated to remove paraffin before DNA extraction. Briefly, one 10 µm section was dissolved in 800 µl lysis buffer (bioMérieux) and incubated for 10 min at 100 °C in a dry bath under agitation. The lysate was centrifuged at 14462 g for 5 min, and 400 µl of the lower phase (the cellular components) was recovered and stored at -20 °C until DNA extraction using an automated system for total nucleic acid extraction (NucliSENS[®] easyMAG[®]; bioMérieux) according to the manufacturer's instructions.

The paired samples of tumour and adjacent intact tissue were simultaneously subjected to extraction, together with a positive control (paraffin-embedded tissue from an HPV-16-positive cervical cancer) and a negative control (water). The concentration and purity of the extracted DNA were evaluated using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific). DNA integrity was assessed by amplification of a 268 bp fragment in the ubiquitous β-globin gene (Puranen *et al.*, 1996).

HPV DNA was detected using in-house nested PCR amplification of a 150 bp segment of the L1 ORF. Degenerate ELSI-f/ELSI-r (outer) and GP5+/GP6+ (inner) primer pairs were used for the first and second amplification steps, respectively. Each PCR run included negative (water) and positive controls (DNA extracted from Caski cells) (de Roda Husman *et al.*, 1995; Tanzi *et al.*, 2013).

To obtain 450 bp fragments suitable for genotyping, DNA extracted from HPV-positive samples was subjected to 40

cycles of amplification using the degenerate primer pair ELSI-f/ELSI-r (Tanzi *et al.*, 2013).

Amplicons obtained were purified with NucleoSpin[®] Extract II (Macherey-Nagel). L1 gene nucleotide sequences were aligned with the reference sequences in GenBank (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and Papillomavirus Episteme (PaVE; <http://pave.niaid.nih.gov/#home>) using the public bioinformatics program BLAST.

Furthermore, phylogenetic analysis was performed using the optimal substitution model HKY+G, selected by jModelTest (version 2.0) and three different approaches: a neighbour-joining (NJ) method implemented in the MEGA package (version 4.0), a maximum-likelihood method by PhyML (version 3.0) and a Bayesian method using MrBayes (version 3.2). Bootstrap analysis (number of replicates: $n=1000$ using NJ and $n=200$ using PhyML) was performed to test tree robustness. Posterior probabilities (PPs) were used for internal node significance on Bayesian trees.

Strict precautions were taken to avoid potential contamination at each step of the study, including specimen preparation and analytical processing.

The purity, quality and integrity of the extracted DNA were confirmed for all samples.

Overall, HPV DNA was detected in 12.3% [14/114; 95% confidence interval (CI) 7.2–19.3] of the samples analysed: specifically, in 15.8% (9/57; 95% CI 8.5–27.4) of tumour tissues and 8.8% (5/57; 95% CI 3.3–18.4) of non-cancerous tissue samples (Table 1). HPV DNA was never detected in both types of sample from the same patient.

Twelve out of 14 (85.7%) HPV DNA-positive samples were genotyped successfully by sequencing and analysed using BLAST. Five (41.7%) of the sequences showed four genotypes of the HR clade Group 1: HPV-16 ($N=1$), HPV-18 ($N=1$), HPV-33 ($N=1$) and HPV-58 ($N=2$) (Table 1).

Table 1. HPV DNA detection and genotyping in tumour (T) and adjacent intact non-cancerous tissues (P), broken down by anatomical site

