SPANDIDOS PUBLICATIONS

Single or multiple HPV types in cervical cancer and associated metastases

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Received May 26, 2005; Accepted July 13, 2005

Abstract. Almost all cervical cancers are human papillomavirus (HPV)-positive. Some aspects of HPV carcinogenesis, such as factors involved in the transformation process and the mono- or polyclonal origin of the carcinogenic process, need to be defined. The latter aspect is addressed in our study. Cervical samples were collected from 102 patients with squamous cell carcinoma. The HPV positivity was established by PCR analysis performed using consensus and specific primers for the L1 and E6/E7 regions, respectively. Eighty-seven samples were positive for the L1 gene and 5 for the E6/E7 genes. Overall, 92 samples contained segments of HPV-DNA (90.2%). HPV-16 was most frequently found either alone or associated with other genotypes (63%). All genotypes identified as a single infection, except HPV-73, belonged to the high-risk HPV group. Among multiple infections, the HPV-31+54 couple was the most frequent. The presence of two genotypes in a primary tumor raises the question of their distribution in a single tumor cell. We attempted to answer this question by comparing the HPV patterns in primary tumors and metastases, considering that metastases derive from cell clones released from the primary tumor. The HPV patterns of primary tumors and metastases overlapped in most patients, even when primary tumors contained a double genotype, thus suggesting that single tumor cells may contain multiple HPV genotypes.

Introduction

To date, nearly 118 HPV genotypes have been identified (1), about 40 of which can infect the genital tract (2). Clinical, epidemiological and molecular evidence indicate that some HPV genotypes are the principal cause of both invasive cervical cancer and cervical intraepithelial neoplasia: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56 and 58, and have been classified as high-risk HPVs. Another twelve genotypes were classified as low risk: 6, 11, 40, 42, 43, 44, 54, 61,70, 72 and 81, and CP6108. Integration of the oncogenic HPV types into the cell genome is a critical event in malignant transformation. The mechanism of integration requires linearization of circular DNA and disruption of the E2 gene with consequent overexpression of the E6/E7 oncogenes, a key factor in oncogenic progression (3,4). Since HPV integration is an irreversible genetic alteration, it can be regarded as a marker of HPV-related tumors. HPV positivity allows us to distinguish metastases of cervical carcinoma from HPV-unrelated tumors (5). Luft *et al* demonstrated the monoclonal origin of cervical cancer by investigating human genomic sequences adjacent to integrated HPV (6).

Women with cervical dysplastic lesions can harbor single or multiple HPVs (7-9). The presence of multiple genotypes in cervical cancer has also been reported (10-13). However, in these reports, the physical state of multiple genotypes is not indicated. A study by Badaracco *et al* demonstrated that, one genotype was present in episomal form, while the second was integrated in some double-infected tumors (14).

The eventual presence of multiple genotypes integrated in the tumor cell genome raises the question as to how they are distributed in single tumor cells. Since metastases derive from clones of primary tumors, studies on HPV genotypes in primary tumors and their associated metastases could shed light on this issue. We investigated the presence and type of HPV in 102 cervical tumors. In some, we also compared the HPV patterns of primary tumors with those in related metastases.

Materials and methods

Study group. We analyzed 102 paraffin-embedded samples of squamous cell carcinoma (SCC) and the metastases related to 11 of these primary tumors. The histological material was provided by the Pathology Department of the University of Trieste. The average age of the patients was 67 years, and all were from the northeast region of Italy. Thirty-six patients presented metastases at diagnosis or during progression of disease.

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Key words: cervical cancer, human papillomavirus, multiple genotypes, metastases

