Detection of Oncogenic DNA Viruses in Colorectal Cancer

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Abstract. *Background: As a part of our search for oncogenic viruses as potential etiological agents in human malignancies, our studies on human papillomaviruses (HPV) were extended to analysis of the 3 polyomaviruses (SV40, BKV and JCV) in colorectal carcinomas. Patients and Methods: Archival tumour samples from 71 patients with colorectal cancer were analyzed for the sequences of SV40, BKV, JCV and HPV using PCR-based techniques. HPV genotypes were determined using sequencing and reverse blot hybridization (InnoLipa). Results: Amplification of BKV and JCV with the primer pair PEP-1 and PEP-2 and subsequent restriction digestion of the amplified products with BamH I disclosed BKV in 6/66 (9%) of the samples, whereas none contained JCV. SV40 was amplified in 10/66 (15.1%) samples and confirmed by sequencing analysis. In pair-wise analysis for co-infections, the samples were significantly different in their BKV-JCV and JCV-SV40 status, in contrast to their BKV-SV40 co-infection status. HPV DNA was detected in 22/66 (33.3%) of the samples analysed with either the MY09/11 or SPF primer mix. Of these 22 HPV infections, 7 were single-type infections and 15 contained multiple HPV types. HPV detection or type distribution showed no relationship to the gender of the patients or histological grade of the tumour. HPV status was not significantly related to detection of BKV, JCV or SV40. Similarly, in pair-wise analysis for co-infections, the samples were significantly different in their status of HPV-BKV ($p=0.0006$), HPV-JCV ($p=0.0001$), and HPV-SV40 ($p=0.019$), implicating that HPV and the 3 polyomaviruses are rarely detected concomitantly in the same samples. Conclusion: Taking the known molecular mechanisms of action of these individual viruses, there is a chance that these viruses could alter the mechanisms of cell cycle control and inhibit apoptosis, thus potentially causing chromosomal instability and promoting colorectal oncogenesis.*

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Colorectal cancer is the third leading cause of cancer-related deaths in the world, with about 1 million new cases diagnosed per year (1). In recent years, several risk factors have been identified as important in colorectal carcinogenesis including environmental and lifestyle factors, sequential genetic alterations (2) and viral infections (3-7). Viral aetiology of human malignancies is an intriguing subject, and so far only a few viral species have been recognized as oncogenic tumour viruses in the human. These include HTLV-1 (a T-cell leukaemia virus), Epstein-Barr virus (EBV), human herpesvirus type 8 (HHV8), hepatitis C (HCV) and hepatitis B virus (HBV), and human papillomavirus (HPV). Other viruses such as polyomaviruses (BKV and JCV) have been implicated as potential human carcinogens, but their definite role as causal agents of human cancer still awaits final confirmation.

HPV is the recognized aetiological agent of cervical cancer. More than 40 HPV types can infect the anogenital tract. A distinct group (13-18 HPV types) is associated with the development of cervical cancer (HR-HPV), while the remaining types fall into a low-risk category (LR-HPV) and cause mainly benign lesions (*i.e.* genital warts). The oncogenic potential of HPV is due to its ability to interfere, upon viral integration into the host cell DNA, with the cell cycle and tumour suppressive function of the p53 and pRb proteins. Indeed, the E6 and E7 oncoproteins of the HR-HPV, unlike those of LR-HPV, exhibit a higher binding affinity for p53 and pRb, inducing their degradation and inactivation, respectively (8). Several recent studies have also reported HPV infections in a variety of non-genital human malignancies, including bladder, head and neck, oral, oesophageal, breast and lung cancer. More recently, HPV has been detected by polymerase chain reaction (PCR) and using *in situ* hybridization in colorectal cancer (3-5).

Polyomaviruses are oncogenic in animal models and readily transform animal and human cells *in vitro* (9, 10).

Table I. *Localisation of the colorectal carcinomas analysed.*

Anatomic site	No. of cases	%
*Proximal colon	39	54.9
†Distal colon	21	29.6
Rectum	3	4.2
Unknown	8	11.3

*Cecum, ascending colon, transverse colon; †Descending colon, sigmoid colon.

