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COORDINATORE Prof. Cuneo Antonio

Association between colorectal carcinoma and the oncogenic polyomavirus JCPyV

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Dottoranda
Dott.ssa Pietrobon Silvia

Tutore
Prof.ssa Martini Fernanda

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ABBREVIATIONS

Ab	Antibody
ABTS	2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid
ATCC	American type culture collection
BKPyV	BK Polyomavirus
BSA	Bovine serum albumin
CRC	Colorectal carcinoma
DAPI	4',6-diamidino-2-phenylindole
DNA	Deoxyribonucleic acid
E.L.I.S.A.	Enzyme linked immunoadsorbent assay
EB	Elution buffer
FITC	Fluorescein isothiocyanate
H.A.	Hemagglutination assay
H.I.A.	Hemagglutination inhibition assay
H.U.	Haemagglutinating units
HCT-116	Human colon cancer cell line
HM	Healthy mucosa
HNPCC	Hereditary nonpolyposis colon cancer
hNPS	Human peptide
HPyV	Polyomaviruses
HPyV10	Malawi Polyomavirus/Human Polyomavirus 10
HPyV12	Human Polyomavirus 12
HPyV13	Human Polyomavirus 13
HPyV6	Human Polyomavirus 6
HPyV7	Human Polyomavirus 7
HPyV9	Human Polyomavirus 9
HS	Healthy subject
IgG	Immunoglobulin G
JCPyV	JC Polyomavirus
KIPyV	Karolinska institute Polyomavirus
MCPyV	Merkel cell Polyomavirus
MNF116	Monoclonal mouse anti-human cytokeratin
mRNA	Messenger RNA

NAC	Not amplify control
NTC	Not template control
OD	Optical density
P	P-value
p53	Tumor suppressor protein 53
PBS	Phosphate-buffered saline
PML	Progressive multifocal leukoencephalopathy
pRb	Retinoblastoma protein
RNA	Ribonucleic acid
RPA	Replication protein A
RT-qPCR	Quantitative real time - polymerase chain reaction
SS	Salmon sperm
STLPyV	Saint Louis Polyomavirus
Tag	Large T antigen
tag	Small t antigen
TSPyV	Trichodysplasia spinulosa-associated Polyomavirus
VLPs	Virus-like particles
VP1 K	Viral protein 1 K mimotope
VP1 N	Viral protein 1 N mimotope
VP1	Viral protein 1
VP2	Viral protein 2
VP3	Viral protein 3
WUPyV	Washington University Polyomavirus

ABSTRACT

Colorectal carcinoma (CRC) can be sporadic, familial or inherited. The sporadic form represents about 70% of CRC cases. DNA alterations detected in CRC somatic cells are due to several factors. Different cancerogenic agents are involved in the CRC onset/progression, including tumor viruses, such as herpes-, adeno-, pox-, papilloma-, hepatitis-, retro- and polyoma-viruses. Among the oncogenic Polyomaviruses, JC polyomavirus (JCPyV) was found to be associated with CRC cases.
JCPyV virus is a polyomavirus identified in 1971 as the causative agent of progressive multifocal leukoencephalopathy (PML). In recent years, several studies reported on the association between JCPyV and different human cancers, mainly brain and colorectal tumors. However, confliting data were published. Indeed, several investigations found the association between colorectal cancer and JCPyV, whereas other studies reported negative results.
On this ground, in this study I investigated the association between CRC and JCPyV with new technical approaches. To this purpose, JCPyV DNA sequences were investigated (i) in CRC and the adjacent healthy mucosa (HM) biopsies; (ii) in primary cell cultures derived from CRC and HM; (iii) in serum samples from CRC patients and controls, represented by healthy subjects (HS) with the same median age and gender of the patients. Then, (iv) the prevalence of JCPyV-antibodies was investigated in serum samples of the two cohorts, CRC and HS.
JCPyV sequences were detected in 22/53, 41.5%, of CRC biopsies and in 11/53, 21%, of HM tissues, being the different prevalence statistically significant. It is interesting to note that 11 CRC samples tested JCPyV-positive, whereas the matched HM sample were found to be JCPyV-negative. Furthermore, JCPyV sequences are more prevalent in the cecum and in the splenic flexure samples, both in tumor and normal mucosa biopsies. This data suggests an association between CRC and JCPyV with a particular focus in these two anatomical districts.
It is known that B- and T-lymphocytes represent vehicles for JCPyV to reach different tissues of the host. To verify if JCPyV-positive CRC tumors are due to the presence of the virus in the cancer cells, or to the lymphocytes infiltrating the tumor *in vivo*, primary cultures derived from CRC cells were set up. Indeed, B- and T-lymphocytes do not grow *in vitro* during the cell culture passages, thus allowing the transformed epithelial cells to multiply in monolayers. Primary CRC cell cultures (n=13) from the biopsies were set up and characterized. Among these 13 randomly chosen biopsies, 4/13 (31%) CRC tested JCPyV-positive. The 4 cell samples found JCPyV-positive and the 9 other cell cultures tested JCPyV-negative had the same results when analyzed as biopsies. This result suggests that the presence of JCPyV sequences in human colorectal cancer biopsy is not due to the lymphocytes infiltrating the tumor.

In recent studies, circulating JCPyV DNA sequences were detected in human sera. However, in my investigation among 53 CRC and 89 HM sera only one sample, from CRC patient, tested JCPyV-positive.
Subsequently, the prevalence of serum IgG antibodies against JCPyV was comparatively investigated in CRC tumor patients and HS controls. Mimotopes from JCPyV structural peptides were tested to investigate for specific reactions to human sera antibodies. An indirect ELISA with synthetic peptides from JCPyV viral capsid protein 1 (VP1) was set up and employed to test 53 CRC serum samples and 89 healthy subject. Data from immunologic assays indicate that the overall prevalence of JCPyV-VP1 antibodies in CRC patients is 26%, whereas in HS is 51%. As a control of the data obtained by indirect E.L.I.S.A., hemagglutination inhibition assay (H.I.A.) was used. Data from H.I.A. overlapped the results obtained by the indirect ELISA with synthetic peptides. In conclusion, the new indirect ELISA is reliable, faster, sensitive, specific and with affordable costs.
This investigation found an association between colorectal cancer and JCPyV infection. Indeed, JCPyV sequences were detected in CRC samples with a higher prevalence than that revealed in HM of the same patients. Interestingly, the majority of sera from CRC patients, tested JCPyV-positive, did not react with JCPyV VP antigens. It is possible that these CRC patients, as oncologic patients, were at least partially immunodepressed or non-responders and therefore they were unable to neutralize the JCPyV infection and consequently its oncogenic ativity. This data are innovative in this field and they may represent a starting point to investigate further the putative role of JCPyV in CRC onset/progression and its mechanism of transformation.

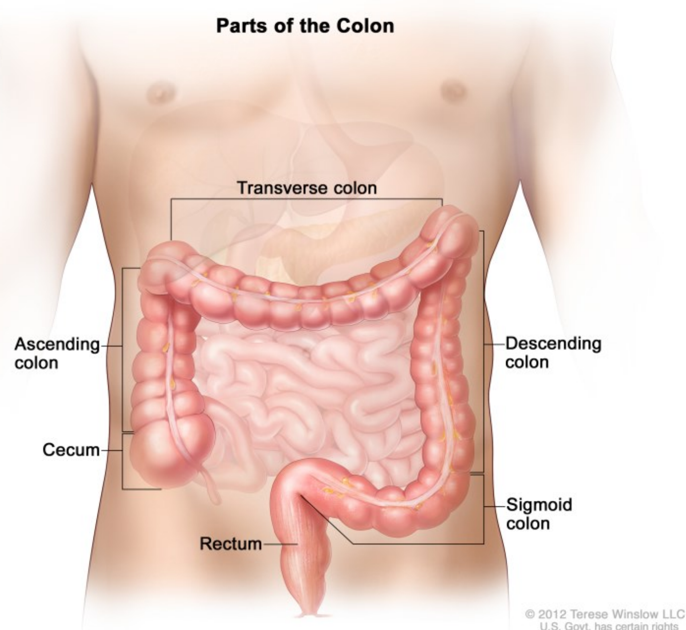
INTRODUCTION

COLORECTAL CARCINOMA

Colorectal anatomy and histology

The colon is part of the digestive system. The digestive system removes and processes nutrients (vitamins, minerals, carbohydrates, fats, proteins, and water) from foods and helps pass waste material out of the body. The digestive system is made up of the esophagus, stomach, and the small and large intestines. The colon (large bowel) is the first part of the large intestine and is about 1.5 meters. Together with the rectum and anal canal make up the last part of the large intestine, which are about 15-20 centimeters. The anal canal ends at the anus (Bethesda 2002). The large intestine includes the cecum, colon (ascending colon or right colon, hepatic flexure, transverse colon, splenic flexure, descending colon or left colon, sigmoid colon or sigma), rectum and anus (figure 1).

Figure 1: Anatomy of the colon and rectum.



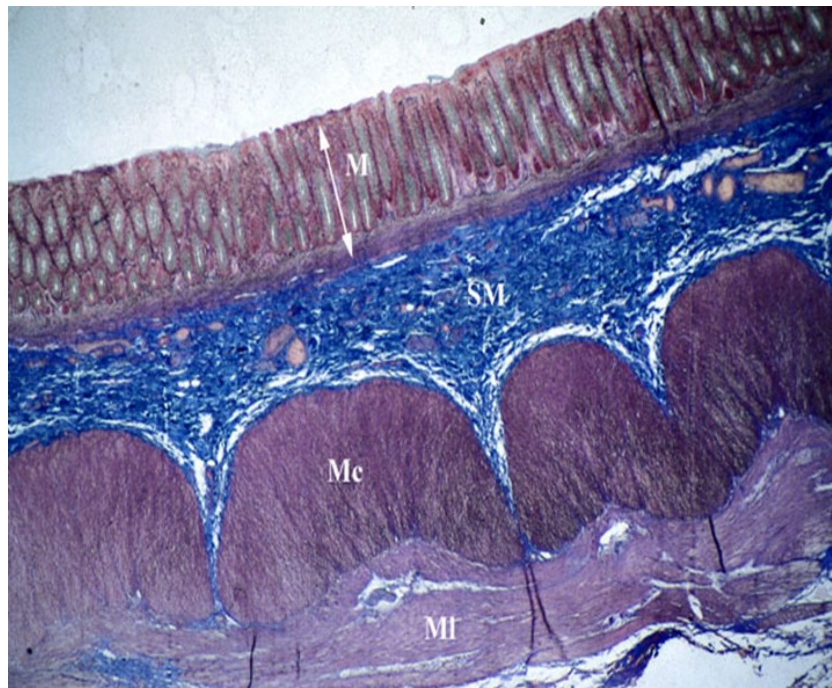
In the figure, they are shown the different sites of large intestine. The large intestine includes the cecum, colon (ascending, transvers, descending and sigmoid), rectum and anus.

The cecum and colon, histologically, not stand out. For this reason, they are considered together as a colon.

Histologically, we can distinguish four distinct layers, called tunic, present throughout the digestive tract: mucosa, submucosa, muscularis e serous (Figure 2); modification and specializations are in the individual segments.

Mucosa is a layer composed of epithelium, lamina propria, and muscularis mucosa. Epithelium is based on the lamina propria, a layer of loose connective tissue containing glands, lymphatic vessels and lymph nodes. Under the lamina propria there muscularis mucosae, a layer of smooth muscle cells arranged in a circular internally and externally in the longitudinal direction. The mucosa is surrounded by submucosa, a layer of dense irregular connective tissue-type fibroelastic. A thick muscle layer, the muscularis externa or muscular tunica surrounds submucosa. In the colon, this layer is composed of an inner circular layer and one outer longitudinal muscle layer is not continuous over the entire surface. In fact, it is divided into three cords of muscle bundles, the taenia coli. The muscularis externa is surrounded by a thin connective tissue layer that is coated by simple squamous epithelium of the visceral peritoneum (Gartner 2007).

Figure 2. Longitudinal section of human colon.



Longitudinal section of human colon. There are different tunic: mucosa (M); submucosa (SM); muscularis, circular (Mc) and longitudinal (Ml); and serous.

Colorectal carcinoma

Colorectal carcinoma (CRC) is a genetic disease of the somatic cells of this anatomical region. Humans (5%), without distinction between the genders, develop colorectal cancer. CRC is one of the most common cancers worldwide and the third leading cause of death in the United States (Siegel, Ma et al. 2014). In 2004 in Europe, there were an estimated 2,886,800 incident cases of cancer diagnosed. The most common incident form of cancer was lung cancer (13.3% of all incident cases), followed by colorectal cancer (13.2%) and breast cancer (13%). Lung cancer was also the most common cause of cancer death (341,800 deaths), followed by colorectal (203,700), stomach (137,900) and breast (129,900) (Boyle and Ferlay 2005). The high prevalence of this cancer is considered highly relevant for the public health.

Colon cancer can be sporadic, familial or inherited. Sporadic form, comprising about 70% of CRC cases and occurs mainly in people with an age over 50 years old. Alterations of the DNA of somatic cells are due to several factors, such as environmental carcinogens present in the diet, and genetic mutations and / or epigenetic alterations, which mostly accumulate with aging. Less than 10% of colon cancer have inherited origin. The inherited cases occur both in the absence and in the presence of colic polyposis. The cases are subdivided into familial adenomatous polyposis and hamartomatous polyposis syndromes. The nonpolyposis predominant syndromes include hereditary nonpolyposis colon cancer (HNPCC), or Lynch syndrome I, and the syndrome familial cancer or Lynch syndrome II. Syndrome Type I only affects the colon, whereas in type II involvement of the colon is always predominant, but in association with other cancers, such as cancer of the uterus, ovaries, hepatobiliary tract, pancreas, urinary tract. These syndromes although uncommon provide important explanations on the biology of all types of colorectal cancers. The third type understood pattern of colon cancer development is known as familial colon cancer. In affected families, colon cancer develops too frequently to be considered a sporadic cancer, but the observed cases are less than expected compared to the syndromic hereditary. Up to 25% of all cases of colon cancer should fall into this category (Calvert and Frucht 2002). Among the different oncogenic agents/factors involved in the colorectal cancer onset, i.e. physical, chemical and biological causes agents, oncogenic viruses are enclosed in the biological agents (Dalianis and Hirsch 2013). Indeed, several human viruses own oncogenic properties. Examples are the herpes-, adeno-, pox-, papilloma-, hepatitis-, retro- and polyoma-viruses (Hattori and Ushijima 2016).

