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Human Papillomavirus DNA and Oncogene Alterations in Colorectal Tumors

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Abstract The aim of the present study is to determine the presence and molecular integrity of high-risk HPV types in colorectal adenocarcinomas and to assess whether viral DNA is related to common proto-oncogene alterations, such as k-*ras* mutations and c-*myc* gene amplification, in colorectal cancer. Seventy-five colorectal adenocarcinomas were screened for HPV infection using nested-PCR (MY09/11-GP5+/6+). HPV typing was performed by type-specific PCR for HPV 16 and HPV 18 DNA. Unidentified samples were subsequently sequenced to determine the viral genotype. The physical status of HPV was determined by a nested PCR approach for type-specific *E2* sequences. C-*myc* amplification was assessed by co-amplification with β-*globin* as control locus, and mutation in k-*ras* codons 12 and 13 by ARMS-PCR. Overall, HPV was detected in thirty-three colorectal specimens (44%). HPV 16 was the prevalent type (16/75), followed by HPV 18 (15/75), HPV 31 (1/75) and HPV 66 (1/75). *E2* disruption was detected in 56.3% of HPV 16 and in 40% of HPV 18 positive tumors. C-*myc* amplification was detected in 29.4% of cases, while k-*ras* mutations in 30.7%. There was no significant trend for HPV infection in tumors harboring either k-*ras* or c-*myc* alterations. This study demonstrates HPV DNA and viral integration in colorectal tumors, suggesting a potential role of this virus in colorectal carcinogenesis. There was no concurrence, however, of k-*ras* and c-*myc* activation with viral infection.

Keywords Human Papillomavirus · Colorectal carcinoma · Viral integration · Proto-oncogene activation · k-*ras* · c-*myc*

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Introduction

Colorectal cancer is the second leading cause of cancer deaths in the Western World with more than 1 million of new cases diagnosed per year [1]. The current model of colon carcinogenesis is characterized by a progressive accumulation of genetic alterations, accompanied by a progression from early adenomatous polyps to primary carcinoma. Genetic predisposition explains a small proportion of cases, whereas more than 70% of cases develop sporadically. The influence of external factors, such as lifestyle and/or biological agents, is widely recognized, although the molecular mechanism underlying such influence is not well understood. Consumption of alcohol, red meat, high fat and low fiber food and sedentary lifestyle has been associated with increasing risk [2–4]. Recently, several studies have suggested that some viruses, especially Papillomavirus (HPV), may play a role in colorectal cancer.

HPV DNA was detected in colorectal tissues by several methodologies [5–9]. However, the significance of such findings remains controversial [9, 10].

Human Papillomavirus is the causative agent of cervical and ano-genital cancers [11]. It comprises more than 100 types and certain variants, such as HPV types 16, 18, 31 and 45, are considered high risk types. The oncogenic potential is exerted by onco-proteins that are able to immortalize and transform cells. Low-risk HPV types include 6, 11, 40, 42, etc. and are commonly associated with benign lesions [12, 13]. Papillomavirus has been associated with anal cancer and it was recently found in squamous cell carcinoma (SCC) of the colon [5, 10]. An evaluation of the potential link between colorectal adenocarcinoma and HPV should involve the analysis of specific factors in HPV positive tumors. Integration of the viral genome into the host chromosome is a known pathogenic factor, commonly associated with the rearrangement or amplification of host sequences [14, 15]. An undesired outcome of HPV integration is the inactivation of *E2* gene, a negative regulator that controls the expression of E6 and E7 oncoproteins. These proteins antagonize the function of the *p53* and *pRb* tumor suppressor genes, respectively [16]. Therefore, the insertion of HPV sequences may have consequences in the generation of the cancerous cell, inducing genomic instability and cell transformation [17, 18].

Studies revealed that HPV DNA appears to be preferentially integrated in certain locations, and this might result in the alteration of nearby genes or regulatory sequences. The cooperation of HPV and the activation of oncogenes, such as c-*myc* and k-*ras*, have been widely reported. HPV DNA sequences have been repeatedly observed in common fragile sites and regions surrounding the c-*myc* locus [15, 19]. C-*myc* is a member of the multigenic family Myc, and it is located in the chromosome 8q21. It encodes for a 62 kD transcription factor that is involved in the cell cycle regulation, differentiation and apoptosis [20]. The most common mechanism for *c-myc* activation is deregulation of expression followed by gene amplification. Excess of gene copies is characteristic of several malignant human formations and it increases with tumor severity [21–23]. To date, the coexistence of c-*myc* amplification and HPV DNA has been reported in three cell lines from colon adenocarcinomas derived from Chinese patients [24]. In the present study we analyze such association in a larger number of samples.