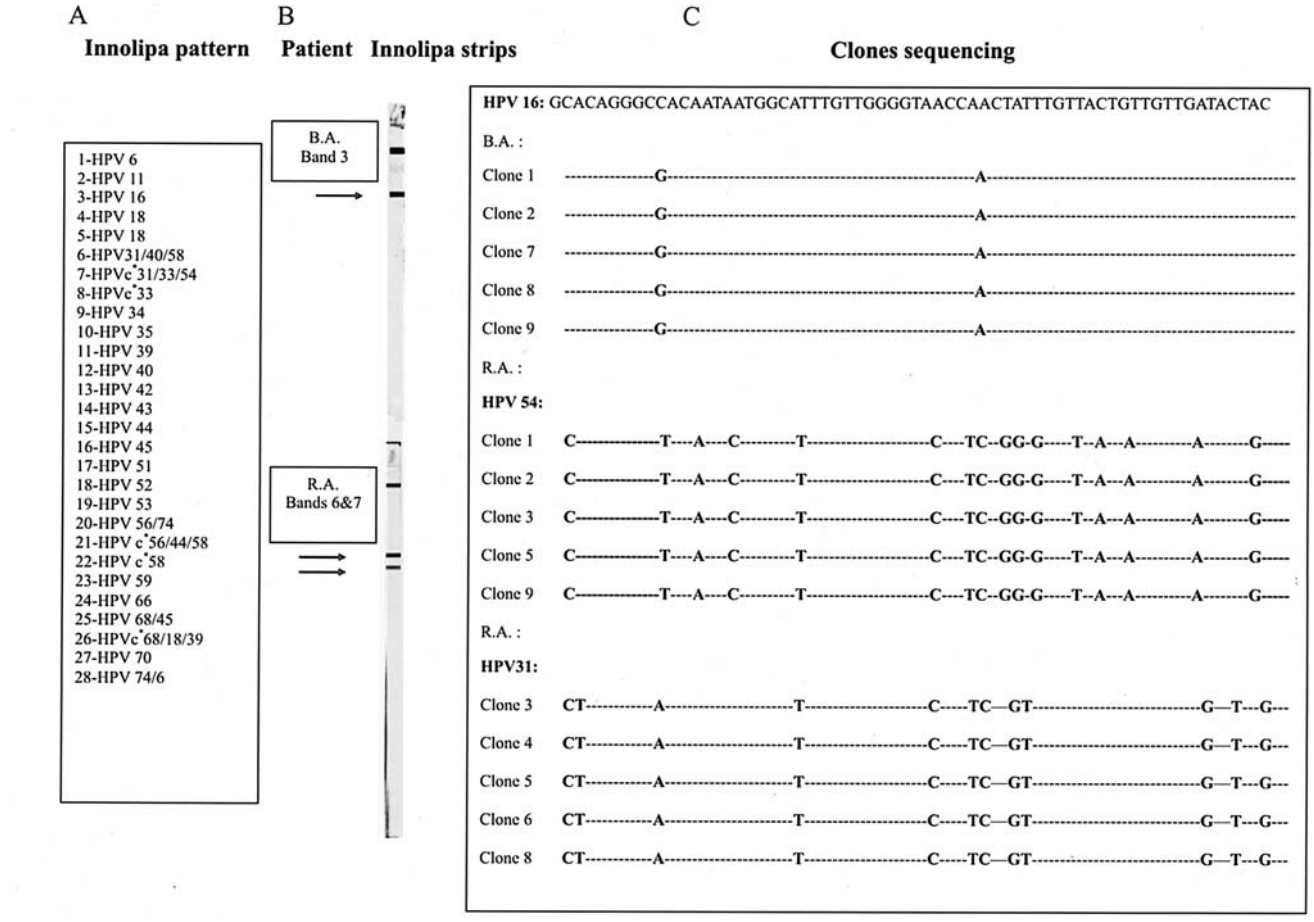

Figure 1. (A) Innolipa pattern. (B) Innolipa strips of metastases in patients with a single (B.A.) or double infection (R.A.). (C) Sequencing clones from metastases of patients, B.A. and R.A. Nucleotide sequence alignments were matched against HPV-16 (positions 6582 to 6646; GenBank accession no. K02718). Dashed lines indicate the presence of nucleotides identical to the HPV-16 consensus sequence.

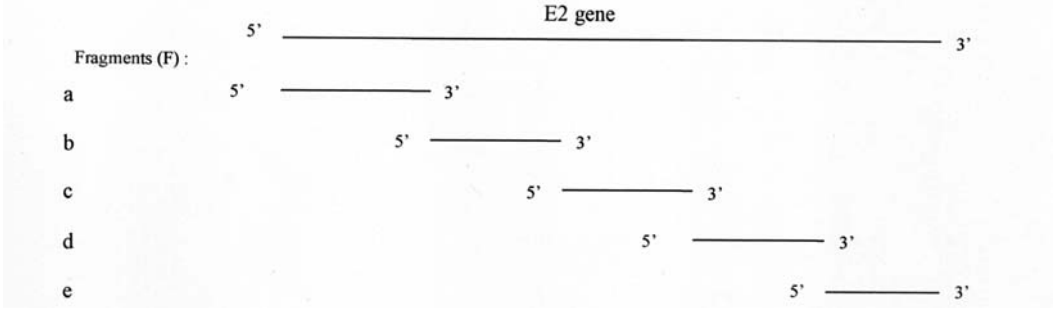

Figure 2. E2 analysis by PCR of E2 overlapping fragments. Each E2 fragment is indicated by a letter (a to e).

DNA extraction. Cervical sections of tumor tissues selected by our pathologist (5 μm-thick paraffin-embedded) were treated with xylene to remove paraffin, digested with ATL buffer and proteinase K o/n at 56˚C in a thermomixer, followed by DNA extraction according to the manufacturer's instructions (QIAamp DNA Mini kit, QmbG, Germany).