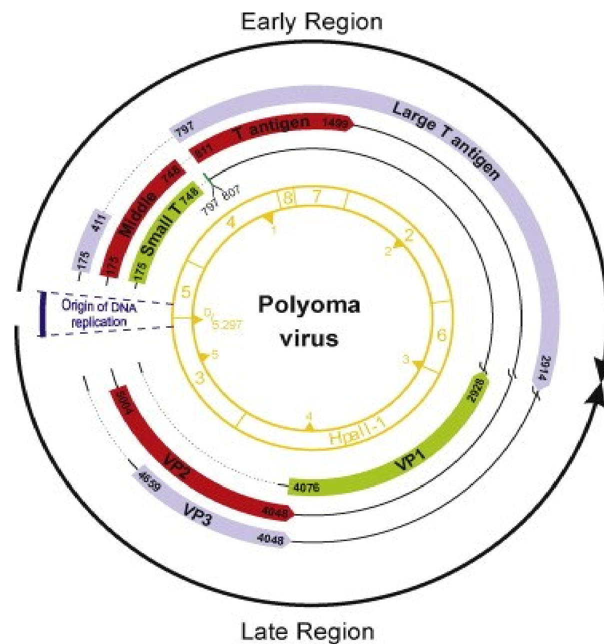
Among the oncogenic Polyomaviruses, JC polyomavirus (JCPyV) has been found to be associated with CRC cases.

POLYOMAVIRUSES

Genome

Polyomaviruses have a double-stranded DNA genome of about 5.2 kb, with regions characterized by an early, a late and non-coding sequences. The early region is characterized by sequences encoding the large T antigen (Tag) and small t antigen (tag), which are the viral oncoproteins. The late region contains the sequences encoding for the structural proteins named the viral capsid proteins (VP) VP1, VP2 and VP3; the synthesis of these proteins occurs through the production of a single messenger RNA (mRNA). The non-coding region contains the origin of replication, a promoter, and other sequences regulating the synthesis of early and late genes. The mechanism of DNA replication of Polyomavirus is bidirectional (Figure 3) (Van Ghelue, Khan et al. 2012) .

Figure 3. Polyomavirus genome.



The figure represents a circular map of the Polyomavirus. The nucleotide (nt) sequence and numbers refer to the about 5,000 nucleotide genome of Polyomavirus. Ori marks the origin of viral DNA replication (0/5243 nt). Polyomavirus early and late genes are transcribed in anti-clockwise and clockwise directions, respectively (black arrows); numbers indicate nt. The large T antigen (T) and small t antigen (t) are encoded by the early region, whereas VP1-2-3 are encoded by the late region.

The replication cycle

Polyomaviruses are characterized by oncogenic properties. Indeed, they are able to interact with the host genome determining alterations that may impact at the level of the cell cycle and cell proliferation.

This action occurs only in cells that appear to be permissive to the viral infection. Indeed, not all cells allow the virus multiplication exploiting the cellular systems of the host.

Once Polyomaviruses enter into permissive cells, the replication cycle occurs with two phases, the early and late, which are characterized by the protein synthesis. During the early phase, Tag and tag are expressed. It has been shown that the Tag is a multifunction protein with different polypeptide domains. Tag is able to interact with different cellular enzymes, including DNA polymerases, primase, topoisomerase I, the protein A of replication (Replication Protein A , RPA) and the protein 70 similar to those of heat shock protein family (Heat-Shock Cognate Protein 70, Hsc70). Tag is able to induce the cell to enter into the S phase of the cell cycle. In this way, Tag changes the normal cell cycle favoring the cell multiplication and the viral proliferation. Tag induces the expression of the late genes encoding VP1, VP2 and VP3, which are the structural viral proteins that will form the viral capsid. Through these mechanisms, the viral DNA replication and multiplication occur within the permissive host cells (Atkin, Griffin et al. 2009).

Polyomaviruses routes of infection

The routes of infection of the Polyomaviruses are both horizontal and vertical. Polyomavirus footprints were detected in different biological fluids, such as urine, feces, serum, etc. Thus, Polyomaviruses may spread through the sexual, blood, respiratory, urine and fecal-oral routes. Together with horizontal infections, i.e. human-to-human contacts, vertical polyomavirus transmission has been demonstrate by the presence of the virus in placenta samples of the mother and in the embryo/fetus specimens (Boldorini, Allegrini et al. 2010).

Oncogenic properties

The cells can be permissive or non-permissive to the virus infection. In the case of the occurrence of the Polyomaviruses infection in a non-permissive cell, the replication of the virus is not allowed and the infection is abortive with the expression of only Tag and tag proteins. These viral oncoproteins are able to determine alterations in cellular DNA and to bind the tumor suppressor gene products p53 and pRb family proteins (Dilworth 2002). Specifically, Tag/tag are characterized by transforming properties and are named viral oncoproteins. They can interact into the cell by different mechanisms inducing alterations and chromosomal aberrations, interfering with DNA repair systems and determining genomic instability.

It has been highlighted that the large T antigen is capable of binding family proteins of the tumor suppressor genes, such as tumor suppressor protein 53 (p53) and the retinoblastoma protein (pRb). Tag is also able to trans activate proto-oncogenes (c-myc , c-fos, c-jun) (Hollanderova, Raslova et al. 2003). Other factors, such as insulin-like growth factor 1 (Insulin-like Growth Factor-1, IGF-1), the platelet-derived growth factor α and β (Piastin-Derived Growth Factor α and β), VEGF and HGF, are activated through the interaction with large T antigen (Ali and DeCaprio 2001).

The alterations of the normal cell cycle operated by Tag/tag and the interactions of this oncoproteins with cell factors involved in the cell growth program, altogether can cause the cell proliferation and transformation.

Studies carried out in experimental animals, such as mice, rats, hamsters demonstrated the tumorigenic properties of Polyomaviruses. Indeed, these oncogenic viruses inoculated by different routes induce tumors of distinct isotypes. In these rodent tumor tissues, Polyomavirus DNA was found integrated into the cell genome.

Classification of Polyomaviruses

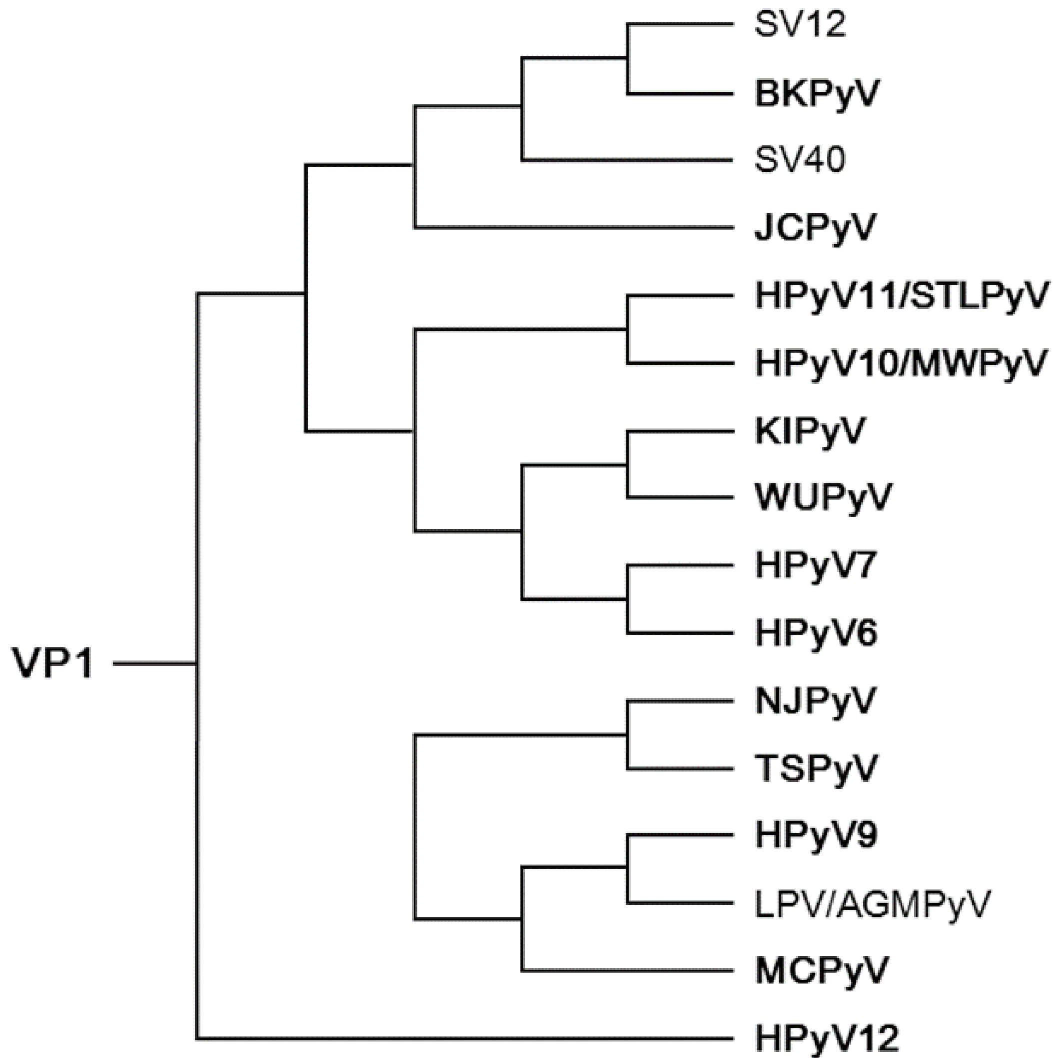
Until now, sixteen Polyomaviruses have been identified (HPyV 1-15). They can be divided into two groups according to primate hosts: human and monkey Polyomaviruses. Other Polyomaviruses infect different animal. Among human Polyomaviruses, 13 HPyV have been identified: JC (JCPyV) (Padgett, Walker et al. 1971), BK (BKPyV) (Gardner, Field et al. 1971), Washington University Polyomavirus (WUPyV), Karolinska Institute Polyomavirus (KIPyV) (Nguyen, Le et al. 2009), Merkel Cell Polyomavir (MCPyV) (Feng, Shuda et al. 2008), Human Polyomavirus 6 (HPyV6) and Human Polyomavirus 7 (HPyV7) (Schowalter, Pastrana et al. 2010), Trichodysplasia spinulosa-associated Polyomavirus (TSPyV) (van der Meijden, Janssens et al. 2010), Human Polyomavirus 9 (HPyV9) (Scuda, Hofmann et al. 2011), Malawi Polyomavirus/Human Polyomavirus 10 (HPyV10) (van der Meijden, Janssens et al. 2010), Saint Louis Polyomavirus (STLPyV) (Pastrana, Fitzgerald et al. 2013), Human Polyomavirus 12 (HPyV12) (Ehlers and Wieland 2013), Human Polyomavirus 13 (HPyV13) (Mishra, Pereira et al. 2014). Considering this trend, it is possible that in the future other Polyomaviruses will be discovered.

HPyV10 was identified in 2012, being its genome is different from other Polyomaviruses already identified. Therefore, it is difficult to insert it within existing this viral group. This virus was isolated in stool samples, but not in the blood or urine of immunosuppressed patients. At present it is not known if this Polyomavirus is associated with human diseases.

The Polyomaviruses (i) Simian Virus 40 (SV40) (Barbanti-Brodano, Sabbioni et al. 2004), (ii) Simian Virus Agent -12 (SA-12) and (iii) Lymphotropic Polyomavirus (LPyV) infect monkeys. The classification has undergone several changes over time, according to the discoveries of new Polyomaviruses, resulting in a readjustment of the classes.