We have extended our analysis to the proto-oncogene k-*ras*. This gene encodes for a 21 kD protein involved in signal transduction, proliferation and differentiation. It is located on chromosome 12 and mutations at codons 12 and 13, and less frequently at codon 61, are common alterations in colorectal cancer, leading to uncontrolled proliferation and malignant transformation [25]. According to the published data, mutation of k-*ras* leads the transition from adenoma to carcinoma, ranging from 10–20% for advanced adenomas to 30–50% for early carcinomas [26, 27]. To date, only one study has examined the co-existence of HPV and k-*ras* mutations in colorectal cancer, reporting a mutation prevalence of 56% in HPV positive tumors [28].

In order to examine the role of HPV in colorectal cancers, a group of colorectal tumors from all stages was evaluated for high-risk HPV DNA and its physical status, while addressing the question whether two specific colorectal cancer-related mutations, c-*myc* amplification and k-*ras* mutations, may be associated.

Materials and Methods

Patients and Samples

A series of 75 sporadic colorectal cancers were collected between 2002 and 2004 at the "Hospital Interzonal de Agudos General San Martin" and the Faculty of Medicine from the "National University of La Plata, Argentina". The gender of the patients was: 39 females and 36 males (mean age 63 years old). The tumors comprised: 22 classified as Dukes A-B1; 18 classified as Dukes B2-B3; 26 as C1-C3; and 9 as D. The anatomical location of samples corresponded to 27 surgical pieces from the rectum and rectosigmoid and 48 from the colon.

DNA Extraction and Sample Processing

The samples were archival formaldehyde-fixed and paraffin-embedded biopsies. Before PCR analysis, paraffin embedded samples were washed twice in xilol, then twice in ethanol 100%, suspended in 300 µL of digestion buffer (50 mM Tris-HCL pH 8.5; 1 mM EDTA; 1% Triton X100 and 0.5% Tween20) with proteinase K (250 µg/ml), and incubated overnight at 56°C. DNA was extracted and purified by the salting out procedure [29], using 2.5 M ammonium acetate as the concentrated salt. Purified DNA was eluted in 150 µL of distilled water and kept frozen until used. A fragment of the *β-globin* gene was amplified to verify the quality of the DNA samples.

HPV Detection and Genotyping

Human Papillomavirus DNA was detected by a nested PCR methodology, using My09/11 as outer primers and GP5+/6+ as inner primers, according to the methods previously described [30, 31]. Extreme precautions were taken in order to avoid cross-contamination between samples: Separate spaces, new gloves per experiment,

decontamination of materials and surfaces with UV light, filter tips for protection against aerosol, and the use of special work-coats. Each experiment was done in duplicate and a negative control was included every six samples. After cycling, PCR products were analyzed in 2% agarose gels and stained with ethidium bromide. Positive samples were subsequently genotyped by specific PCR for HPV 16 and 18. All primers used in this study are detailed in Table 1.

Amplification reactions were performed in a 25 μL mixture containing 0.75 U Taq Polymerase (Invitrogen, USA), 15 pmol of each HPV E6 primer, 3 mM $MgCL_2$, 200 mM of each dNTP and 1X buffer. The PCR comprised an initial step of 3 min at 92°C, followed by 35 cycles for 30 s at 92°C, 1 min at 58°C (HPV 18) or 61°C (HPV 16), and 45 s at 72°C. A final elongation step was done for 5 min at 72°C. The obtained PCR products were 134 bp and 163 bp long for HPV 16 and 18, respectively.

DNA sequencing was performed in Human Papillomavirus positive samples that were not identified by type-specific PCR. Briefly, PCR products were purified by polyethilenglicol precipitation (20% PEG, 2.5 mM NaCl), and quantified by spectrophotometry. Direct sequencing was performed (primers GP+) by the dye-terminator method using a MegaBACE™ 1000 device (DNA Sequencing Service of IGEVET-CONICET, UNLP).