Table II. *HPV types detected in colorectal carcinomas.*

HPV type	Number	%
Single HPV		31.8
HPV18	5	22.7
HPV58	2	9.1
Multiple HPVs		68.2
HPV6/18	1	4.5
HPV18/56	2	9.1
HPV18/66	1	4.5
HPV11/18	2	9.1
HPV16/58	1	4.5
HPV16/31	2	9.1
HPV6/16	1	4.5
HPV6/43	1	4.5
HPV6/33	2	9.1
HPV6/31	2	9.1

The early (E) region of a polyomavirus genome encodes two transforming proteins known as large T antigen (T-Ag) and small T antigen (t-Ag). T-Ag interacts with p53 and pRb tumour suppressor proteins altering cell cycle control and inducing malignant transformation. Moreover, SV40 and JCV are capable of inducing chromosomal instability (11). JCV and BKV are widespread in the human population, with a large proportion of people exhibiting antibodies against these two viruses. Recently, the presence of BKV and JCV sequences was repeatedly demonstrated in colon cancer, suggesting that these viruses could have a role in the early stages of colorectal carcinogenesis (6, 7, 12, 13).

To cast further light on the detection of potential oncogenic viruses in colorectal cancer, we studied the presence of HPV and the 3 polyomaviruses BKV, JCV and SV40 in a series of 71 colorectal cancer samples.

Patients and Methods

Study group. A series of 71 paraffin-embedded samples of colorectal adenocarcinoma was provided by the Pathology Department of the University of Trieste, Italy. All cases (33 women and 38 men) were derived from the North-East region of Italy and the mean age of the patients was 75 years. The TNM stage was as follows: 1 case of pT1N0, 1 pT2NX, 9 pT2N0, 2 pT2N1, 1 pT3NX, 15 pT3N0, 14 pT3N1, 2 pT3N2, 6 pT4N0, 6 pT4N1, 5 pT4N2, 1 pT4N1M1. The TNM stage was unknown for 8 patients. The anatomic location of the tumours is shown in Table I.

DNA extraction. Prior to DNA extraction, 5 Bm paraffin-embededed sections of each tumour were deparaffinised in xylene (Carlo Erba Reagenti, Milano, Italy), digested with ATL buffer and proteinase K o/n at 56˚C (Qiagen GmbH, Germany) in a thermomixer. The following day, DNA was extracted using QIAamp DNA mini kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. In order to verify the quality of the extracted DNA, 5 Bl of each sample were amplified with primers recognizing the β-actin gene (14). All samples but five were suitable for molecular analysis.

HPV screening and typing. Extracted DNA was amplified with the consensus primers MY09/11 (15). In cases of negative amplification with this primer pair, the samples were further amplified with the SPF primer mix which amplify a shorter fragment within the L1 gene (16). HPV genotyping was performed by sequencing all positive bands obtained with the MY09/11 primers. In this case, the obtained sequence was matched against all sequences deposited in Genebank (nucleotide-nucleotide BLAST; http://www.ncbi.nlm.nih.gov/BLAST) and the HPV type assigned based on the highest score obtained and confirmed with two alignment sequences (BLAST 2 sequence) against the consensus sequence for that specific genotype. Because multiple infections can be missed by sequencing, all MY09/11-positive samples were further characterized by reverse hybridization (InnoLipa; Innogenetics, Ghent, Belgium). Similarly, all samples amplified with the SPF primers were also typed by reverse hybridization assay (InnoLipa) according to the manufacturer's instructions (Innogenetics). A confirmatory genotype-specific PCR against the E6/E7 region was performed for each HPV type identified (17).

Polyomavirus JCV, BKV and SV40 screening. Human polyomaviruses JCV and BKV and the simian SV40 were detected by a qualitative PCR as reported elsewhere (18, 19).

Statistical analysis. Statistical analyses were performed using the SPSS® (SPSS for Windows, version 14.1.; SPSS, Inc., Chicago, USA) software package. Frequency tables were analyzed using the Chi-square test, with likelihood ratio (LR) or Fischer's exact test being used to assess the significance levels between the categorical variables. Wilcoxon signed ranks test and/or McNemar test were used for analysis of the paired data, *i.e.*, co-infections of the different viruses in the same samples. Odds ratios (OR) and their 95% confidence intervals (95% CI) were calculated where appropriate, using the exact method. In all tests, values $p<0.05$ were regarded statistically significant.