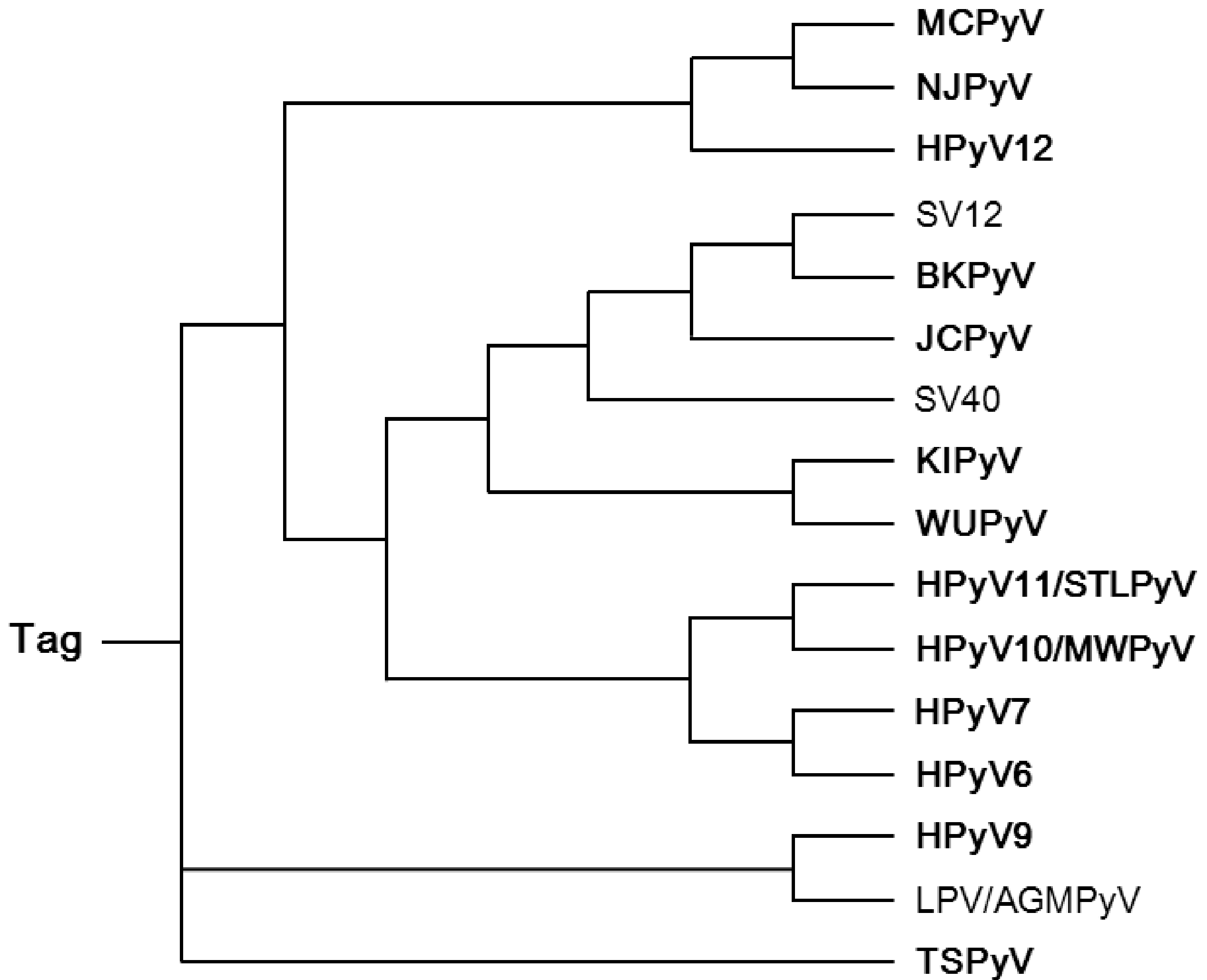
The different symbols with which they are identified derived from the initials of the patient in which they were identified or isolated. Examples are the JCPyV from the patient John Cunningham affected by progressive multifocal leukoencephalopathy (PML); from different institutions in which they have been identified as KIPyV is derived by the Swedish Karolinska Institute; or WUPyV for the Washington University; and in other cases the names are derived from the animal host, as in the case of SV40 (where SV stands for Simian Virus) or from cell lines in which the virus was found (LPV, Lymphotropic Polyomavirus).

Figure 4. Polyomavirus phylogenetic tree based on the VP1 a.a. sequences.



The similarity of VP1 sequences among different polyomaviruses is shown. Note that JCV (JCPyV, JC polyomavirus) VP1 is more closely related to those of SV40 (simian virus 40), SV12 (simian agent virus 12) and BKV (BKPyV, BK polyomavirus) than to the VP1 of other polyomaviruses: KIV (KIPyV, KI polyomavirus), WUV (WUPyV, WU polyomavirus), HPyV11/STLPyV (human polyomavirus 11), HPyV10 (human polyomavirus 10), HPyV7 (human polyomavirus 7), HPyV6 (human polyomavirus 6), HPyV9 (human polyomavirus 9), LPV/AGMPyV (B-lymphotropic polyomavirus), TSV (TSPyV, Trichodysplasia spinulosa-associated polyomavirus), MCPyV (Merkel cell polyomavirus), HPyV12 (human polyomavirus 12), and NJPyV (New Jersey polyomavirus, not shown)

Figure 5. Polyomavirus phylogenetic tree based on the Tag a.a. sequences.



The similarity of Tag sequences among different polyomaviruses is shown. Note that JCV (JCPyV, JC polyomavirus) Tag is more closely related to those of SV40 (simian virus 40), SV12 (simian agent virus 12) and BKV (BKPyV, BK polyomavirus) than to the Tag of other polyomaviruses: KIV (KIPyV, KI polyomavirus), WUV (WUPyV, WU polyomavirus), HPyV11/STLPyV (human polyomavirus 11), HPyV10 (human polyomavirus 10), HPyV7 (human polyomavirus 7), HPyV6 (human polyomavirus 6), HPyV9 (human polyomavirus 9), LPV/AGMPyV (B-lymphotropic polyomavirus), TSV (TSPyV, Trichodysplasia spinulosa-associated polyomavirus), MCPyV (Merkel cell polyomavirus), HPyV12 (human polyomavirus 12), and NJPyV (New Jersey polyomavirus, not shown)

Human Polyomaviruses

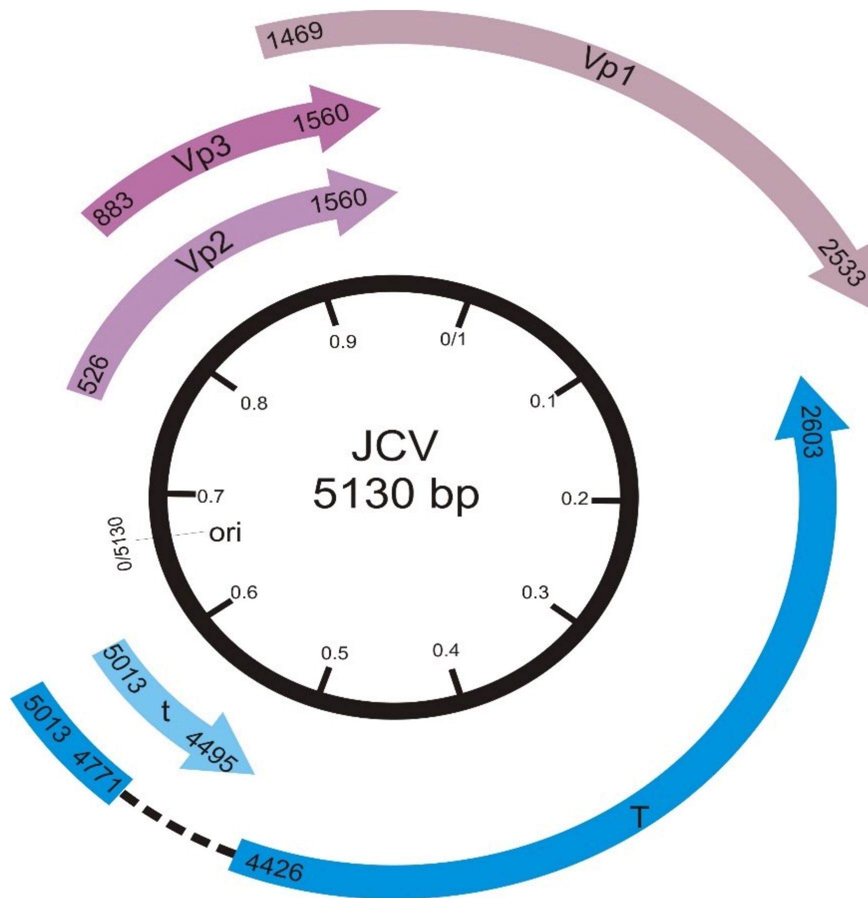
Human is the natural host for at least 13 Polyomaviruses, such as: BKPyV, JCPyV, KIPyV, WUPyV, MCPyV, HPyV6, HPyV7, TSV, HPyV9, HPyV10, STLPyV, HPyV12 and HPyV13 (NJPyV).
In order to assess the prevalence of Polyomaviruses, their serum antibody was investigated in the different age groups. Polyomaviruses are ubiquitous or wide spread in the human populations, being most of them detected since the childhood. From this result, it can be assumed that the first contact between the virus and the individual occurs in the early age. Subsequently the virus is inactivated entering a phase of latency. Then, it reactivates in a phase of immune deficiency. It is well established that in the population there are all Polyomaviruses, but with a different rate of infection. Immunohistochemistry studies carried out with antibodies against the major human Polyomaviruses provided a different seroprevalence in early childhood (1-3 years) and in young age (1-21 years): BKV (73%), JCV (21%), MCPyV (23-34%), KIV (56%) and WUV (54%) (Kean, Rao et al. 2009). Human polyomaviruses HPyV6, HPyV7, TSPyV, HPyV9, MWPyV, and KIPyV have been discovered between 2007 and 2012, in healthy blood donors. Serum samples were examined by enzyme-linked immunoassay (E.L.I.S.A.), using virus-like particles (VLPs) based on the major VP1 capsid proteins of these viruses. Overall, serum antibodies against HPyV6, HPyV7, TSPyV, HPyV9, MWPyV, and KIPyV were found in 88.2%, 65.7%, 63.2%, 31.6%, 84.4%, and 58%, respectively, of this population (Sroller, Hamsikova et al. 2015). The reactivation of the virus may be related to some pathological conditions, especially in immunosuppressed or immunocompromised individuals. Infections by Polyomavirus have been associated with different diseases. BKPyV infections have been associated with cystitis and kidney diseases, infections TSV to trichodisplasia spinulosa (hence the name), the reactivation of JCPyV has been associated with progressive multifocal leukoencephalopathy (PML), while MCPyV is associated with Merkel cell carcinoma. Considering these associations Polyomaviruses can be opportunistic, in particular condition of the host such as the immune deficiency. BKPyV and JCPyV, isolated in 1971, are the most common Polyomaviruses in different human populations, with a few exceptions represented by isolated populations in Brasil, Paraguay and Malaysia (Barbanti-Brodano, Sabbioni et al. 2006). It was observed that infected individuals develop a high titer antibody against these viruses, both for the late proteins (VP1, VP2 and VP3) or even early (Tag and tag).

JC Polyomavirus (JCPyV)

JC virus (JCPyV) is a polyomavirus identified in 1971 as the causative agent of progressive multifocal leukoencephalopathy (PML) in a patient with Hodgkin lymphoma (Padgett, Walker et al. 1971). JCPyV is a virus belonging to the group of Polyomavirus, characterized by a circular genome DNA of 5.13 Kb, usually identified in episomal state (Pinto and Dobson 2014). The JCPyV circular genome contain two regions of approximately equal size known as the early and late transcription unit (figure 6). Transcription of both units is initiated from a common non-transcribed regulatory region which contains the origin of DNA replication. Starting from this region, early transcription proceeds in a counterclockwise direction while late transcription proceeds clockwise on the opposite strand of the DNA. The JCPyV late region encodes the capsid structural proteins VP1, VP2 and VP3 that are encoded by alternatively spliced mRNAs derived from the same primary late transcript and a small regulatory protein known as agnoprotein. The JCPyV early region encodes the alternatively spliced transforming proteins large T-antigen and small t-antigen. These proteins are important in promoting transformation of cells in culture and oncogenesis *in vitro*. Unlike rodent polyomaviruses, primate polyomaviruses do not encode a middle T-antigen. However, in the case of JCPyV, three additional forms of T-antigen (T'_{135}, T'_{136}, T'_{165}) have been identified that are generated by alternative splicing (Trowbridge and Frisque 1995, Bollag, Prins et al. 2000, Prins and Frisque 2001, Frisque, Bollag et al. 2003).

It found an association between JCPyV and tumors of brain, liver and gastrointestinal tract (Blake, Pillay et al. 1992). The primary infection of JCPyV is asymptomatic, generally followed by a latent phase that persists long life in the host, until the virus does not reactivate. This usually occurs when the subject is immunocompromised, or during a phase of immunosuppressed. JCPyV infection rate may vary from 20 to 60% (Knowles, Pipkin et al. 2003, Stolt, Sasnauskas et al. 2003, Knowles 2006, Kean, Rao et al. 2009). The reactivation of JCPyV has been associated with progressive multifocal leukoencephalopathy (PML) (Padgett, Walker et al. 1971). Investigations carried out to assess the viral infection showed a high titer of antibodies against the large T antigen (Tag). It is known that this viral oncoprotein is capable to induce genome instability, chromosome aberration and gene mutations. These genotypic alterations can be detected in patients affected by colon cancer. It should be noted that a few antiviral compounds are available against JCPyV infections such as the interferons (Huang, Skolasky et al. 1998) and the nucleotide analog Cidofovir (De Clercq 2003).

Figure 6. Structural organization of the JCPyV genome.



Schematic representation of the JCPyV genome: showing the early and late regions separated by the non-coding regulatory region. In blue the early region (2.6 kb), coding for Tag and tag; in purple it is instead identified the late region (2.5 kb), coding for the structural proteins VP1, VP2 and VP3.

Association between colorectal carcinoma and JCPyV

JCPyV is able transform cells in culture, particularly cells of glial origin including human fetal glial cells and primary hamster brain cells.

In recent years, the role of specific oncogenic viruses in the onset of specific human tumors was investigated. Although JCPyV is ubiquitous in the human population, its characteristics indicate that this virus owns some oncogenic potentials. Several studies found JCPyV to be associated with different human cancers (Imperiale 2000), such as brain (Del Valle, Gordon et al. 2002, Noch, Sariyer et al. 2012) and colorectal tumors (Enam, Del Valle et al. 2002).

The etiological role of JCPyV in the onset/progression of human tumors is not completely understood. Some studies indicate that Tag, the viral oncoprotein, plays a critical role in malignant transformation through interaction with the tumor suppressor proteins, products of the p53 and pRb genes. Tag is a multifunctional protein with helicase and ATPase activities, which are necessary for DNA replication. It binds to and inactivates the p53 and pRb proteins, that control the cell cycle, and apoptosis. This coordinated action allows the replication and proliferation of cells with genetic aberrations. In addition, Tag can act on the control of cell proliferation by altering the Wnt signaling through the stabilization of β-catenin (Enam, Del Valle et al. 2002).

JCPyV Tag may induce colorectal cancer. Evidences are based on: (i) 90% of colorectal cancer tissues and their adjacent normal tissues contain sequences of DNA of JCPyV, but the number of viral copies is higher in tumors (ten times) than normal cells (Theodoropoulos, Panoussopoulos et al. 2005). (ii) the expression of viral proteins, including Tag and the agnoprotein , was detected in adenomas and invasive cancers, while absent in adjacent healthy tissue. These data suggest that JCPyV has a role in the early stages of the tumor development and progression (Laghi, Randolph et al. 1999). (iii) the gastrointestinal tract represents a tank for JCPyV, whereas its infection could be due to oral-fecal transmission during childhood. In fact, JCPyV was isolated in tissue obtained from biopsies of the esophagus, stomach, duodenum, colon and rectum (Theodoropoulos, Panoussopoulos et al. 2005). (iv) Tag, stabilizing the β-catenin, induces a super expression of genes that alter the cell cycle including c-MYC and cyclin D1 (Ripple, Parker Struckhoff et al. 2014).