HPV 16-18 Integration

The physical status of HPV 16 and 18 was examined by a nested PCR approach, using two sets of primers for each *E2* sequence. Oligonucleotides were designed with the software FastPCR® (University of Helsinki, Finland), based on the complete sequences of HPV 18 genome (Accession number X05015), and HPV 16 genome (Accession number AY686584).

The first round of HPV 16-18 *E2* DNA amplification was performed in a final volume of 25 μL, containing 1X reaction buffer, 3.125 pmols of each outer primer, 200 mM

Table 1 Oligonucleotides used for PCR analyses

Gene	Sequence
HPV 16 *E6*	
HPV16E6F	5′-GAG AAC TGC AAT GTT TCA GGA CC-3′
HPV16E6R	5′-CCT CAC GTC GCA GTA ACT GTT GC-3′
HPV18 *E6*	
HPV18E6F	5′-AGA GAC AGT ATA CCC CAT GCT-3′
HPV18E6R	5′-GTT TCT GGC ACC GCA GGC ACC T-3′
HPV 16 *E2* outer	
16P3300	5′-AGT ATG GGA AGT TCA TGC GGG TG-3′
16P3694	5′-TGC CAT GTA GAC GAC ACT GC-3′
HPV 16 *E2* inner	
16P3362	5′-AAC GAA GTA TCC TCT CCT GAA ATT ATT AG-3′
16P3533	5′-TGG AGC ACT GTC CAC TGA GTC-3′
HPV 18 *E2* outer	
18P3277	5′-CTA CCT GTG TAA GTC ACA GGG GA-3′
18P3728	5′-GGT CGC TAT GTT TTC GCA ATC TG-3′
HPV 18 *E2* inner	
18P3423	5′-TCT ATG TGC AGT ACC AGT GAC G-3′
18P3601	5′-TGC ACC GAG AAG TGG GTT GAC AG-3′
k-*ras* codon 12	
K12MF	5′-ACT GAA TAT AAA CTT GTG GTA GTT GGA CCT-3′
K12MR	5′-ACT CAT GAA AAT GGT CAG AGA AAC CTT TAT-3′
k-*ras* codon 13	
K13MF	5′-GTA CTG GTG GAG TAT TTG ATA GTG TAT TAA-3′
K13MR	5′-GTA TCG TCA AGG CAC TCT TGC CTA GG-3′
C-*myc*	
mycF	5′-AAA GAG GCA GGC TCC TGG CA-3′
mycR	5′-TCT CGT CGT TTC CGC AAC AA-3′
B-*globin*	
globinaF	5′-ACA CAA CTG TGT TCA CTA GC-3′
globinaR	5′-CAA CTT CAT CCA CGT TCA CC-3′

of each dNTP, 3 mM $MgCl_2$, 0.75 units of Taq DNA polymerase (Invitrogen, USA), and 5 μL of sample DNA. The cycling program consisted of 25 cycles of 1 min at 92°C, 1 min 30 s at 58°C, and 1 min at 72°C. Five μL of each PCR product was transferred to another tube, for a second round of amplification, using the HPV 16-18 *E2* inner primers (18.75 pmol each). Concentration of PCR buffer and reagents were the same as used in the first round. The cycling conditions were: 35 cycles of 1 min at 92°C, 1 min at 57°C, and 1 min at 72°C.

The Hela cell line, containing 20–40 copies of HPV 18 per cell, and HPV 18 and 16 purified plasmids were used as positive and negative controls for viral integration. All reactions were done in duplicates, in order to confirm the obtained results.

C-*myc* Amplification Analysis

The analysis of C-*myc* amplification was assessed by a semi-quantitative approach. A fragment of c-*myc* DNA and a reference gene, β-*globin*, were co-amplified by PCR in a single tube reaction, as described previously by Lönn et al. [32], with few modifications. The single copy reference: target gene relative ratio system has been proved to be an efficient method to determine gene amplification in a semi-quantitative manner. Detection of PCR products was made by electrophoresis in 10% poly-acrylamide gels followed by a silver staining protocol. The ratio between net intensity bands of c-*myc* and β-*globin* was determined using the Kodak Digital Science 1D Image Analysis Software™. Samples were considered to be c-*myc* amplified when the ratio c-*myc*/ β-*globin* exceeded two standard deviations from the value obtained in normal DNA.