Anatomical site	T-P pairs [N (%)]	HPV DNA-positive [N (%)]/T;P [n (%)]	HPV genotypes in tumour tissue (N)	HPV genotypes in adjacent intact tissue (N)
Colon*	82 (71.9) (41 pairs)	11 (13.4) 7;4 (17.1;9.8)	HPV-16 (1), HPV-33 (1), unclassified β-HPV (4), untypable HPV (1)	HPV-58 (1), unclassified β-HPV (2), untypable HPV (1)
Rectum	20 (17.6) (10 pairs)	2 (10) 2;0 (20;0)	HPV-58 (1), unclassified β-HPV (1)	
Sigmoid rectum	12 (10.5) (six pairs)	1 (8.3) 0;1 (0;16.7)		HPV-18 (1)
Total	114 (100) (57 pairs)	14 (12.3) 9;5 (15.8;8.8)	HPV-16 (1), HPV-33 (1), HPV-58 (1), unclassified β-HPV (5), untypable HPV (1)	HPV-18 (1), HPV-58 (1), unclassified β-HPV (2), untypable HPV (1)

*Proximal colon (caecum, ascending, transverse) and distal colon (descending, sigmoid).

Notably, the L1 fragment of the remaining seven sequences (58.3%) showed a high similarity with both the clone DL369 (de Villiers *et al.*, 1999) and Italy/1552*/2011 (La Rosa *et al.*, 2013) sequences (similarity range 86–99%), matching an unclassified genotype of the genus *Betapapillomavirus*. Furthermore, a similarity range of 72–75% with β -HPV-107 was obtained by using PaVE.

To better characterize these seven unclassified isolates, we also performed a phylogenetic analysis with 43 *Betapapillomavirus* reference sequences in GenBank and PaVE, and clone DL369 and Italy/1552*/2011 to reconstruct three phylogenetic trees (Fig. 1, Table S1, available in the online Supplementary Material). The unclassified HPV did not cluster with the genotype HPV-107, but formed a highly

significant clade with clone DL369 and Italy/1552*/2011 in all three phylogenetic analyses, supported by bootstrap values of 99 (NJ) and 87.5% (PhyML), and PP=1 (Bayesian tree).

Identifying the involvement of a viral infection in colorectal tumours could improve our understanding of tumour pathogenesis, widen diagnostic capabilities and identify prevention strategies.

We found HPV infection in 24.6% (14/57) of patients with colorectal cancer. In particular, considering the patients broken down by anatomical site of the tumour (cancerous tissue together with adjacent intact tissue), the highest frequency of HPV positivity was detected in the colon (11/