Currently, it is believed that JCPyV, after the first infection, remains latent lifelong in the majority of individuals; then in elderly subjects because of the immune system impairment to due to the age, reactivated JCPyV may cause devastating diseases such as PML or fatal cancers. It has been suggested that JCPyV can play an important role in colorectal tumors during the multistep process of carcinogenesis, which foresee the onset of adenomatous polyps evolving in invasive cancers. If a

causal role for JCPyV in the onset of colorectal carcinoma will be demonstrated, up new strategies for the prevention of colorectal cancer such as the use of immunotherapy can be applied.
However, in the literature, there is a strong debate on the association between colorectal cancer and JCPyV. In fact, some studies have shown the association between CRC and JCPyV (Laghi, Randolph et al. 1999, Shadan, Cunningham et al. 2002, Goel, Li et al. 2006, Coelho, Gaspar et al. 2013), while other studies did not confirm this association (Newcomb, Bush et al. 2004, Lundstig, Stattin et al. 2007).
On this ground, in this study I investigated the association between CRC and JCPyV with new technical approaches.

AIMS

It is well established that JCPyV infects approximately the 60% of the human population worldwide. This high prevalence indicates that JCPyV is an ubiquitous virus. It has been demonstrated that in experimental conditions JCPyV induces cancers in animal models, whereas in tissue cultures transforms *in vitro* different cell types. Many studies associated JCPyV with human cancers, such as brain tumors of different isotypes and colorectal carcinomas (CRC). It should be noted that the association is not a prove of a JCPyV role in the onset/progression of human tumors.

My study was carried because in the scientific community there is not a general consensus on the association between CRC and JCPyV. At present, it is not clear if JCPyV has a role in the onset/progression of CRC. Some studies showed an association, while on the other hand other reports found no associations between CRC and JCPyV.

In my research, I investigated with novel approaches the association between the human colorectal carcinoma (CRC) and JCPyV. To this purpose, I analyzed the presence of JCPyV DNA sequences (i) in CRC and the adjacent healthy mucosa (HM) biopsies; (ii) in primary cell cultures derived from CRC; (iii) in serum samples from CRC patients and controls, represented by healthy subjects (HS) with the same median age and gender of the patients. Then, (iv) the prevalence of JCPyV-antibodies was investigated in serum samples of the same two cohorts, CRC and HS.

MATERIALS AND METHODS

Colorectal carcinoma and healthy mucosa biopsies

Patients affected by colorectal carcinomas (CRC) were admitted at the Department of Surgery, University Hospital of Ferrara, Italy. CRC patients (n=53) were included in this study, after informed written consent, according to standardized diagnostic criteria.

CRC biopsies were collected from 53 patients (n=33 male, n=20 female, age 39-89 years). CRC biopsies collects for this investigation were of high grade (III°-IV°). The histopathology of each specimen was analyzed in details to determine the percentage of necrotic, non-malignant and neoplastic cells. If necrotic cells were more of 80% specimens were not collected. CRC biopsies were derived from different anatomical sites of the colorectal tract. Specifically, they were collected from: cecum (5/53), ascending colon or right colon (23/53), hepatic flexure (1/53), transverse colon (2/53), splenic flexure (2/53), descending colon or left colon (2/53), sigmoid colon or sigma (9/53) and rectum (9/53).

From each patient were collected two CR biopsies, one from CRC and one from healthy mucosa (HM) adjacent to the carcinoma. From a fraction of each biopsies, primary cell cultures were derived and from another fraction total DNA were extracted.

Human primary cell cultures

To carry out the study of association between CRC and JCPyV was set up primary cell cultures derived from CRC and HM biopsies. These cell cultures were set up to analyze the JCPyV sequences in the isolated epithelial cells derived from the biopsies.

CRC tissues are composed by different cells, such as, fibroblasts, infiltrating lymphocytes and other blood cells, together with the epithelial cells. Cell cultures were employed because it is well established that B- and T-lymphocytes are vectors for JCPyV and they are responsible of the infection of other tissues of the host by JCPyV. To avoid that JCPyV-sequences detected in our experiments are simply due to the carry over effect of B- and T-lymphocytes JCPyV-positive infiltrating the tumor, epithelial cell cultures were set up to eliminate the other cells present in the original biopsies. Indeed, after few passages in cultures, epithelial cells are the only cells which may grow *in vitro*, thus enriching the cultures with epithelial cells and eliminating passage-by-passage the other cells. As control, the human colon cancer cell line HCT-116 was grown *in vitro* (Li, Wang et al. 2012). These cells were a kind gift from Prof. Luigi Ricciardiello, Dept. Gastroenterology, School of Medicine, University of Bologna.

CRC and the corresponding HM biopsies from patients were transferred immediately from the Surgery Department to the research laboratories, Unit of Cell Biology and Molecular Genetics, School of Medicine, University of Ferrara in sterile plastic disposable tubes with a specific transport medium. In particular, the collected tissues were placed in the transport medium, kept on ice and transported to the research laboratories for additional processes. The transferred medium avoids, during the time course, the degradation of the tissue and the growth of microorganisms in the sample. Specifically, in RPMI 1640 medium (Lonza, Biowhittaker, Belgium), without fetal bovine serum (FBS), was added the antibiotics Penicillin/Streptomycin (P/S) mixed to a final concentration of 500 U/ml, together with the Gentamicin at a final concentration of 100 µg/ml and the Amphotericin B to a final concentration of 12.5 µg/ml. Each tissue sample, in a final volume of approximately 10 ml of medium, was stored at +4°C for 15 minutes before setting up the primary cell cultures, as reported elsewhere (Failli, Consolini et al. 2009).

The transport medium was removed and then the biopsy was washed three times with the PBS solution containing P/S at a final concentration of 500 U/ml. Then, the biopsy was placed in a Petri dish of 3.3 cm diameter. The tissue was cut and minced to set up the primary cell culture. The tissue pieces were approximately of 3 mm of diameter. This process was done with a sterile disposable scalpel. A small volume, 4 ml, of RPMI 1640 medium without FBS, was added to the minced tissue

and transferred to the cell vessel of 25 cm^2 (T25), together with collagenase (2000 U/ml). Cells were incubated overnight (ON) at 37°C in a CO_2 (5%) incubator. After 24 hours, the collagenase was removed, while the medium/cells/tissues in suspension were centrifuged in a Falcon tube of 15 ml for 10 min at 2,000 rpm at room temperature. Then, the cell pellet, suspended in RPMI 1640 medium with 10% FBS, was placed in the same T25 cell culture vessel. After 48 hours, the cell medium was removed and replaced with the new one. During these two days of culture, cells formed colonies which appear at the light microscope observation as a small islets. The culture medium was changed twice a week, until adherent cells, grown in a T25 vessel, reached their confluence in approximately one month. This monolayer of epithelial cells, both from CRC and HM biopsies, were counted with a final amount of $4x10^6$ cells. Furthermore, in this study human colon cancer cell line (HCT-116) was enclosed in the experiments as control. This cell line was grown and then maintained in McCoy's 5A medium (Gibco, Life Technologies) with 10% FBS and 1% penicillin-streptomycin.

Immunofluorescence

Primary cell cultures from CRC and HM, together with the positive control represented by human colon cancer cell line (HCT-116), were analyzed by the immunofluorescence technique to detect the specific epithelial markers.

Briefly, the Cytokeratin, a marker of the epithelial cells, was analyzed with the primary antibody which forms the first immunocomplex reaction, then the immunocomplex is revealed with the second antibody labelled with fluorescein, followed by the visualization with the UV light microscope, (Nikon ECLIPSE TE2000-E).

The two immune reactions were carried out with the following antibodies: monoclonal Mouse Anti-Human Cytokeratin (Dako-MNF116) 1:50 diluted in PBS-BSA 3% was used as primary antibody; whereas the Goat-Anti-Mouse IgG (Fab-specific)-FITC (conjugate with fluorescein isothiocyanate), (Sigma) 1:50 diluted in PBS-BSA 3% was employed as secondary antibody.

The technique involves several steps. The first step is to clean the glass coverslip (slides) with ethanol and place them in a 6 well plate. Subsequently, cells were grown on flask and at confluence cells were removed by trypsin from the flask and they were collected with 5 ml of culture medium. Using a pipet, cell suspension was pulled down drop by drop on glass slides to form a hemisphere by surface tension and slides were incubate ON at 37°C with 5% CO_2.

Once cells were grown on the slide they were fixed with Acetone / Methanol (1:1). In the first step, three washes with PBS 1X at room temperature were performed for 5 min in order to remove the culture medium present on the slides. Subsequently, 1 ml of Acetone/Methanol (1:1) solution was added in each well for 7 min at -20°C. The fixative was removed and the slides were placed on paper for 30 minutes at room temperature, to dry out; the slides were kept at -20°C until the time of the analysis.

For the immunofluorescence a wet petri dish, called wet room, was set up. The slides were thaw at room temperature for 30 min. The primary antibody was diluted 1:50 in PBS- BSA 3%. For each slide a 100 µl drop of diluted primary antibody was prepared on parafilm inside the wet room. With the help of a scalpel, the slide was taken and turned upside down on the drop of primary antibody and incubated ON at 4°C.

With the help of a scalpel or tweezers each slide was left to laid in a well of a 4 well plate, and then three washes with 1X PBS were performed for 5 minutes at room temperature.

In the second step, the secondary antibody was diluted 1:50 in PBS- BSA 3%; for each slide a 100 µl drop of diluted secondary antibody was prepared on a new cleaned parafilm inside the wet room. With the help of a scalpel, the slide was taken and turned upside down on the drop of primary antibody

and incubated 1 hour at RT in the dark. At the end of the incubation time three washes were performed with 1X PBS for 5 minutes at room temperature in the dark.
The last step is the assembly of slide. The mounting medium used was DAPI solution: 21 µl glycerol, 9 µl PBS 1x, 1x 3 µl DAPI (4',6-diamidino-2-phenylindole). A drop of mounting medium was placed on a cleaned microscope slide and the immunofluorescence glass coverslip was turn over the drop on slide making a slight pressure. Finally the edges were sealed with wax or nail polish. Slides were maintained in the dark. Microscope observation/analysis was performed immediately or within 24 hours.

DNA extraction from biopsy

DNA was extracted from CRC and HM biopsies, using the QIAamp DNA Mini Kit (QIAGEN), following the manufacturer protocol. For each DNA extraction, two negative controls were used, represented by sterile H_2O and Salmon Sperm (SS) DNA. Tissue (25 mg) was cut into small pieces and placed in a 1.5 ml microcentrifuge tube together with 180 µl of Buffer ATL. Proteinase K (20 µl) were added, mixed by vortexing, and incubated at 56°C until the tissue was completely lysed.
Samples were mixed occasionally by vortex during incubation to disperse the sample. Buffer AL (200 µl) was added to the sample, mixed by vortexing, and incubated at 70°C for 10 min. Ethanol (200 µl, 96–100%) were added to the sample, and mixed by vortexing. Carefully the mixture (including the precipitate) was applied to the QIAamp Spin Column (in a 2 ml collection tube) without wetting the rim. The column was centrifuged at 8,000 rpm for 1 min.
The QIAamp Spin Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. Buffer AW1 (500 µl) was added to the QIAamp Spin Column without wetting the rim. The column was centrifuged at 8,000 rpm for 1 min. The wash was repeated with 500 µl Buffer AW2 without wetting the rim. The column was centrifuged at full speed (14,000 rpm) for 3 min.
For the elution of the DNA 200 µl Buffer AE were added. After incubation at room temperature for 1 min, the column was centrifuged at 8,000 rpm for 1 min. This step was repeated for the second DNA elution. To verify whether cross-contamination had occurred during the DNA extraction procedure, each sample was extracted simultaneously with a salmon sperm DNA specimen and a mock sample lacking DNA (distilled water).

DNA extraction from cells

DNA was extracted from CRC and HM human primary cell cultures, using the QIAamp DNA Mini Kit (QIAGEN), following the manufacturer protocol. For each DNA extraction, two negative controls were used, H_2O and Salmon Sperm (SS) DNA specimen.
Cell pellet was resuspend in PBS 1X to a final volume of 200 µl. Then 20 µl QIAGEN Protease or Proteinase K were added to it. Then 200 µl of Buffer AL were added the sample and mix by Vortex for 15 second. Samples were incubated at 56°C for 10 min.
To remove drops inside the cap, the 1.5 ml tube was centrifuged briefly. Ethanol (200 µl, 96-100%) was added, the sample was mix by vortexing for 15 seconds. After mixing, 1.5 ml microcentrifuge tube was centrifuged briefly to remove drops inside the cap.
The sample was placed in the QIAamp Spin Column (in 2 ml collection tube) and centrifuged at 8,000 rpm for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
The QIAamp Spin Column was opened with caution, with 500 µl of Buffer AW1 added without wetting the rim and centrifuged at 8,000 rpm for 1 min. The QIAamp Spin Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded.
The QIAamp Spin Column was opened with caution and 500 µl Buffer AW2 added without wetting the rim. The sample was centrifuged at maximum speed (14,000 rpm) for 3 minutes. The QIAamp Spin Column was placed in a clean 2 ml collection tube, and the tube containing the filtrate was discarded. Buffer AE or distilled water (200 µl) was added to QIAamp Spin Column and incubated at room temperature for 1 minute, then centrifuged at 8,000 rpm for 1 minute.