K-*ras* 12 and 13 Mutations Detection

Mutations at codons 12 and 13 of the k-*ras* gene were detected using the Artificial Refractory Mutation System (ARMS-PCR) according to the protocol described by Hatzaki et al, with minor modifications [33]. In this method, PCR amplification was performed using a mismatched right primer for codon 12 amplification and a mismatched left primer for codon 13 amplification which introduces a *BstNI* and a *HaeIII* restriction site in the normal allele, respectively.

The expected PCR products are 192 bp and 159 bp for codon 12 and 13, respectively. The primers used in this study are the same as those reported by Hatzaki et al., except for a different antisense codon 12 primer that extended the amplicon 30 bp downstream, from 162 bp to 192 bp.

Digestion of the codon 12 amplicon by *BstNI* yields DNA fragments of 162 bp and 30 bp, whereas the mutant type remains undigested. On the other hand, digestion of codon 13 amplicon by *HaeIII* results in 85 bp, 48 bp and 26 bp DNA fragments, but the mutant type produces only 85 bp and 74 bp bands.

Statistical Analyses

The prevalence of HPV was compared among groups by chi-square ($\chi 2$) test, with confidence levels of 0.05. The correlation of ordinal variables was calculated by Kendall's tau (τ) c test. Descriptive data and statistical processes were computed with the SPSS® software.

Results

High-Risk HPV Distribution

All 75 samples analyzed in this study were adequate for PCR analysis, as it was revealed by *β-globin* amplification. HPV DNA was detected in 33 samples (44%). Most of the infections harbored HPV 16 and 18 DNA (94%), sixteen tumors were positive for HPV 16 and fifteen for HPV 18. One sample was co-infected with HPV16-18, and two were unidentified by type-specific PCR. Sequence analysis revealed that HPV DNA from the unidentified samples corresponded to single infections of the high-risk types HPV 31 and HPV 66. All colorectal stages exhibited HPV DNA detection, as detailed in Table 2. Regarding histo-pathological features, overall HPV prevalence increases according to disease severity, although this trend was not significant (τ=0.16; P=0.2). There was no association between HPV positivity and differentiation grade (P=0.46).

The analysis of global infection according to anatomic location showed that high-risk HPV detection was uniformly distributed in colorectal carcinomas (OR=1.2, $IC_{95\%}$=0.47–3.1, P>0.05). In this sense, Table 3 illustrates the distribution of HPV prevalence: 40% of rectal tumors and 46% of colon tumors were HPV positive by PCR. However, type-specific detection revealed that HPV 16 infection was highest in the colon (14/22); whereas most of viral detections in the rectum corresponded to HPV 18 (8/11).

HPV 18 and 16 Integration

The physical status of high-risk HPV types 16 and 18 was analyzed by examining the *E2* sequence integrity in colorectal cancers. All tumors harboring HPV 16 (n=16) and HPV 18 (n=15) DNA were studied. The absence of PCR product was interpreted as *E2* sequence disruption and therefore integration of the viral sequences into the host genome. Table 4 shows the results for type-specific *E2* amplification according to tumor staging. As it is shown in the table, a significant proportion of HPV positive tumors

Table 2 A summary of the obtained results in this study

	Dukes A-B1 $N=22$	Dukes B2-3 $N=18$	Dukes C1-3 $N=26$	Dukes D $N=9$
Differentiation				
Well-differentiated	15 (68.2%)	3 (16.7%)	9 (34.6%)	3 (33.3%)
In-differentiated	1 (4.5%)	2 (11.2%)	8 (30.8%)	2 (22.3%)
Semi-differentiated	4 (18.2%)	12 (6.7%)	9 (34.6%)	2 (22.3%)
Missing	2 (0.09%)	1 (0.06%)	–	2 (22.3%)
Location				
Rectum and Recto-sigmoid	9 (40,9%)	9 (50,0%)	6 (23,1%)	3 (33,3%)
Colon	13 (59,1%)	9 (50,0%)	20 (76,9%)	6 (66,7%)
HPV DNA				
HPV 16	4 (18.2%)	2 (11.2%)	9 (34.6%)	1 (11%)
HPV 18	3 (13.7%)	4 (22.3%)	4 (15.4%)	4 (45%)
HPV 31	–	–	1 (3.8%)	–
HPV 66	1 (4.5%)	–	–	–
k-*ras* 12 mutation	6 (27.3%)	8 (44.5%)	3 (11.5%)	3 (33.3%)
k-*ras* 13 mutation	–	–	2 (7.6%)	1 (11%)
Total k-*ras* mutations	6 (27.3%)	8(44.5%)	5 (19.1%)	4(44.5%)
c-*myc* amplified	3 (13.6%)	4 (22.2%)	12 (46.2%)	3 (33.3%)

showed disruption of *E2* gene, although the frequency of HPV 16 DNA integration was higher (56%) than that observed for HPV 18 (40%).