HPV screening by polymerase chain reaction. To verify the quality of DNA extracted from paraffin-embedded tissues, 5 μl of each sample was amplified with a set of primers recognizing the ß-actin gene, as described (15).

Each sample (primary tumor and metastasis) was tested for the presence of HPV using three sets of primers specific for the L1 gene: My09/My11 (16), $GP5^{+}/GP6^{+}$ (17) and the SPF primer mix (7). Positive and negative controls were included in each amplification.

Finally, in 5 samples that were negative for the L1 gene, HPV screening was carried out using PCR with primers encompassing the E6/E7 region of the most representative human papillomaviruses, according to Fujinaga *et al* (18).

HPV typing. All samples shown to be positive by PCR with SPF primers were typed by reverse hybridization assay (Innolipa) according to the manufacturer's instructions (Innogenetics, Ghent, Belgium). When reverse hybridization indicated the presence of multiple genotypes, the PCR product

2 primer list.

HPV type	PCR primers	Fragment length
16		
16FE2	5'-ATGGAGACTCTTTGCCAACG-3'	268
16RE2	5'-TCATTACTATATTGTGAGTTATA-3'	
16F2E2	5'-TATAACTCACAATATAGTAATGA-3'	279
16R2E2	5'-CTGCATCATCTTTAAACTGC-3'	
16F3E2	5'-GCAGTTTAAAGATGATGCAG-3'	280
16R3E2	5'-CACTGAGTCTCTGTGCAACA-3'	
16F4E2	5'-TGTTGCACAGAGACTCAGTG-3'	191
16R4E2	5'-GACACTGCAGTATACAATGTA-3'	
16F5E2	5'-TACATTGTATACTGCAGTGTC-3'	150
16R5E2	5'-TCATATAGACATAAATCCAGTAG-3'	
18		
18FE2	5'-ATGCAGACACCGAAGGAAACCCTT-3'	253
18RE2	5'-CCTTGTAGGGCCATTTGCAGTT-3'	
18F2E2	5'-AACTGCAAATGGCCCTACAAGG-3'	252
18R2E2	5'-ATCCCCTGTGACTTACACAGGT-3'	
18F3E2	5'-ACCTGTGTAAGTCACAGGGGAT-3'	242
18R3E2	5'-CCACGGACACGGTGCTGGA-3'	
18F4E2	5'-TCCAGCACCGTGTCCGTGG-3'	250
18R4E2	5'-GGTCGCTATGTTTTCGCAATCT-3'	
18F5E2	5'-AGATTGCGAAAACATAGCGACC-3'	186
18R5E2	5'-TTACATTGTCATGTATCCCACC-3'	
31		
31FE2	5'-GAGACTCTTTCTCAACGTTTA-3'	245
31RE2	5'-TTTAATGTTTCCAACATCATTTG-3'	
31F2E2	5'-CAAATGATGTTGGAAACATTAAA-3'	263
31R2E2	5'-CCTTCATGTACATAATAAATGC-3'	
31F3E2	5'-GCATTTATTATGTACATGAAGG-3'	272
31R3E2	5'- TGGTCGCTTAGTAGACGTC-3'	
31F4E2	5'-GACGTCTACTAAGCGACCA-3'	250
31R4E2	5'-GACACTTGTTCATACAATTGTTT-3'	
31F5E2	5'-AAACAATTGTATGAACAAGTGTC-3'	171
31R5E2	5'-AATAGTCATATATCCTGTTGA-3'	

Table I. Continued.

HPV type	PCR primers	Fragment length
52		
52FE2	5'-ATGGAGTCGATACCGGCA-3'	258
52RE2	5'-TTGTGTTTTGTTTAATGCCTCC-3'	
52F2E2	5'-GGAGGCATTAAAGAAAAGAGAA-3'	232
52R2E2	5'-CCCATAGTAATCTACTTGTC-3'	
52F3E2	5'-GACAAGTAGATTACTATGGG-3'	250
52R3E2	5'-TGTAGGTGTGTGTCTTTGGC-3'	
52F4E2	5'-GCCAAAGACACACACCTACA-3'	249
52R4E2	5'-GTGTTTTTACCCTATATCTTAA-3'	
52F5E2	5'-TTAAGATATAGGGTAAAAACAC-3'	201
52R5E2	5'-TCACAATGACATGACACCTTG-3'	
53		
53FE2	5'-ATGGAGACGCTATGCCAACG-3'	250
53RE2	5'-TACAAAGTGATTCCAATGCTATT-3'	
53F2E2	5'-AATAGCATTGGAATCACTTTGTA-3'	273
53R2E2	5'-TACGTTTTATGGCCGTCATG-3'	
53F3E2	5'-CATGACGGCCATAAAACGTA-3'	260
53R3E2	5'-TCTGTGGTTCTGGGACGTTT-3'	
53F4E2	5'-AAACGTCCCAGAACCACAGA-3'	250
53R4E2	5'-GTTTTTGAAACCGATACCTTAAA-3'	
53F5E2	5'-TTTAAGGTATCGGTTTCAAAAAC-3'	208
53R5E2	5'TTACATATCAACACATGTCATATG-3'	
54		
54FE2	5'-GAGACCCTAGCAACACGTTTA-3'	267
54RE2	5'-TTCGGTGCTGTATACTGTCT-3'	
54F2E2	5'-AGACAGTATACAGCACCGAA-3'	270
54R2E2	5'-TAAAATCCACATAATACACTTTG-3'	
54F3E2	5'-CAAAGTGTATTATGTGGATTTTA-3'	273
54R3E2	5'-GTTGCTGGTCTGTGCTGTAG-3'	
54F4E2	5'-CTACAGCACAGACCAGCAAC-3'	200
54R4E2	5'-CAAACAAATGTTTATACTTT-3'	
54F5E2	5'-AAAGTATAAACATTTGTTTG-3'	181
54R5E2	5'-TAATGACATTACCCCTAATGA-3'	