DNA extraction from serum

For DNA extraction from human serum ChargeSwitch® gDNA 1 ml Serum kit (Life Technologies) was used, following the manufacturer protocol. For each DNA extraction, two negative controls were used, sterile H_2O and Salmon Sperm (SS) DNA specimen.The optimal amount of human serum to be used to obtain a good yield is 1 ml. Before starting, the Lysis Mix was prepared. For each sample of human serum (800 µl), 560 µl of ChargeSwitch® Lysis Buffer were added, with 30 µl of proteinase K to prepare the Lysis Mix.The serum sample (800 µl) was put into a 2 ml microcentrifuge tube in presence of the previously prepared Lysis Mix (590 µl). Sample was mixed for 5 times with the pipette. The sample was incubated at room temperature for 20 minutes to lyse the sample.
Before the DNA binding procedure, the tube containing the magnetic beads (ChargeSwitch® Magnetic Beads) was vortexed to completely re-suspend and evenly distribute the beads in the storage buffer.
At the digested sample, ChargeSwitch® Purification Buffer (200 µl) was added with the addition of ChargeSwitch® Magnetic Beads (24 µl). The sample was mixed gently 5 times and incubated at room temperature for 2 minutes to allow the DNA to bind to magnetic beads.
The sample was placed in MagnaRack™ for 3 minutes. Without removing the tube from MagnaRack™, the supernatant was removed and discarded.
The tube containing the magnetic beads pellet was removed from MagnaRack™. They were added to 800 µl of ChargeSwitch® Wash Buffer and was mixed by gently pipetting up and down 5 times to resuspend the magnetic beads. The sample was placed in MagnaRack ™ for 2 minutes. Without removing the tube from MagnaRack™, the supernatant was removed and discarded. This procedure was repeated.
The tube containing the magnetic beads pellet was removed from MagnaRack™. They were added 50 µl of ChargeSwitch® Elution Buffer (or TE Buffer, pH 8.5) and was mixed by pipetting gently up and down 10 times to resuspend the magnetic beads. It was incubated at room temperature for 2 minutes.
The sample was put in MagnaRack ™ for 1 min. Subsequently, without removing the tube from the MagnaRack ™, it has been removed and the supernatant containing the DNA was placed into a sterile tube. Finally, the magnetic beads have been removed.
The purified DNA was quantified with the Spectrophotometer (NanoDrop, ThermoScientific) and then the extract was confirmed by electrophoretic run. Finally, the purified DNA was stored at -20°C until use for the RT-qPCR.

Quantitative PCR (Real Time PCR)

To investigate JCPyV sequences in biopsies, primary culture cells and serum of colorectal cancer patients, it was used the technique of absolute quantitative RealTime PCR (RT-qPCR), with TaqMan chemistry.

Standard curve was obtained through the use of pMITCR1A plasmid containing the complete JCPyV genome. Since pMITCR1A plasmid is composed by 9,491 bp, it was calculate that 10.02 ng contains 10^9 molecules of plasmid. The prep was quantified (47.5 ng/µl) with NanoDrop (ThermoScientific) and it was diluted to 10.02 ng/µl so that 1 µl contained 10^9 molecules.

To build the standard curve was prepared serial dilutions of the plasmid from 10^9 to 10^{-1} molecules/µl. For the RT-qPCR, an assay (ThermoScientific) containing primers and probe specific to the Tag DNA region was used. Forward primer sequences was 5'-AGTGTTGGGATCCTGTGTTTTCA-3' (JCV_AT_F); reverse primer sequences was 5'-GTGGGATGAAGACCTGTTTTGC-3' (JCV_AT_R); and probe sequences was 5'-FAM–CATCACTGGCAAACAT–NFQ-3' (JCV_AT_M).

For each experimental plate, 4 NTC (Not Template Control) in duplicate were inserted. In each well must be inserted: 9 µl of DNA, 10 µl of Master Mix and 1 µl of assay containing primers and probe. For the run, was used Bio-Rad CFX96 Touch, set as following : 10 minutes at 95°C (initial setup),15 seconds at 95°C (denaturation) and 1 minute at 60°C (annealing/extension). Denaturation, annealing/extension were repeated for 40 cycles.

Serum samples

To investigate the association between CRC and JCPyV sera, collected from CRC patients and healthy subjects (HS) employed as a control, were analyzed for the antibodies against the polyomavirus. Indeed, we envisioned that JCPyV-positive CRC sera may are more prevalent and with high titer in patients than in HS sera.

Specifically, collected era from patients with CRC (n=53) and HS (n=89) for a total of 142 samples were harvested by the Clinical Laboratory Analysis, University Hospital of Ferrara. Briefly, each CRC patient and HS underwent to a venous blood puncture in the left harm. Blood sample was collected in sample tubes "vacutainer" containing a gel that promotes the clot formation. The serum was obtained at room temperature after the clot was formed. Then, the sample was centrifuged at room temperature at 2,000 rpm for 10 minutes. Supernatant with a volume of approximately 500 ul-1,000 µl was collected each time. Serum aliquots were anonymously coded, marked and stored at -80° C until the time of the analysis.

Serum samples were analyzed by two immunological techniques, the indirect Enzyme-Linked Immunosorbent Assay (E.L.I.S.A.) and the Hemagglutination Inhibition Assay (H.I.A).

Synthetic peptides

Computer-assisted analyses enabled 2 specific JCPyV peptides (Figure 7A) to be selected from the late viral region by comparing viral capsid proteins 1(VP1) from JCPyV, with amino acids from BK (BKPyV) and SV40 polyomaviruses, which are highly homologous to JCPyV, as well as with other less homologous polyomaviruses (http://blast.ncbi.nlm.nih.gov). The aa sequences of the two peptides VP1 K and VP1 N are as follows: (a) VP1 K: NH_2 – KSISISDTFESDSPNRD – COOH (17 amino acids, aa 1646 – 1696) and (b)VP1 N: NH_2 – LMNVHSNGQATHDNGAGK - COOH (18 amino acids, aa 1820-1873). VP1 K and VP1 N mimotopes were selected because they reacted specifically in indirect E.L.I.S.A. testing (see below) with rabbit hyperimmune serum that had been experimentally immunized with JCPyV (positive control serum). BKV and SV40 hyperimmune sera did not react with VP1 K and VP1 N peptides (negative control sera). The amino acid residues of the 2 specific JCPyV peptides exhibit low homology with the BKV and SV40 VPs (Figure 7B). The human peptide hNPS, amino acid (aa) sequence NH_2 – SFRNGVGTGMKKTSFQRAKS – COOH (20 amino acids), was employed as a negative control peptide (Guerrini, Salvadori et al. 2010). The synthetic peptides were synthesized by standard procedures and purchased from UFPeptides s.r.l. (Ferrara, Italy).

Figure 7. Synthetic peptides.

A

Pep VP1 K	KSISISDTFESDSPNRD
Pep VP1 N	LMNVHSNGQATHDNGAG

B

Pep VP1 K		Similarity (%)	Pep VP1 N		Similarity (%)
VP1 K	KSISISDTFESDSPNRD		VP1N	LMNVHSNGQATHDNGAGK	
JCPyV	KSISISDTFESDSPNRD	100	JCPyV	LMNVHSNGQATHDNGAGK	100
BKPyV	QHLSAENAFESDSPDRK	47	SV40	MLNLHSGTQKTHENGAGK	61
LPV/AGMPyV	NSINTAFSKASDTPNKD	41	MCPyV	LINVHYWDMKRHDYGAGI	50
SV40	KSLAAEKQFTDDSPDKE	35	HPyV12	LMNVHDYMT--TDNGVGH	50
SV12	QRVTCDVTFENDAPQKK	29	SV12	MLNLHAEAQKVHDNGAGR	50
HPyV9	ADINVASSKASDNPNAT	29	BKPyV	MLNLHAGSQKVHENGGGK	44
KIPyV	SAIHDKESKVEETPDAD	18	TSPyV	LVNVHMATKRMDDKGIGF	39
HPyV12	EQVTVATARETDRPPKE	18	HPyV9	LVNLHQGGKYIGSSSGTI	22
HPyV10	DPITVT-----NTPGIN	12	HPyV10	LITLHTPGVADPATAPAL	22
NJPyV	EVIHHADGYD-NNLLSV	12	HPyV11/STLPyV	LISGHTPGTPPADRAPTN	22
MCPyV	YDLQP-KGSSPDQPIKE	12	NJPyV	LISVHLLEAKMNSDGPSR	22
TSPyV	EKVTVANSSDQDKPTSG	12	LPV/AGMPyV	LVNLHQGGKYIGSSSGCV	22
HPyV7	------SRPMSDTLGPG	12	HPyV7	MASSGY----QRANGTLA	11
HPyV11/STLPyV	DPVTVT-----ATPTAP	6	KIPyV	VNALG------NTNGVVH	11
HPyV6	------SRTFTDTLDAG	6	WUPyV	LNAGI------LTTGGVG	11
WUPyV	LKTAEA--NVTPDADTT	0	HPyV6	VGSAGY----IRAQGTPA	5

(A) Amino acid sequences of 2 different peptides of viral proteins 1 (VP1) of JC Polyomavirus (JCPyV). The peptides were selected on the basis of their low homology with the corresponding peptides from other human/simian polyomaviruses. Preliminary enzyme-linked immunosorbent assay indicated that only VP1 K and VP1 N peptides reacted with human serum antibodies without cross-reaction with the BKPyV and SV40 immune-sera employed as controls. (B) Similarity between synthetic peptides specific to JCPyV and other polyomavirus sequences: BKPyV (human polyomavirus BK), JCPyV (human polyomavirus JC), SV12 (simian virus 12), MCPyV (Merkel cell polyomavirus), TSPyV (Trichodysplasia spinulosa-associated polyomavirus), LPV (B-lymphotropic polyomavirus), KIPyV (human polyomavirus KI), WUPyV (human polyomavirus WU), HPyV 6 (human polyomavirus 6), HPyV 7 (human polyomavirus 7), and HPyV 9 (human Polyomavirus 9), HPyV10 (Malawi Polyomavirus/Human Polyomavirus 10), STLPyV (Saint Louis Polyomavirus), HPyV12 (Human Polyomavirus 12), NJPyV (New Jearsy Polyomavirus/Human Polyomavirus 13).

Indirect Enzyme Linked Immunosorbent Assay (E.L.I.S.A.)

An indirect E.L.I.S.A. (Enzyme Linked ImmunoSorbent Assay) was developed to detect specific antibodies (Ab) against JCPyV VP1 in human sera. E.L.I.S.A. plates were coated with synthetic peptides, which were employed as mimotopes, corresponding to JCPyV VP1 encoded by the early viral DNA region. Synthetic peptides, VP1 K (17 a.a.) and VP1 N (18 a.a.), were described above.

Peptide coating. 96 flat-bottom well plates (Nunc-immuno plate PolySorp, CelBio, Milan) were coated with 5 µg of the selected peptide in each well, diluted in 100 µl of Coating Buffer, pH 9.6 (Candor Bioscience, Weissensberg, Germany). The plates were incubated at 4°C for 16 hours, allowing the peptide to cover the bottom of each well completely. Plates were rinsed three times with Washing Buffer (Candor Bioscience, Germany) to remove unattached peptide, using a washing apparatus (Thermo Electron Corp., model Wellwash 4MK2, Finland).

Peptide blocking. Blocking was accomplished with 200 µl/well of Blocking Solution containing casein (Candor Bioscience, Germany) at 37°C for 90 min to allow well saturation. To eliminate residual blocking solution, the plates were rinsed three times with Washing Buffer using a washing apparatus.

Primary antibody. Test human serum samples were diluted 1:20 in Low Cross-Buffer pH 7.2 (Candor Bioscience, Germany) and were added to the plate. In each plate were included several controls (100 µl): positive control represented by immune rabbit serum containing anti-JCPyV Tag antibodies; negative controls represented by immune sera with anti-BKPyV and anti-SV40 antibodies and three human serum samples found to be JCPyV negative in previous investigations. The plate was incubated at 37°C for 90 min.

Secondary antibody. After 90 min of incubation, a triple rinsing cycle was repeated and then the secondary antibody solution was added to each well. The solution contained goat anti-human or goat anti-rabbit IgG heavy (H) and light (L) chain specific peroxidase-conjugate (Calbiochem-Merck, Germany) diluted 1:10,000 in Low Cross-Buffer. The reaction mixture was incubated at room temperature for 90 min.

Optical density reading. At the end of the incubation period, the plate was rinsed three times with Washing Buffer and then treated with 100 µl of 2,2'-azino-bis 3-ethylbenzthiazoline-6-sulfonic acid (ABTS) solution (Sigma-Aldrich, Milan) which reacted with the peroxidase enzyme to yield the color reaction. The plate was then read spectrophotometrically (Thermo Electron Corp., model Multiskan EX, Finland) at a wavelength (λ) of 405 nm. This optical density (OD) reading reflected the extent of immunocomplexes formed by the presence of specific antibodies which bound to the JCPyV synthetic peptide/epitopes/mimotopes.

The cut-off was determined for each experimental plate. For cut-off calculation, a tendency curve was drawn from a second–degree polynomial regression for VP1 K peptide and VP1 N peptide, as published for MCPyV and BKPyV virus-like particles (VLPs) (Touze, Gaitan et al. 2010). The three human serum samples used as negative controls, were selected because they were below the cut-off of previous experiments.

During the cut-off calculation, OD of these negative controls must be under the new cutoff.

At the end of the analysis, sample that was positive for both peptides (VP1 K and VP1 N) was considered JC-positive. The differences between the prevalence of anti-JCPyV antibodies in the sera of the different groups of subjects studied (CRC and HS) were analyzed statistically. Chi-square test was used to compare the prevalence of anti-JCPyV antibodies in sera of different cohorts of subjects. The comparison was statistically significant when p value was less than 0.05 ($P < 0.05$) All statistical analyzes were performed using the Prism software (GraphPad, San Diego, CA, USA).

Hemagglutination Assay (H.A.) and Hemagglutination Inhibition Assay (H.I.A.)