C-*myc* Amplification

In this study, 29.4% (22/75) of the tumors presented amplified c-*myc* gene copies. Table 2 summarizes the findings according to Duke staging grade. It is interesting that most of the samples showing amplification of the oncogene c-*myc* belonged to the C stage. C-*myc* amplification prevalence increased from tumors in Duke A (13.6%) to those with Duke C (46.2%). However, the trend of amplification by stage of colorectal neoplasia was not significant, as c-*myc* amplification prevalence dropped to 33.3% in Duke D. In addition, C-*myc* amplification was not significantly higher in tumors with HPV DNA than those without HPV DNA ($P=0.15$) (Table 5).

K-*ras* Codon 12 and 13 Mutations

Table 2 shows the distribution of k-*ras* mutations among the 75 colorectal tumors. In this study, the overall prevalence of k-*ras* mutations was 30.7%. Whereas 87% belonged to k-*ras* codon 12 mutations, only 13% of the analyzed samples exhibited codon 13 mutations. As seen in Table 2, the frequency of *k-ras* mutations did not correlate with Duke staging.

The prevalence of k-*ras* mutations was slightly higher in HPV DNA positive (40%) than HPV negative carcinomas (24%), but this difference was not significant ($P=0.5$). It is interesting to mention that the three tumors presenting codon 13 mutations also harbored HPV 16 DNA. On the other hand, there was not statistical difference in HPV integration prevalence when k-*ras* mutated tumors (45.5%) and those without mutations (47.4%) ($P>0.05$) were compared.

Discussion

Colorectal cancer is a multi-step process characterized by a sequential accumulation of genetic alterations in specific proto-oncogenes and tumor suppressor genes. While many factors included in the inherited predisposition of colorectal cancer have been elucidated, the molecular changes

Table 3 HPV prevalence according to anatomic location of tumors

Anatomic location	HPV DNA Status				
	HPV 16	HPV 18	HPV 31	HPV 66	NEG
Rectum and rectosigmoid region	2 (7.4%)	8 (29.6%)	1 (3.7%)	–	16 (59.3%)
Colon	14 (29.2%)	7 (14.6%)	–	1 (2.1%)	26 (54.1%)
Total	16 (21.3%)	15 (20%)	1 (1.3%)	1 (1.3%)	42 (56.1%)

Table 4 HPV 16 and HPV 18 E2 positivity in colorectal adenocarcinomas

Colorectal stage	HPV 16 E2 positive/ total	HPV 18 E2 positive/total
Dukes A	3/4	2/3
Dukes B	2/2	2/4
Dukes C	2/9	2/4
Dukes D	0/1	3/4
Total	7/16 (43.7%)	9/15 (60%)

underlying sporadic cancer are not well-understood. In this sense, clinic-pathological examination is still the best marker for patient prognosis [3]. Within the sequence of changes, additional co-factors and genetic aberrations have been described, and many of them have been suggested as carcinogenic contributors. Assessing such candidates is an enormous task, first, because colorectal cancer arises as a result of different molecular pathways; and second, because each path may be relevant to a subset of adenocarcinomas very similar in histology, but different in genotype [34]. Recognition of genetic aberrations and description of novel pathogenic changes are, therefore, particularly important for assessing the aggressiveness of tumor phenotypes.

The link between human Papillomavirus infection and tumor-genesis is clear in cervical cancer, and accumulating data suggest that this virus may have impact in some extra-genital cancers [13]. Several studies have found HPV DNA in colon adenocarcinomas and suggested a potential association with carcinogenesis [5–8]. Debate has arisen about the meaning of these findings. To address this issue, we have analyzed the integration status of HPV in colorectal tissues by examining E2 ORF integrity after universal HPV detection. In this sense, insertion of viral sequences near oncogenes may lead to chromosomal alterations, like gene rearrangements or sequence amplifications [16].