Results

Detection of HPV DNA and typing. HPV-DNA was detected in 22 out of 66 colorectal cancer samples analysed (33.3%) with either MY09/11 or SPF primer mix. HPV typing revealed the presence of a single infection in 7 cases and

Table III. *Detection of BKV, JCV and SV40 in colerectal carcinomas.*

Polyomavirus	Number	%	Total carcinomas
BKV	6	9	66
JCV	0	0	66
SV40	10	15.1	66

multiple infections in the remaining 15 cases (Table II). These results were confirmed by nested-PCR targeting the E6/E7 region of the types identified (17). HPV detection was slightly more frequent among women (39.4%) than men (23.7%), but the difference was not significant. The same is true with type distribution and multiple/single infections. The histological grade of the tumour did not show any association with HPV detection ($p=0.789$) or their type distribution ($p=0.507$).

Detection of polyomaviruses BKV, JCV and SV40. Amplification of the human polyomaviruses BKV and JCV with the primer pair PEP-1 and PEP-2 and subsequent restriction digestion of the amplified products with *BamH I* showed the presence of 6/66 BKV (9%) only. SV40 was amplified in 10/66 samples (15.1%) and confirmed by sequencing analysis. No JCV was detected (Table III). When analysed pair-wise for co-infections (Wilcoxon signed ranks test or McNemar test), the samples were not significantly different in their status of BKV-SV40 ($p=0.439$), implicating that co-infections of these two viruses are not uncommon. (JCV-BKV and JCV-SV40 pairs were excluded, because no JCV was found).

Association of HPV and polyomaviruses. Associations of HPV with the 3 polyomaviruses were tested using different statistical approaches. In the whole series, detection of HPV (+) was not significantly related to detection of BKV ($p=0.423$, Fisher's exact test) or SV40 ($p=0.942$). When analysed pair-wise for co-infections, the samples were significantly different in their status of HPV-BKV ($p=0.0006$) and HPV-SV40 ($p=0.019$), implying that HPV and the 2 polyomaviruses (JCV not found in any sample) were rarely detected in the same samples.

Discussion

Since the discovery by Gross of viral causation of murine leukaemia, the search for oncogenic viruses in human malignancies has exploded. Based on the current understanding, it has been estimated that some 15% of the global cancer burden can be linked to oncogenic tumour viruses (20). With the exception of HCV, all the known human tumour viruses contain DNA as their genetic material. In addition to HPV, the other DNA viruses consistently found associated with human tumours include HBV, EBV and HHV8, while the role of polyomaviruses BKV, JCV and SV40 is still controversial. (10). Genetic instability is a feature common to many human malignancies and it seems to play a role in tumour progression allowing the emergence of cell clones with growth advantages over normal cells (21). Oncogenic viruses may contribute to human carcinogenesis favouring genetic instability and inducing chromosomal aberrations (22).

HPV is the established aetiological agent of cervical cancer (23). In addition, several studies have suggested the involvement of HPV in other human malignancies such as breast, respiratory tract, bladder and oesophageal cancer (24-27). Colorectal cancer is one of the most common malignancies in developed countries (1). Most likely, dietary and other environmental factors constitute a major component of the risk, but detection of HPV DNA in colorectal cancer by *in situ* hybridization (28) and PCR (29, 30) has suggested a potential role of HPV in colorectal carcinogenesis as well.

In this study, we examined 66 paraffin-embedded samples from 71 colorectal cancer patients derived from the North-East region of Italy for the presence of four known or implicated human tumour viruses: HPV, BKV, JCV and SV40. We found HPV DNA in 22/66 samples (33.3%). Single infections were present in 7 cases and multiple infections in the remaining 15 samples, as shown in Table II. All positive samples contained at least one high-risk HPV type, except one sample that was co-infected with two low-risk types (HPV6/43). HPV18 was the most prevalent viral type, being detected in 16.6% (11/66) of positive cases, as also reported by Lee *et al.* (31). This contrasts with three other reports where the HPV16 was the most frequently found virus type (3-5). HPV detection or type showed no relationship to the gender of the patients or histological grade of the tumor. HPV status was not significantly related to the detection of BKV, JCV or SV40. Similarly, in pair-wise analysis for co-infections, the samples were significantly different in their status of HPV-BKV ($p=0.0006$), and HPV-SV40 ($p=0.019$), implying that HPV and the 2 polyomaviruses are rarely detected concomitantly in the same samples. As mentioned before, JCV was not present in any of the samples, making analysis of JCV pairs redundant.