Hemagglutination Inhibition Assay (H.A.I.) is an immunoassay which need a solution of human red blood group 0 Rh+. For the experiments the solution of red blood cells has been provided by the transfusion center of the University-Hospital of Ferrara. The solution of red blood cells was subjected to 3 washes with PBS 1X in the following way: 1:1 dilution in PBS 1X, centrifugation at 1500 rpm for 8 minutes, elimination of the supernatant and storage of red blood cells pellet. which was subsequently again diluted 1:1 in PBS 1x to repeat washing. At the end of 3 washes you are obtained a precipitate/pellet containing only red blood cells, which then are diluted with PBS 1x to obtain a 10% solution, which is then stored at 4°C until the time of the experiment. The solution thus diluted can be used within a week. After this period begins the process of hemolysis, so it must obtain new red blood cells. In the case in which the day of the experiment is noted that the 10% solution showing little clear, you can perform a further washing before proceeding with the experiment.
To perform H.I.A., a viral stock is needed. JCPyV stock, used in our laboratory, was purchased by American Type Culture Collection (ATCC, VR-1583).
The viral titer was determined by Hemagglutination Assay (H.A.) and found to be 3,2 x 10^3 H.U. (Haemagglutinating Units), corresponding to 3,2 x 10^7 PFU/ml (infectious units in 1 ml). The virus is used as antigen in both the H.A. and H.I.A.
To carry out the H.A.I. is necessary to determine the viral titer; this is determined by the H.A. that exploits the ability haemagglutinating of the virus. In fact, the JC Polyomavirus has the ability to agglutinate human red blood cells of 0 Rh+ group and this is manifested by the formation of a grid of cells in the wells of the plates. Conversely, if the virus is not present the red blood cells they do not agglutinate and precipitate to the bottom of the well. To carry out this assay 96 well plates to round bottom have been used, in which it is possible to evaluate the possible formation of the grid cell due to the presence of the virus. The titration of JCPyV is based on serial 1:2 dilutions virus in PBS 1x, starting from the stock. Typically the dilutions used are 10 (from 1:10 to 1: 5120), performed in a scalar way directly into the wells of the plate. The next step is the addition of red blood cells diluted to 1% of the stock solution (10%) using a multichannel pipette. Finally, the plate is incubated at 4° C for 2-4 hours and at the end of this incubation will be possible to assess the effect (and therefore the power) haemagglutinating viruses concerned. For viral titer means the highest dilution at which the virus still has capacity haemagglutination, that is shows the formation of the grid of the cells. Over this dilution the capacity is not enough and it can be seen the formation of the precipitate of red blood cells on the bottom of the well.

The H.I.A. is based on the ability of JCPyV to agglutinate human red blood cells of 0 Rh+ group and the ability of the antibodies may be present in the sera to inhibit the action of the virus. This test can determine the presence of antibodies against the virus in serum samples examined. Before to the experiment, it is necessary to proceed with the removal of complement present in the serum, which could cause interactions in degrees of inhibiting the hemagglutination phenomenon, thus altering accordingly the results of the experiment.

To do this, serum is incubated in a water bath at 56° C for 30 minutes. Then aliquots of serum decomplemented are stored at -20° C until the time of testing.

The assay involves the use of round bottom plates, such as those for H.A., allowing the display of the grid cell or the precipitate of red blood cells.

The evaluation of the appearance of the well allows to discriminate the samples in which antibodies against JCPyV are present. Positive serum sample forms a precipitate of red blood cells because antibodies are able to inhibit haemagglutinating ability of the virus. Negative samples does not contains antibodies against the JC virus, and therefore it does not inhibit the haemagglutinating ability of the virus, thus allowing the formation of red cell nets. The assay includes several scale dilutions of serum. Three plates were employed in each experiment.

The first plate includes 1:2 serum dilution. The serum (30 µl) was diluted with 15 µl of sodium periodate ($NaIO_4$) 0,1 M and, after incubating at room temperature for 30 minutes, was added 15 µl of Glycerin 5% in PBS 1x.

The second plate includes four different serum dilutions (1:16 - 1:32 - 1:64 and 1:128.). For each sample, were prepared four wells. In the first well, were added 140 µl of PBS 1X and in the three subsequent wells were added 30 µl of PBS 1x. Serum sample (20 µl) was transferred from the first plate to the first well of the second plate (1:16 dilution). Subsequently, the serum sample (1:16 dilution) was diluted three times and were carried out 1:2 dilutions. Then, 30 µl of serum sample (1:16 dilution) were transferred from the first well to the second well of the same plate (1:32 dilution). This last step was repeated for the third (1:64 dilution) and fourth (dilution 1:128) well. At each step, the serum sample was mixed with the pipet.

The third plate shown the results of the examined samples. Diluted serum sample (25 µl) was transferred from the second plate to the third plate. The virus solution (25 µl) was added in each well, 8 H.U. in each well. The plate was incubated for 60 minutes at room temperature.

Red blood cells solution 1% diluted in PBS 1X (50 µl) was added in each well. The plate was incubated for 4 hours at 4°C.

Finally, the result was observed: positive serum sample forms a precipitate of red blood cells (bottom) because antibodies are able to inhibit haemagglutinating ability of the virus; negative samples does

not contains antibodies against the JC virus, and therefore it does not inhibit the haemagglutinating ability of the virus, thus allowing the formation of red cell nets. The experiments were repeated three times for each sample.

In the plates, was added two negative controls and one positive control, in duplicate. The negative controls, were represented by (i) $NaIO_4$ (0.1M) and Glycerin (5% in PBS 1X) solution (25 μl); (ii) PBS 1X solution (25 μl). The virus solution (50 μl) and the red blood cells (50 μl), were added into these wells, just like in the samples. PBS 1X (50 μl) with red blood solution (50 μl), without virus, represented the positive control. The controls were included in each plate and were served to check the correct performance and reliability test.

Statistical Analysis

It was made a statistical analysis to compare the prevalence of JCPyV DNA sequences in two different types of tissues and sera sample (CRC vs. HM biopsies and CRC vs HS sera). Specifically, both for the analysis of the sequences in the biopsy, in primary culture cells and in serum, it chose the test of Chi-square (χ^2). The confidence interval (CI) is 95% and then the p-value considered for the determination of statistical significance is $P < 0.05$.
It was made a statistical analysis to compare the prevalence of serum antibodies anti-JCPyV in the different cohort of subjects (CRC vs. HS sera). In this case it chose the test of Chi-square (χ^2). The serologic profile of serum antibody reactivity to JCPyV mimotopes was statistically analyzed using t test. The confidence interval (CI) is 95% and then the p-value considered for the determination of statistical significance is $P < 0.05$.
Instead, to control the comparability of the groups according to age and gender distribution, it was made the t-student test.
Analyses were performed using the Prism software 6 (GraphPad, San Diego, CA, USA).

RESULTS

Cohorts of colorectal carcinoma patients and healthy subjects, biopsy and serum samples

Patients affected by colorectal cancers (CRC) were admitted at the Department of Surgery, University Hospital of Ferrara. Their biopsies and blood samples were analyzed at the Department of Anatomical Pathology and Clinical Laboratory Analysis, University Hospital of Ferrara, respectively. Patients were enrolled in this study, in agreement with the standard criteria, after informed written consent. The study was approved by the County Ethical Committee.

Collected CRC (n=53) and HM (n=53) biopsies were from CRC patients (n=33 male, n=20 female), with an age in the range of 39-89 years old (table 1). All CRC patients enrolled in this study were affected by a tumor of high grade. Since the aim of our study is to analyze both at molecular and cellular levels CRC samples, tumor specimens with only a high prevalence of neoplastic cells were collected. To this purpose the histopathology analysis of CRC and HM specimens was carefully carried out to establish the extension of the tissue necrosis, the presence of normal tissue and of the neoplastic cells. Cancer and HM biopsies were collected from different anatomical sites of the colorectal district. Specifically, specimens were collected from cecum (5/53), ascending colon or right colon (23/53), hepatic flexure (1/53), transverse colon (2/53), splenic flexure (2/52), descending colon or left colon (2/53), sigmoid colon or sigma (9/53) and rectum (9/53).

As indicated above per each CRC case two biopsies were selected, one biopsy from the colorectal cancer and one biopsy from the normal mucosa adjacent to the tumor.

In addition, a small aliquot of serum was harvested for each CRC patient (n=53), as well as serum samples were collected from healthy subjects (HS, n=89) employed as controls, with the same median age of CRC patients, for a total of 142 sera (Table 2). Specifically, sera were collected from patients affected CRC localized in the district described above, i.e. cecum (5/53), ascending colon or right colon (23/53), hepatic flexure (1/53), transverse colon (2/53), splenic flexure (2/52), descending colon or left colon (2/53), sigmoid colon or sigma (9/53) and rectum (9/53).

Table 1. CRC and HM biopsy sample.

Biopsy Type	Number	Male (%)	Mean age ± SD (range)	Subtype	Number
§**CRC**	53	33 (62)	69 ± 12 (39-89)	Cecum	5
				Ascending or right colon	23
				Hepatic flexure	1
				Transverse colon	2
				Splenic flexure	2
				Descending or left colon	2
				Sigmoid colon or sigma	9
				Rectum	9
§§**HM**	53	33 (62)	69 ± 12 (39-89)	Cecum	5
				Ascending or right colon	23
				Hepatic flexure	1
				Transverse colon	2
				Splenic flexure	2
				Descending or left colon	2
				Sigmoid colon or sigma	9
				Rectum	9

§*CRC (colorectal carcinoma);* §§*HM (healthy mucosa adjacent of the tumor).*

Table 2. CRC patient and healthy subject serum.

Biopsy Type	Number	Male (%)	Mean age ± SD (range)	Subtype	Number
§**CRC**	53	33 (62)	69 ± 12 (39-89)	Cecum	5
				Ascending or right colon	23
				Hepatic flexure	1
				Transverse colon	2
				Splenic flexure	2
				Descending or left colon	2
				Sigmoid colon or sigma	9
				Rectum	9
§§**HS**	89	58 (65)	51 ± 11 (39-89)	Healthy subject	89

§*CRC (Colorectal Carcinoma);* §§*HS (Healthy Subject).*

Analysis of JCPyV DNA sequences in CRC and HM biopsy sample

To carry out the study of association between CRC and JCPyV sequences, the colorectal carcinoma biopsies (CRC) and healthy mucosa (HM) adjacent to cancer were tested by quantitative RealTime PCR (RT-qPCR) for the presence of JCPyV DNA sequences encoding for the large T antigen (Tag). DNA from CRC and HM tissue samples (n=53) were analyzed by a quantitative Real-Time PCR. In our experimental conditions, JCPyV sequences were detected in 22/53, 41.5% of CRC biopsies and in 11/53, 21% of HM tissues. Statistical analysis performed by chi square test indicates that the different prevalence of JCPyV sequences in CRC and HM is significant *(* $P < 0.05$)*. It is interesting to note that one DNA sample found to be JCPyV-positive among HM tissues, in its matched CRC sample from the same patient was instead detected JCPyV-negative. All other 10 matched CRC and HM samples were found JCPyV-positive. In addition the remaining 11 CRC samples were JCPyV-positive, whereas in its matched HM sample no JCPyV sequences were detected.

Biopsies of cancer, which I collected for this study, derived from the various districts of colorectal. As illustrated in table 3 distinct CRC types, among the 53 samples, tested JCPyV-positive with a different prevalence. Indeed, we found cecum 5/5, 100%; ascending colon or right colon 9/23, 35%, hepatic flexure 0/1, 0%, transverse colon 0/2, 0%, splenic flexure 2/2, 100%, descending colon or left colon 0/2, 100%, sigmoid colon or sigma 2/9, 22% and rectum 4/9, 44%. In the healthy mucosa biopsy, collected adjacent to cancer, the samples tested JCPyV-positive were: cecum 3/5, 60%; ascending colon or right colon 4/23, 17%; hepatic flexure 0/1; transverse colon 0/2; splenic flexure 2/2, 100%; descending colon or left colon 0/2; sigmoid colon or sigma 1/9, 11%; and rectum 1/9, 11%.

The RT-qPCR technical approach allowed us to quantify the amount of JCV DNA sequences in positive samples. It turned out that in our DNAs, without any difference between the CRC and HM DNA, the viral DNA copy numbers were very low, ranging from 10 to 100 copies/10^5 cells, being the cycle threshold values with a mean Ct = 36.

Table 3. JCPyV sequences in colorectal carcinoma and healthy mucosa biopsy.

Biopsy Type	Number	Male (%)	Mean age ± SD (range)	Subtype	JC-positive biopsy/biopsy Analyzed (%)
§**CRC**	53	33 (62)	69 ± 12 (39-89)	Cecum	5/5 (100)
				Ascending or right colon	9/23 (39)
				Hepatic flexure	0/1
				Transverse colon	0/2
				Splenic flexure	2/2 (100)
				Descending or left colon	0/2
				Sigmoid colon or sigma	2/9 (22)
				Rectum	4/9 (44)
TOT.				**CRC**	**22/53 (41.5) ***
§§**HM**	53	33 (62)	69 ± 12 (39-89)	Cecum	3/5 (60)
				Ascending or right colon	4/23 (17)
				Hepatic flexure	0/1
				Transverse colon	0/2
				Splenic flexure	2/2 (100)
				Descending or left colon	0/2
				Sigmoid colon or sigma	1/9 (11)
				Rectum	1/9 (11)
TOT.				**HM**	**11/53 (21)**

*§CRC (colorectal carcinoma biopsy); §§HM (healthy mucosa biopsy adjacent of the tumor). Statistical analysis was performed using the Chi-square test. The difference in prevalence of JCPyV sequences between CRC and HM was statistically significant (*P < 0.05).*