We have detected high-risk HPV in 44% of the patients with colorectal cancer, with one sample co-infected. E2 type-specific PCR revealed that 56% of HPV 16 positive tumors and 40% of HPV 18 positive tumors had integrated DNA. The overall prevalence of HPV was lower than that previously reported by our group (70%), although in a smaller series of carcinomas [35]. A well-controlled study from USA recently reported that 51% (28/55) of colorectal tumors had HPV DNA, being HPV 16 the most frequent type. In the same study, the authors reported viral integration in most of the HPV positive tumors [6]. In contrast to these findings, HPV 18 was more commonly detected in colorectal tumors from Turkey [28], and Taiwan [36].

In the present study we have detected a differential distribution of HPV types: HPV 16 was prevalent in tumors from the colon (14/22), whereas HPV 18 in tumors from the rectum (8/11). Although this could be a spurious association caused by small sample size, the preferential location of HPV types in colorectal neoplasias may be related to a variable susceptibility to infection by tumor site. In addition to these findings, we also reported HPV 31 DNA in a rectal invasive carcinoma and HPV 66 DNA in an advanced colonic adenocarcinoma, both biopsies deriving from elderly women.

Regarding the methodology, we have used nested PCR to assess E2 integrity. This is a sensitive and reliable method to detect viral integration in cases when the virus genome is in low copy numbers, like colorectal cancer. However, we are aware of potential limitation: First, this method does not discriminate between pure episomal and mixed forms; and second, the possibility of viral integration without losing the E2 gene fragment was not considered.

Elevated c-*myc* expression and gene amplification are typical events in tumorgenesis of colorectal cancer. Globally, c-*myc* is over-expressed in 70% of primary colorectal adenocarcinomas, although gene amplification seems to be less frequent [37]. The highest prevalence of c-*myc* amplification (50–70%) is detected at later stages of colorectal cancer, while primary tumors are characterized by low rates [22, 38–40]. In this study we found increasing prevalence of c-*myc* amplification with increasing severity of tumor staging, ranging from 13% in primary tumors to 46% in Dukes C, and falling to 33% in the metastatic stage. These data indicate that accumulation of c-*myc* copies is associated with more aggressive phenotypes. Recent studies

Table 5 HPV prevalence according to oncogene alterations in colorectal samples

	HPV Negative $n=42$	HPV Positive $N=33$
K-*ras* status[a] (SR/C)[b] n Normal	(13/19) 32	(7/13) 20
Mutated	(3/7) 10	(4/9) 13
C-*myc* status[a] (SR/C)[b] n Normal	(15/18) 33	(10/10) 20
Amplified	(1/8) 9	(1/12)13

[a] *P value*>0.05

[b] SR = rectal and sigmoid-rectal location, C = colonic location

have reported that the mutation of the *APC* gene induces accumulation of a fraction of the β-catenin protein, leading to c-*myc* over-expression at early stages [41]. This situation brings up the question whether c-*myc* enhanced expression may actually contribute to genome destabilization. The molecular mechanisms underlying gene amplification are, however, still unclear. The relationship of HPV infection and c-*myc* amplification has been recently investigated in cell lines derived from genital cancers. High-risk HPV integration is associated with genomic instability and rearrangement of flanking cellular sequences [42]. In this sense, HPV 16 genome seems to integrate randomly into the host genome and preferentially in *common fragile sites* (CFS) of chromosomes, whereas HPV 18 is found in CFS and in areas surrounding c-*myc* region [15–19]. In this study, statistical analysis did not reveal an association between *c-myc* status and global HPV infection. In contrast with genital carcinomas, only two colorectal tumors with c-*myc* amplified had HPV 18 integrated.