The human polyomaviruses BKV and JCV are widespread among the general population. About 63-80% of the population shows antibodies against these two viruses. They can persist in the organism in the latent form and reactivate in the presence of immunosuppressive conditions. They are commonly associated with rejection of transplanted kidney (BKV) and progressive multifocal leukoencephalopathy (JCV). More recently, they have

been linked to colorectal carcinogenesis (6, 7, 12, 13), although with conflicting results (32). In our series, polyomavirus DNA was amplified using the PEP-1/PEP-2 primer set (7, 18) which amplifies a region of DNA where the nucleotide sequence is identical in BKV and JCV. The JCV sequence targeted for this amplification contains a *BamHI* site, whereas BKV DNA is not cleaved by this enzyme. In our series, BKV DNA was found after restriction digestion in 6/66 tumour samples, while none contained JCV DNA. These results confirm the previous reports on the presence of BKV DNA in some colon cancers, but fail to confirm the association between JCV and colon cancer reported by other groups (6, 7, 12, 13), in line with Newcomb *et al.* (32).

To evaluate the possible differences, it is important to assess the prevalence of JCV infection among the general population. In fact, JCV infection rates vary among populations, being influenced *e.g.* by geographical area, ethnic group and age ranging from 20 to 80% in urine samples (33). In our population, the JCV infection rate is known to be around 30% (34). Sensitivity of the technique used (PCR, immunohistochemistry, Southern blot hybridization) and selection of the genome region are other factors to be considered while weighing up these discordant results. PCR is the most sensitive technique (7, 18) and amplification of the early region of the JCV genome, T-Ag, gives more reliable results than the amplification of VP1 or agnoprotein regions which are often not detected (7). However, the lack of detection of JCV T-Ag cannot rule out a "hit and run" mechanism as demonstrated by Ricciardiello *et al.* in an *in vitro* model of colonic cells (35).

SV40 is a monkey virus that was probably introduced in the human population in the early 1960's by contaminated polio vaccines produced in monkey kidney cells where the virus can be present in a latent form. It probably continued to spread among humans through the orofecal, sexual and haematogenic routes, since it was found in urine and sewage samples (36, 37). SV40 was first linked to human malignancies by Soriano *et al.* who detected SV40 DNA in a metastatic melanoma (38). Since then, SV40-related sequences have been found in mesotheliomas, brain tumors and osteosarcoma (39-41). We recently described the presence of SV40 genome in bronchial carcinomas (19). In the present study, SV40 T-Ag was amplified in 10 out of 66 samples screened (15.1%) and the results were confirmed by direct sequencing. When analysed pair-wise for co-infections, it was shown that BKV and SV40 frequently co-existed in the same samples (*i.e.*, test for paired samples indicated no significant difference between the BKV and SV40 status. To our knowledge, this is the first report describing the presence of SV40 DNA in colorectal carcinomas. However, *in vitro* and animal studies have been performed that showed how the expression of SV40 T-Ag can alter the cell phenotype and induce tumor growth at the intestinal level (42-44). These tumors were rapidly growing, multifocal and invasive, resembling human small cell carcinoma of the colon (42).

Interestingly, HPV and the 3 polyomaviruses share many properties in common that might be linked with malignant transformation of the target cells. Accordingly, the T-Ags of all three polyomaviruses as well as the E6 and E7 oncoproteins of high-risk HPVs interfere with the suppressive functions of two key regulators of the cell cycle, p53 and pRb (8, 10). It is well established that an aberrant stimulation of the cell cycle leads to oncogenic transformation of the cell. The T-Ag and E7 of hig-hrisk HPVs bind to pRb, displacing the trancription factor E2F, thereby promoting cell proliferation. The p53 gene is found mutated or disrupted in many human tumors. The encoded protein acts as a checkpoint in the cell cycle and regulates apoptosis. Polyomavirus T-Ags and the E6 oncoprotein of high-risk HPVs bind and degrade p53 through the ubiquitin pathway thus preventing inhibition of the cell cycle and apoptosis. Furthermore, all four viruses contribute to chromosomal instability. Gross chromosomal rearrangements, loss of heterozygosity, aneuploidy and disruption of the mitotic checkpoint have been described in colon and cervical cancer (45). In conclusion, apart from confirming the reports on HPV and BKV detection, we here describe for the first time the presence of SV40 in biopsies of colorectal cancer. There was no evidence of JCV infection in these samples, but a "hit and run" mechanism cannot be ruled out for this virus and a possible synergism between the viruses must be considered. Based on the current experimental evidence implicating that all these viruses are capable of altering the cell cycle control and inhibit apoptosis, we can hypothesize that they could induce chromosomal instability and contribute to colorectal carcinogenesis.

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