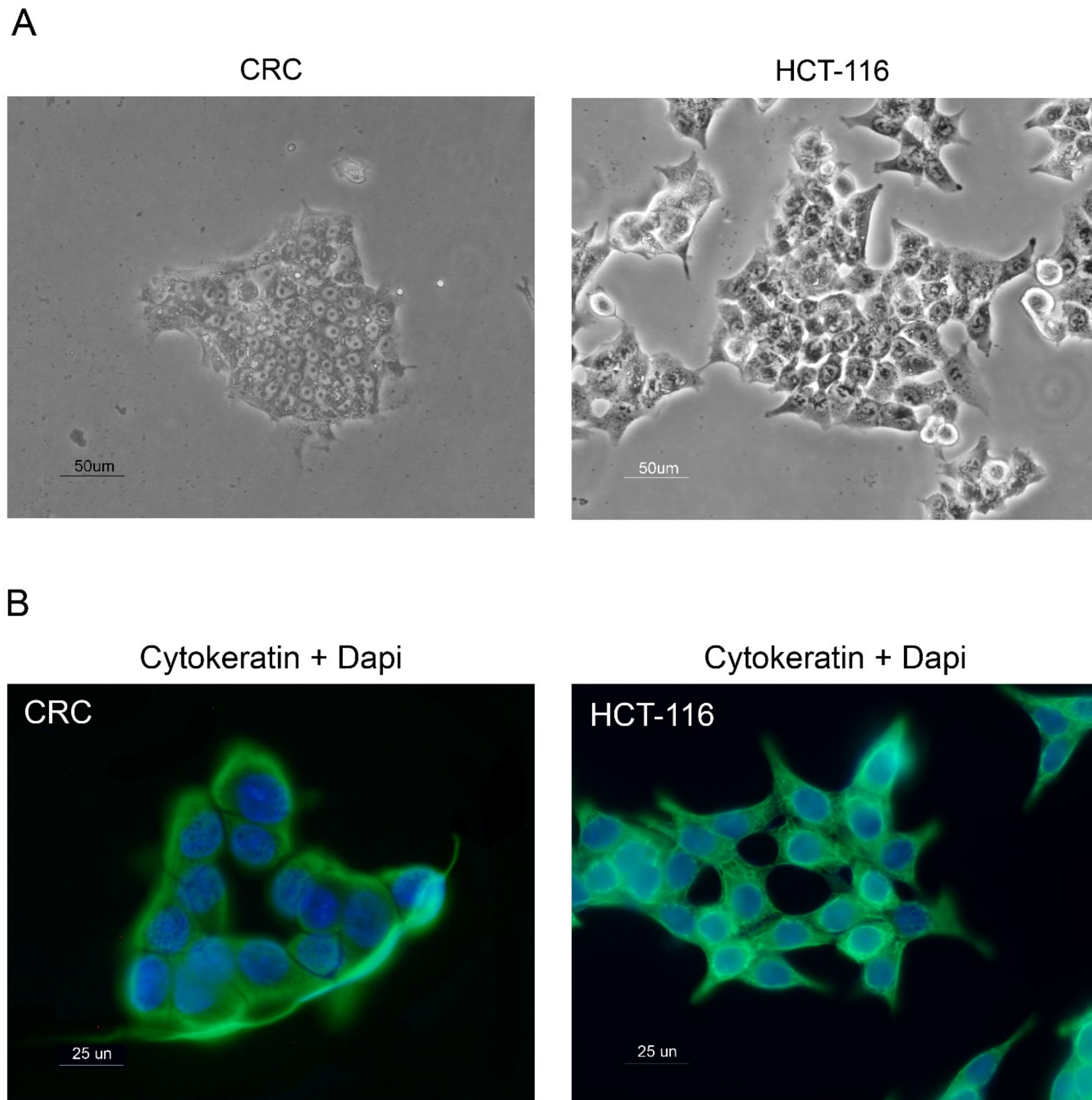
Set up and characterization of human primary colorectal carcinoma cells

To carry out the study of association between CRC and JCPyV has been necessary to set up the primary cultures derived from tumor biopsy. In particular, was set up 13 cell cultures derived from CRC biopsy. Instead, as from the literature, during the first week of culture, all cells freshly isolated from healthy mucosa failed to survive or appears a high contamination of fibroblasts.

The primary colorectal carcinoma cells, in the early phase, grew slowly and the culture was maintained for four weeks before reaching the confluent monolayer. Then, cells were split for the first time, passage 1. Only early passages (passage 1–2) of human primary CRC cells were used in all experiments in this study.

The morphology of CRC cells was first examined using a light phase-contrast microscope. During the first few days after plating, CRC cells proliferated and formed polarized epithelial islets. While in advanced growth (after seven days), CRC cells grew as multilayers and formed tumor-like cell clusters, similar to the morphology of colon cancer cell line HCT-116 (figure 8). The CRC cultures contained epithelial cells and a limited number of fibroblasts, as determined by immunofluorescence staining with anti-pan-cytokeratin.

Figure 8. Characterization of human primary colon cancer cells (CRC).



(A) CRC cells exhibit epithelial-like morphology as human colon cancer cell line HCT-116. During initial growth in culture, CRC cells formed small islets; while in late stage of growth they formed multilayer culture, similar to HCT-116 cells. Scale bar = 50 µm. (B) CRC cells react with cytokeratin as determined using an immunofluorescent staining assay. Nuclei (blue) were counterstained with DAPI. Scale bar = 25µm.

Presence of JCPyV DNA Tag sequences in human primary CRC cell cultures

Primary culture cells (n=13) derived of colorectal carcinoma (CRC) were set up. At the passage 2, when the cell cultures reached the confluence (1-2 x 10^5 cell) cells where harvested and pellet treated for the DNA extraction. Then, DNA form these CRC primary cell cultures, were RT-qPCR analyzed for the presence of JCPyV DNA sequences encoding the Tag oncoprotein. These quantitative RealTime PCR analyses indicated that 4 of 13 (31%) DNA from CRC primary cell cultures tested for JCPyV Tag coding sequences.

The quantitative analysis of JCPyV sequences indicated that the amount of viral DNA present in primary cell cultures the viral DNA copy numbers were very low, ranging from 10-100 copies/10^6 cells, as demonstrated by the cycle threshold values (mean Ct = 37).

Among DNAs from CRC cell cultures analyzed for JCPyV we found that in cecum 100% (1/1), ascending colon or right colon 40% (2/5), descending colon or left colon (0/1), sigmoid colon or sigma (0/2) and rectum 25% (1/4) (table 4).

It is interesting to note that all JCPyV-positive biopsies were found to be JCPyV-positive in the corresponding derived primary cell cultures (table 4).

Table 4. JCPyV sequences in human primary colon cancer cells (CRC).

Cells	Number	Subtype	JC-positive cells cultures/ cells cultures analyzed (%)	JC-positive cells cultures/ JC-positive biopsy (%)
§**CRC**	13	Cecum	1/1(100)	1/1 (100)
		Ascending or right colon	2/5 (40)	2/2 (100)
		Descending or left colon	0/1	0/0
		Sigmoid colon or sigma	0/2	0/0
		Rectum	1/4 (25)	1/1 (100)
TOT.	**13**	**CRC**	**4/13 (31)**	**4/4 (100)**

§CRC (human primary culture cells of colorectal carcinoma).

Circulating JCPyV DNA sequences in serum samples from CRC and HS

In our study, we investigated the association between CRC and JCPyV analyzing the presence of viral sequences in sera from patients and controls, represented by HS with the same median age of the CRC cohort. JCPyV Tag sequences were analyzed by RT-qPCR. CRC (n=53) and HS (n=89) for a total of 142 samples were analyzed (table 5). CRC serum samples were from cancer patients affected by cecum (5/53), ascending colon or right colon (23/53), hepatic flexure (1/53), transverse colon (2/53), splenic flexure (2/53), descending colon or left colon (2/53), sigmoid colon or sigma (9/53) and rectum (9/53) (table5).

Quantitative Real Time-PCR analyses, indicated that only 1 of 53 (2%) DNA samples from sera tested were positive for JCPyV Tag coding sequences. Specifically, the positive sample belongs to a patient with cecum cancer, 20% (1/5) (Table 5), being the viral DNA copy numbers were very low, around 10 copies/10^6 cells, as demonstrated by the cycle threshold values (mean Ct = 38). No HS sera tested JCPyV-positive. The difference in prevalence of JCPyV sequences between CRC and HM was not statistically significant (*P > 0.05*).

Table 5. JCPyV sequences in CRC and healthy subject serum sample.

Serum Sample	Number	Male (%)	Mean age ± SD (range)	Subtype	JC-positive sample/sample Analyzed (%)
§CRC	53	33 (62)	69 ± 12 (39-89)	Cecum	1/5 (20)
				Ascending or right colon	0/23
				Hepatic flexure	0/1
				Transverse colon	0/2
				Splenic flexure	0/2
				Descending or left colon	0/2
				Sigmoid colon or sigma	0/9
				Rectum	0/9
TOT.				**CRC**	**1/53 (2)**
§§HS	89	58 (65)	51 ± 11 (39-89)	Healthy subject	0/89

§CRC (Colorectal Carcinoma serum sample); §§HS (Healthy Subjects serum sample). Statistical analysis was performed using the Chi-square test. The difference in prevalence of JCPyV sequences between CRC and HM was not statistically significant (P > 0.05).

Detection of IgG antibodies against JCPyV in serum samples from CRC patients and HS

Human sera from CRC patients and HS were analysed for the presence of IgG antibodies reacting to JCPyV VP mimotopes. To this purpose, an indirect E.L.I.S.A. was set up and developed employing synthetic peptides, which correspond to specific JCPyV VP 1 antigens. An unrelated human synthetic peptide was employed as a negative control.

In the first step of the investigation, CRC serum samples reacting with the VP1 K mimotope reached an overall prevalence of 28% (15/53), whereas in the control serum samples from HS was 61% (54/89).

Then, the same assay was addressed to detect the IgG class of serum antibodies against VP1 N mimotope. In this assay serum samples reacted with the VP1 N peptide with a prevalence of 30% (16/53) CRC and 67% (62/89) HS. It is interesting to note that the JCPyV peptide K prevalence in these samples is very similar to the data obtained with the same samples for JCPyV peptide N.

In our study, sera were considered JCPyV-positive when reacting with both VP1 K and VP1 N mimotopes/peptides. Combining the data of JCPyV-positive sera, both for the VP1 K and VP1 N mimotopes, the overall prevalence was 26% (14/53) CRC and 51% (45/89) HS (table 6). Statistical analysis was performed using the Chi-square test. The difference in prevalence of JCPyV sequences between CRC and HM was statistically significant (*$**P < 0.01$*).

JCPyV-positive sera tested by indirect E.L.I.S.A., diluted at 1/20, had a general cut-off, by spectrophotometric reading, in the range of 0.17-0.19 OD. This cut-off represents the value that discriminates JCPyV-negative, sample bellow OD 0.17-0.19 from JCPyV-positive samples, above OD 0.17-0.19.

At the beginning of the setup of our indirect E.L.I.S.A. with JCPyV VP 1 mimotopes, the positive control was represented by a rabbit JCPyV hyperimmune serum, which had an OD of up to 1.8, while rabbits BKPyV and SV40 hyperimmune sera were employed as negative controls, with an OD of less than 0.1.

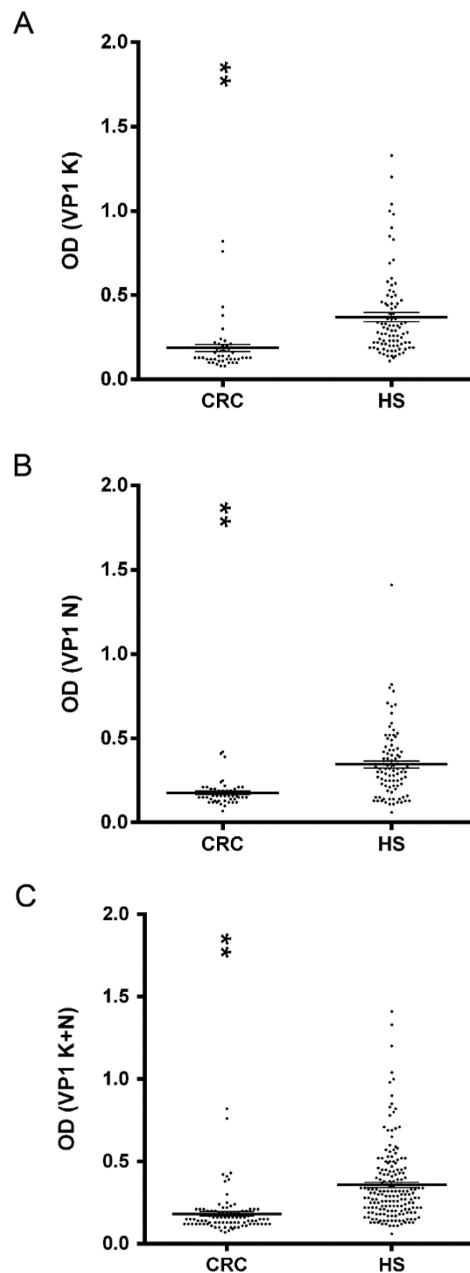
The two indirect E.L.I.S.As., with two distinct VP 1 mimotopes gave overlapping results, thus confirming the presence of anti-JCPyV VP 1 antibodies in human sera from patients affected by colorectal carcinoma (table 6). Serologic profiles of serum antibody reactivity to JCPyV mimotopes are presented in figure 9, while inter-run and intra-run variability is shown in figure 10.

Table 6. Prevalence of immunoglobulin G antibodies in sera from CRC patients and healthy subjects reactive with JCPyV VP1 mimotopes.

Serum	Number	Subtype	Number of positive sample/sample analyzed (%)		
			VP1 K	VP1 N	VP1 (K+N)
§CRC	53	Cecum	2/5 (40)	3/5 (60)	2/5 (40)
		Ascending or right colon	5/23 (22)	5/23 (22)	5/23 (22)
		Hepatic flexure	0/1	0/1	0/1
		Transverse colon	1/2 (50)	1/2 (50)	1/2 (50)
		Splenic flexure	0/2	0/2	0/2
		Descending or left colon	2/2 (100)	2/2 (100)	2/2 (100)
		Sigmoid colon or sigma	1/9 (11)	2/9 (22)	1/9 (11)
		Rectum	4/9 (44)	3/9 (33)	3/9 (33)
TOT.		**CRC**	**15/53 (28)**	**16/53 (30)**	**14/53 (26)****
§§**HS**	**89**	**Healthy subject**	**54/89 (61)**	**62/89 (67)**	**45/89 (51)**

*§CRC (Colorectal Carcinoma serum sample); §§HS (Healthy Subjects serum sample). Statistical analysis was performed using the Chi-square test. The difference in prevalence of JCPyV sequences between CRC and HM was statistically significant (**P < 0.01).*

Figure 9: Serologic profile of human serum antibody reactivity to JCPyV VP1 mimotopes.