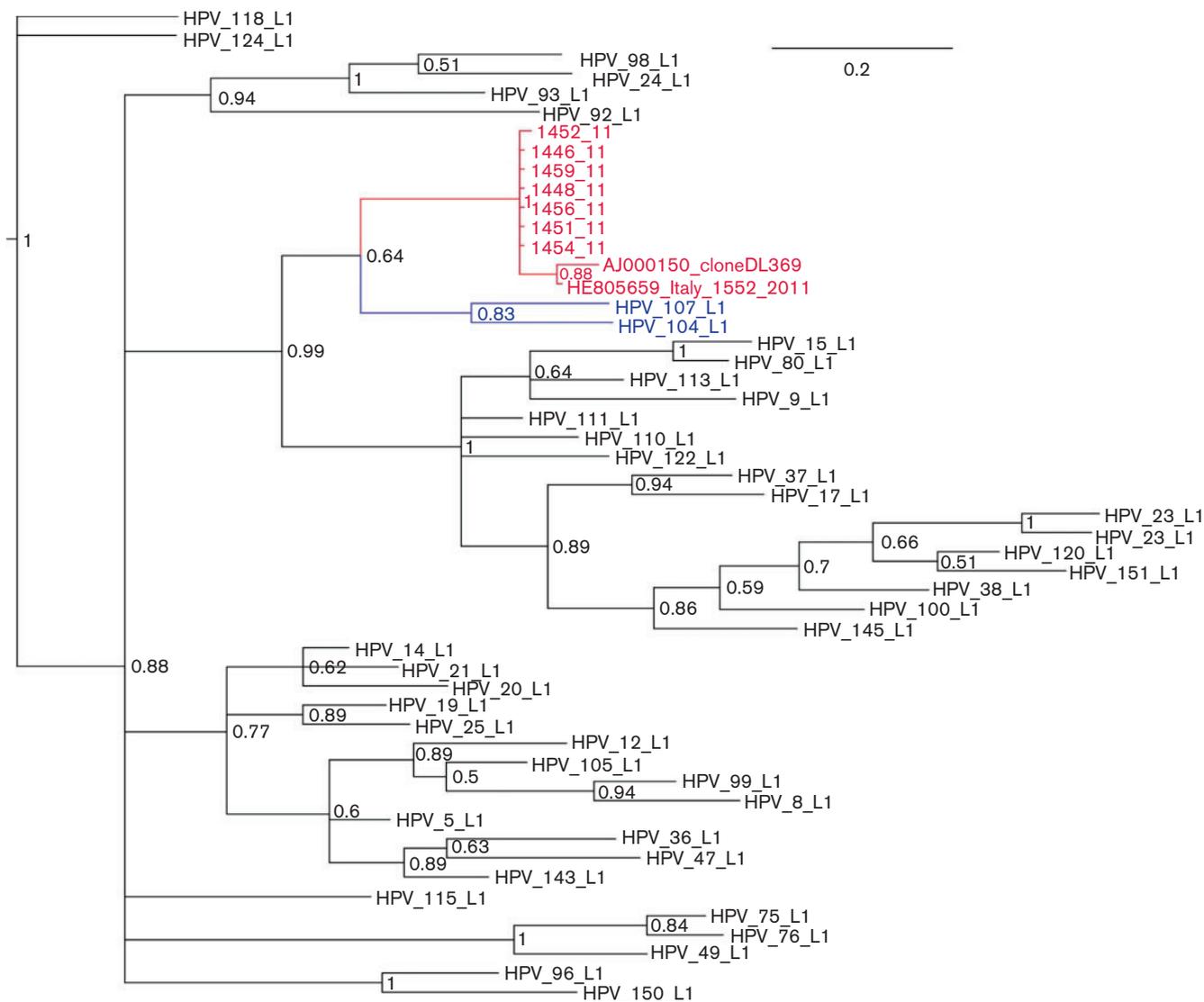


Fig. 1. Bayesian phylogenetic tree depicting the relationships of seven unclassified L1 gene sequences with several reference sequences of β -HPV genotypes. Numbers on the internal nodes indicate PPs.

41, 26.8%). Among the identified sequences, 41.7% belonged to genotypes of the HR clade Group 1, which is classified as carcinogenic to humans. The remaining 58.3% fell into the genus *Betapapillomavirus* and showed a similarity range of 72–75% with the L1 fragment of β -HPV-107. According to the guidelines for papillomavirus nomenclature (<http://www.ictvonline.org/>) and the classification proposed by de Villiers *et al.* (2004), sequences showing a nucleotide similarity of 89–98% throughout the L1 ORF fit within the same genotype. Thus, the β -HPV sequences found in our samples cannot be classified as HPV-107. The close evolutionary relationship with the Italy/1552*/2011 sequence, which is found in urban wastewater in Italy (La Rosa *et al.*, 2013), could support the hypothesis that the spread of HPV in urban wastewater is associated with faecal contamination and the presence of the virus in the human intestine.

The detection of β -HPV was made possible by the use of a degenerate primer pair, designed on the high conserved L1 gene, potentially able to amplify HPV genotypes included in other genera, other than the genus *Alphapapillomavirus*.

Our study is limited by the short fragment (450 bp) of the HPV genome we analysed. Thus, we merely described the identification of sequences of a new unclassified β -HPV genotype. In order to classify our sequences as a new β -HPV genotype, sequencing of the entire genome is necessary. However, it should be noted that we used paraffin-embedded tissues. DNA degradation occurs frequently with this type of sample preservation, resulting in poor amplification of large genomic fragments.

To delineate the significance of our findings, in-depth molecular and phylogenetic analyses will be performed on fresh tissue samples collected from a wider selection of cases from patients with or without colorectal cancer.

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