Regarding the k-*ras* oncogene, we found that 23 colorectal adenocarcinomas had a point mutation in either codon 12 or 13 (30%). Our results support the view that *k-ras* mutations do not necessarily accumulate during tumor progression, as *c-myc* amplification does, emphasizing an early action in carcinogenesis. The obtained prevalence was lower than expected: k-*ras* mutations are found in 30–50% of colorectal adenocarcinomas, and it appears to be more common with increasing degree of dysplasia. The reason for this low prevalence is the possibility of missing mutations at codon 61, although they seem to occur at very low rate [25]. Stage-specific detection ranged from 20% to 33%, without correlation between increasing mutation level and tumor severity. Interestingly, we did not find a significant difference in HPV DNA detection when we compared tumors with different mutation status. However, we found that most of the HPV positive tumors with k-*ras* mutated were C staged, and HPV 16 was the prevalent type in this stage. Although this could be a spurious association, it is widely demonstrated the oncogenic potential of high-risk HPV infection, and we think that it deserves to be further studied. In a previous study, Buyru et al. have reported that half of HPV positive colorectal tumors harbored k-*ras* mutations [28]. In addition, our data indicate that the mutational activation of k-*ras* is independent from viral integration in HPV positive colorectal tumors.

In conclusion, based on our limited number of cases, activation of c-*myc* and k-*ras* are commonly detected in colorectal adenocarcinomas, but HPV infection is not associated with those alterations. Furthermore, the viral genome is integrated in a high proportion of HPV positive tumors. Further studies are needed to elucidate the role of HPV in this tissue.

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References

1. Ferlay J, Bray F, Pisani P et al (2004) GLOBOCAN 2002: cancer incidence, mortality and prevalence worldwide, IARC CancerBase N° 5. IARC, Lyon
2. Beart RW (1995) Colon and rectum. In: Abeloff MD, Armitage JO, Allen SL, Niedehuber JE (eds) Clinical oncology. Churchill Livingstone, New York
3. Gazelle GS, McMahon PM, Scholz FJ (2001) Screening for colorectal cancer. Radiology 221:273–274
4. Vainio H, Miller AB (2003) Primary and secondary prevention in colorectal cancer. Acta Oncol 42:809–815
5. Bognár G, István G, Bereczky B et al (2008) Detection of human papillomavirus type 16 in squamous cell carcinoma of the colon and its lymph node metastases with PCS and southern blot hybridization. Pathol Oncol Res 14:93–96
6. Bodaghi S, Yamanegi K, Xiao SY (2005) Colorectal papillomavirus infection in patients with colorectal cancer. Clin Cancer Res 11:2862–2867
7. Yu HG, Shun LB, Luo HS et al (2002) Deletion of the FHIT gene in human colorectal cancer is independent of high-risk HPV infection. Int J Colorectal Dis 17:396–401
8. Damin DC, Caetano MB, Rosito MA et al (2007) Evidence for an association of human papillomavirus infection and colorectal cancer. Eur J Surg Oncol 33:569–574
9. Shah KV, Daniel RW, Simons JW et al (1992) Investigation of colon cancers for human papillomavirus genomic sequences by polymerase chain reaction. J Surg Oncol 51:5–7
10. Shroyer KR, Kim JG, Manos MM et al (1992) Papillomavirus found in anorectal squamous carcinoma, not in colon adenocarcinoma. Arch Surg 127:741–744
11. zur Hausen H (1999) Viruses in human cancers. Eur J Cancer 35:1878–1885
12. Bosch FX, de Sanjosé S (2007) The epidemiology of human papillomavirus infection and cervical cancer. Dis Markers 23:213–227
13. Giuliano AR, Tortolero-Luna G, Ferrer E et al (2008) Epidemiology of human papillomavirus infection in men, cancers other than cervical and benign conditions. Vaccine 26:17–28
14. Ferber MJ, Montoya DP, Yu C et al (2003) Integrations of the hepatitis B virus (HBV) and human papillomavirus (HPV) into the human telomerase reverse transcriptase (hTERT) gene in liver and cervical cancers. Oncogene 22:3813–3820
15. Ferber MJ, Thorland EC, Brink AA et al (2003) Preferential integration of human papillomavirus type 18 near the c-myc locus in cervical carcinoma. Oncogene 22:7233–7242
16. Chakrabarti O, Krishna S (2003) Molecular interactions of 'high risk' human papillomaviruses E6 and E7 oncoproteins: implications for tumour progression. J Biosci 28:337–348
17. Pett MR, Pett MR, Alazawi WO et al (2004) Acquisition of high-level chromosomal instability is associated with integration of human papillomavirus type 16 in cervical keratinocytes. Cancer Res 64:1359–1368
18. Badaracco G, Venuti A, Sedati A et al (2002) HPV16 and HPV18 in genital tumors: significantly different levels of viral integration and correlation to tumor invasiveness. J Med Virol 67:574–582
19. Thorland EC, Myers SL, Gostout BS (2003) Common fragile sites are preferential targets for HPV16 integrations in cervical tumors. Oncogene 22:1225–1237