*Immunologic data are from serum samples from colorectal carcinoma patients (CRC) and Healthy Subjects (HS). Results are presented as values of optical density (OD) readings at λ 405 nm of serum samples diluted at 1:20, detected in indirect E.L.I.S.A. In scatter dot plotting, each plot represents the dispersion of OD values to a mean level indicated by the line inside the scatter with Standard Error Mean (SEM) for each group of subjects analyzed and statistical analysis was performed using t test. (A) The mean OD of sera (VP1 K ± Std Error) in CRC (0.18±0.02) were lower than that in HS (0.36±0.03). (B) The mean OD of sera (VP1 N ± Std Error) in CRC (0.18±0.01) were lower than that in HS (0.34±0.02). (C) The mean OD of sera (VP1 K+N ± Std Error) in CRC (0.18±0.01) were lower than that in HS (0.36±0.02). Statistical analysis was performed using t test (**P <0.0001).*

Neutralizing antibodies against JCPyV in CRC and HS sera

In order to verify whether our new indirect E.L.IS.A. with synthetic peptides was specific for the JCPyV antibody detection, all sera were analysed by the H.I.A., which is a traditional and very well established assay human sera from cancer patients affected by colorectal carcinoma (CRC) and controls represented by healthy subjects (HS) were preliminary analysed with the technique named Hemagglutination Inibition Assay (H.I.A.). This H.I.A. assay allows the detection of sera containing specific antibodies against JCPyV, which are able to abolish the viral agglutination property.

Serum samples, serially diluted from 1:16; 1:32; 1:64; to 1:128, were analysed by H.I.A. to evaluate both the presence of antibodies against JCPyV and their titre. In order to compare the two techniques, indirect E.L.I.S.A. and H.I.A., I have taken into consideration the dilution 1:128, to eliminate potential non-specific reactivity.

The seroprevalence of JCPyV-positive samples, diluted 1:128, is 27% (8/30) in CRC patients, while in the control groups represented by HS the prevalence is 51% (45/89). The difference was statistically significant ($*P < 0.05$).

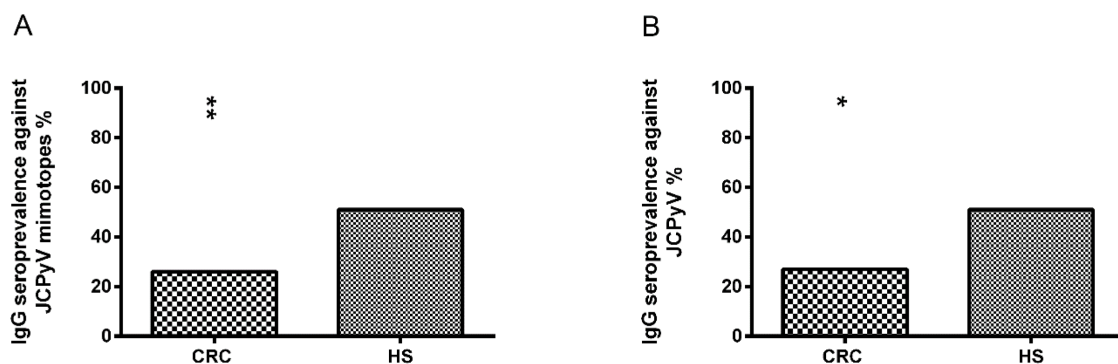
Comparing two techniques, indirect E.L.I.S.A. and H.I.A., it can be observed that the seroprevalence are the same. Indeed, the seroprevalence in CRC is 26% (14/53) by indirect E.L.I.S.A. and 27% (8/30) by H.I.A. This difference is not statistically significant ($P > 0.05$). In the same way, the seroprevalence in HS is 51% (45/89) HS by indirect E.L.I.S.A. and 51% (45/89) by H.I.A. (figure 10). This difference is not statistically significant ($P > 0.05$).

Table 8. Prevalence of immunoglobulin G antibodies in sera from CRC patients and healthy subjects reactive with JCPyV.

Serum	Number	Subtype	Number of positive sample/sample analyzed (%)			
			1:16	**1:32**	**1:64**	**1:128**
§**CRC**	30	Cecum	2/3 (66)	1/3 (33)	1/3 (33)	1/3 (33)
		Ascending or right colon	10/11 (91)	6/11 (54)	5/11 (45)	3/11 (18)
		Transverse colon	1/1 (100)	0/1	0/1	0/1
		Splenic flexure	0/1	0/1	0/1	0/1
		Descending or left colon	0/1	0/1	0/1	0/1
		Sigmoid colon or sigma	4/5 (80)	3/5 (60)	3/5 (60)	3/5 (60)
		Rectum	7/8 (87)	5/8 (62)	5/8 (62)	1/8 (12)
TOT.		**CRC**	**24/30 (80)**	**15/30 (50)**	**14/30 (47)**	**8/30 (27) ***
§§**HS**	**89**	**Healthy subject**	**79/89 (89)**	**78/89 (88)**	**69/89 (77)**	**45/89 (51)**

*§CRC (Colorectal Carcinoma serum sample); §§HS (Healthy Subjects serum sample). Statistical analysis was performed using the Chi-square test. The difference of prevalence of JCPyV antibodies between CRC and HM was statistically significant (*P < 0.05).*

Figure 10: Seroprevalence of human serum antibody reactivity to JCPyV by indirect E.L.I.S.A. and H.I.A.



*Immunologic data are from serum samples from colorectal carcinoma patients (CRC) and Healthy Subjects (HS). Results are presented as values of seroprevalence of antibodies against JCPyV. (A) Seroprevalence was investigated by indirect E.L.I.S.A. Statistical analysis was performed using the Chi-square test (**P <0.01). (B) Seroprevalence was investigated by H.I.A. Statistical analysis was performed using the Chi-square test (*P <0.05). The difference between the two techniques is not statistically significant (P>0.05).*

DISCUSSION

JC virus (JCPyV) is a polyomavirus that was first identified in 1971 as the causative agent of progressive multifocal leukoencephalopathy (PML) in a patient with Hodgkin lymphoma (Padgett, Walker et al. 1971). It is well established that JCPyV infects approximately the 60% of the human population worldwide. This high prevalence indicates that JCPyV is an ubiquitous virus.

Many studies have shown the association between JCPyV and several human cancers. In particular, this association was found between JCPyV and brain tumors. (Del Valle, Gordon et al. 2002, Noch, Sariyer et al. 2012). In addition, JCPyV was associated with the colorectal tumors (Enam, Del Valle et al. 2002). In the literature, there is a strong debate on the putative association between colorectal cancer and JCPyV infection. In fact, some studies have shown the association between CRC and JCPyV (Laghi, Randolph et al. 1999, Shadan, Cunningham et al. 2002, Goel, Li et al. 2006, Coelho, Gaspar et al. 2013), but other studies did not found it (Newcomb, Bush et al. 2004, Lundstig, Stattin et al. 2007).

This investigation was carried out because in the scientific community there is not a consensus on the association between CRC and JCPyV. At present, it is not clear if JCPyV has a role in the onset/progression of CRC. Specifically, in my study the association between the human colorectal carcinoma (CRC) and JCPyV were investigated with novel approaches. To this purpose, the presence of JCPyV DNA sequences was analyzed (i) in CRC and the adjacent healthy mucosa (HM) biopsies; (ii) in primary cell cultures derived from CRC and HM; (iii) in serum samples from CRC patients and controls, represented by healthy subjects (HS) with the same median age and gender of the patients; (iv) the prevalence of JCPyV-antibodies was investigated in serum samples of the same two cohorts, CRC and HS. The survey was conducted after collecting the biopsy and serum samples from CRC patients, together with the control.

JCPyV sequences were detected in 22/53, 41.5% of CRC biopsies and in 11/53, 21% of HM tissues, being the different prevalence statistically significant. This first result indicates an association between CRC and JCPyV infection.

It is interesting to note that 10 matched samples of CRC and HM were found JCPyV-positive. Furthermore, 11 CRC samples tested JCPyV-positive, whereas the matched HM sample were found to be JCPyV-negative. In one HM sample JCPyV sequences were detected, while in the matched CRC the JCPyV sequences were not revealed

JCPyV sequences, as reported in table 3, are more prevalent in the cecum (5/5, 100%) and in the splenic flexure samples (2/2, 100%), both in tumor and normal mucosa biopsies. This data may suggest an association between CRC and JCPyV with a particular focus in these two anatomical districts.

It is worth noting that an association is not a proof of a causal role of JCPyV in the CRC onset/progression. Thus, it remains to verify whether this viral agent with oncogenic potential has role in the development of this human cancer.

It is known that B- and T-lymphocytes represent vehicles for JCPyV to reach different tissues of the host. To verify if JCPyV-positive CRC tumors are due to the presence of the virus in the cancer cells, or to the lymphocytes infiltrating the tumor *in vivo*, primary cultures derived from CRC cells were set up. Indeed, B- and T-lymphocytes do not grow *in vitro* during the cell culture passages, thus allowing the transformed epithelial cells to multiply in monolayers.

Primary epithelial cell cultures from the biopsies were set up. CRC primary cell cultures (n=13) were characterized by morphological and molecular analyses. Among these 13 randomly chosen biopsies, 4/13 (31%) CRC tested JCPyV-positive. The 4 cell samples found JCPyV-positive and the 9 other cell cultures tested JCPyV-negative had the same results when analyzed as biopsies. This result suggests that the presence of JCPyV sequences in human colorectal cancer biopsy is not due to the lymphocytes infiltrating the tumor. JCPyV DNA sequences revealed in cell cultures were found in a lower amount (10-10^2 copies/10^6 cells) compared to the corresponding JCPyV-positive biopsies (10^2-10^3 copies/10^6 cells). The reson of this reduced viral load in cell cultures has not been investigated.

It is possible that cell cultures analyzed for the JCPyV sequences at the first passage are still heterogeneous/mixed, with both JCPyV-positive and JCPyV-negative cells. This cell heterogeneity may account for the lower viral DNA sequence signal in the cell cultures.

In recent studies, circulating JCPyV DNA sequences were detected in human sera with a prevalence of 18% (30/165) (Chehadeh, Kurien et al. 2013).

However, in my investigation among 53 CRC and 89 HM sera only one sample, from CRC patient, tested JCPyV-positive. This result could be due to different technical approaches or to different human subjects enrolled in the studies.

After evaluating the presence of JCPyV sequences in colorectal cancer and normal mucosa biopsies, the prevalence of serum IgG antibodies against JCPyV was comparatively investigated in CRC tumor patients and HS controls. To this purpose an indirect E.L.I.S.A. was designed and set up to investigate the antibody against JCPyV. I wish to recall that this HPyV has a high homology (about 80%) with the other two polyomaviruses, BKPyV and SV40. Serum IgG antibodies against these three Polyomaviruses cross-react when using recombinant VLPs as antigens, thus invalidating the specificity of the immunological data.

For this reason, it was necessary to design and set up a more specific and sensitive immunologic method to assay the specific presence of JCPyV antibodies in human sera.

Investigating by bioinformatics tools the JCPyV VP1, the main viral capsid protein, I detected and selected a small domain with a low a.a. homology compared to the two polyomavirus, BKPyV and SV40. Therefore, two synthetic peptides were designed and selected for the indirect ELISA. These peptides mimic JCPyV VP1 antigens/epitopes and are named mimotopes.

Computational analysis of the linear peptides VP1 K and VP1 N, together with the secondary and tertiary structures of viral capsid protein 1 (VP1) revealed that there is a good statistical confidence level.

On this ground, serum samples from CRC patients and HS were analyzed for their reactivity to JCPyV-VP1 epitopes, using an indirect E.L.I.S.A. with the synthetic peptides K and N. The overall prevalence of JCPyV-VP1 antibodies in CRC patients is 26%, whereas in HS is 51%. No positive results were obtained with the human peptide used as a control.

It is important to note that the seroprevalence of JCPyV antibodies in CRC patients is lower than that detected in HS. The different prevalence between the two cohorts CRC vs. HS (26% vs 51%, $**P < 0.01$) is statistically significant.

It possible that CRC patients, because of their cancer disease, are in part immune-depressed or specifically they are poor responders to JCPyV antigens. The causes of this immune alteration is at present not known.

The data of seroprevalence obtained in healthy subjects, differ from those of other immunological studies where the prevalence of JCPyV antibodies is either very low or absent (Ribeiro, Fleury et al. 2010). It is possible that previous data were affected by technical artifacts. Indeed, earlier results were mainly obtained with recombinant VLPs or VP1 as antigens, which contain many common epitopes with JCPyV, BKPyV and SV40. In these investigations, the immune sera were pre-absorbed with BKV and SV40 (Viscidi and Clayman 2006) in an attempt to give JCPyV specificity to the assay. This procedure may eliminate or drastically reduce the presence of JCPyV antibodies in analyzed sera.

To examine further the serology of JCPyV-positive samples, endpoint titers were determined using an indirect E.L.I.S.A. The highest endpoint titer was observed at a 1/80 dilution for both VP1 K and VP1 N peptides.

In this study, indirect E.L.I.S.As., using JCPyV peptides from VP1 antigens, were set up for the detection of JCPyV antibodies in human sera. E.L.I.S.A. gave reliable results, which can be obtained for many samples in a short period with affordable costs. This E.L.I.S.A. may provide the scientific community with a standardized assay for the study of JCPyV infection in human populations and its association with human cancers.

As a control of the data obtained by the novel indirect E.L.I.S.A., in my study I used another technique which is well consolidated: the hemagglutination inhibition assay (H.I.A.). Data from H.I.A. overlapped the results obtained by the indirect ELISA with synthetic peptides. In conclusion, the new indirect ELISA is reliable, faster, sensitive, specific and with affordable costs.

My investigation found an association between colorectal cancer and JCPyV infection. Indeed, JCPyV sequences were detected in CRC samples with a higher prevalence than that revealed in HM of the same patients. Interestingly, the majority of sera from CRC patients, tested JCPyV-positive, did not react with JCPyV VP antigens. It is possible that these CRC patients, as oncologic patients, were at least partially immunodepressed or non-responders and therefore they were unable to neutralize the JCPyV infection and consequently its oncogenic ativity. My data are innvoative in this field and they may represent a starting point to investigate further the putative role of JCPyV in CRC onset/progression.

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