20. Ryan KM, Birnie GD (1996) Myc oncogenes: the enigmatic family. Biochem J 314:713–721
21. Augenlicht LH, Wadler S, Corner G (1997) Low-level c-myc amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. Cancer Res 57:1769–1775
22. Obara K, Yokoyama M, Asano G et al (2001) Evaluation of myc and chromosome 8 copy number in colorectal cancer using interphase cytogenetics. Int J Oncol 18:233–239
23. Zaharieva B, Simon R, Ruiz C et al (2005) High-throughput tissue microarray analysis of CMYC amplification in urinary bladder cancer. Int J Cancer 117:952–956
24. Cheng JY, Meng CL, Chao CF et al (1991) Human papillomavirus type-related DNA and c-myc oncogene alterations in colon cancer cell lines. Dis Colon Rectum 34:469–474
25. Bos JL (1989) ras oncogenes in human cancer: a review. Cancer Res 49:4682–4689
26. Vogelstein B, Fearon ER, Hamilton SR et al (1988) Genetic alterations during colorectal-tumor development. N Engl J Med 319:525–532
27. Fearon ER, Vogelstein B (1990) A genetic model for colorectal tumorigenesis. Cell 61:759–767
28. Buyru N, Tezol A, Dalay N (2006) Coexistence of K-ras mutations and HPV infection in colon cancer. BMC Cancer 6:115
29. Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215
30. Evander M, Edlund K, Bodén E et al (1992) Comparison of one-step and two-step polymerase chain reaction with degenerate general primers in a population-based studies of human papillomavirus infection in young Swedish women. J Clin Microbiol 30:987–992
31. de Roda Husman AM, Walboomers JM, van den Brule AJ et al (1995) The use of general primers GP5 and GP6 elongated at their 3' ends with adjacent highly conserved sequences improves human papillomavirus detection by PCR. J Gen Virol 76:1057–1062
32. Lönn U, Lönn S, Nilsson B et al (1995) Prognostic value of erb-B2 and myc amplification in breast cancer imprints. Cancer 75:2681–2687
33. Hatzaki A, Razi E, Anagnostopoulou K et al (2001) A modified mutagenic PCR-RFLP method for K-ras codon 12 and 13 mutations detection in NSCLC patients. Mol Cell Probes 15:243–247
34. Ilyas M, Straub J, Tomlinson IP et al (1999) Genetic pathways in colorectal and other cancers. Eur J Cancer 35:1986–2002
35. Perez LO, Abba MC, Laguens RM et al (2005) Analysis of adenocarcinoma of the colon and rectum: detection of human papillomavirus (HPV) DNA by polymerase chain reaction. Colorectal Dis 7:492–495
36. Lee YM, Leu SY, Chiang H et al (2001) Human papillomavirus type 18 in colorectal cancer. J Microbiol Immunol Infect 34:87–91
37. Erisman MD, Rothberg PG, Diehl RE et al (1985) Deregulation of c-myc gene expression in human colon carcinoma is not accompanied by amplification or rearrangement of the gene. Mol Cell Biol 5:1969–1976
38. He QJ, Zeng WF, Sham JS et al (2003) Recurrent genetic alterations in 26 colorectal carcinomas and 21 adenomas from Chinese patients. Cancer Genet Cytogenet 144:112–118
39. Knösel T, Petersen S, Schwabe H et al (2002) Incidence of chromosomal imbalances in advanced colorectal carcinomas and their metastases. Virchows Arch 440:187–194
40. Al-Kuraya K, Novotny H, Bavi P et al (2007) HER2, TOP2A, CCND1, EGFR and C-MYC oncogene amplification in colorectal cancer. J Clin Pathol 60:768–772
41. He TC, Sparks AB, Rago C et al (1998) Identification of c-MYC as a target of the APC pathway. Science 281:1509–1512
42. Herrick J, Conti C, Teissier S et al (2005) Genomic organization of amplified MYC genes suggests distinct mechanisms of amplification in tumorigenesis. Cancer Res 65:1174–1179