was cloned into pGEMT-easy vector (Promega, Madison, WI, USA) and sequenced using the Big Dye Terminator v1.1 Cycle Sequencing kit (Applied Biosystem, Warrington, UK). At least 10 clones were screened each time, and the obtained sequences were matched against all sequences deposited in GeneBank (nucleotide-nucleotide BLAST; http://www.ncbi.nlm.nih.gov/BLAST). The HPV genotype was assigned based on the highest score obtained and confirmed with two alignment sequences (BLAST 2 sequence) against the consensus sequence for that specific genotype.

Two representative cases of single or double genotypes are shown in Fig. 1. The HPV-positive samples based on $GP5^{+}/6^{+}$ PCR were typed by cloning and sequencing of the amplified product as described above. Finally, five samples that were positive for E6/E7 genes with primers specific for the most representative HPVs (18) were also confirmed by cloning and sequencing.

E2 PCR for determination of the physical state. Because the integration of HPV-DNA into the host cell genome disrupts the E2 gene, we investigated the physical state of HPV of this gene using PCR. This study was carried out in 9 out of 11 patients with HPV-positive metastases in different organs. In these patients, the integrity of E2 in HPVs found in primary tumors and metastases was established using PCR analysis of short overlapping fragments along the entire E2 gene, as shown in Fig. 2. Primers used in these experiments are listed in Table I.

The conditions of amplification for all identified HPVs except HPV-18 were: 1 cycle at 94˚C for 10 min, and 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 30 sec, for 40 cycles. A final extension step at 72˚C for 7 min was also performed. In the case of HPV-18 the PCR conditions were the same except for the annealing step, which was performed at 60˚C instead of 55˚C.

Table II. HPV genotypes associated with SCC.

HPV genotype	16	33	18	58	45	56	31	52	51	35	73	31+54	16+33	16+18	33+54	16+53	16+18
No.	52	6	5	4	3	3	2	2	1	1	1	4	3	2	1	1	1
Prevalence[a]	56.5	6.5	5.4	4.3	3.3	3.3	2.2	2.2	1.1	1.1	1.1	4.3	3.3	3.2	1.1	1.1	1.1

[a]Referenced as 92 HPV-positive cases.

Table III. Single and multiple HPV genotypes in primary tumors and related metastases.

		HPV type in metastases											
Patients	HPV type in primary tumor	Lymph node	Larynx	Liver	Large intestine	Small intestine	Perit[a]	Pancreas	Bladder	Pleura	Ovary	Vagina	Lung
A.A.	16	16	-	-	-	-	-	-	-	-	-	-	-
B.A.	16	16	-	-	16	16	16	-	-	-	-	-	-
S.L.	16	-	Neg	-	-	-	-	-	-	-	-	-	-
L.M.	16	-	-	Neg	-	-	-	-	-	-	-	-	-
M. L.	16	-	-	Neg	Neg	-	-	Neg	16	-	-	-	-
M.A.	16	-	-	16	-	-	-	-	-	-	-	-	-
O.M.	52	-	-	Neg	-	-	52	-	Neg	Neg	-	-	Neg
B.M.	16+18	-	-	-	-	-	16/18	-	-	-	-	-	-
Q. L.	31+54	-	-	-	-	-	-	-	-	-	-	31/54	-
T.M.A.	16+53	-	-	16/53	-	16/53	-	-	-	-	-	-	-
R.A.	31+54	31/54	-	-	-	-	-	-	31/54	-	-	31/54	-

[a]Perit, peritoneum.

Table IV. Fragment analysis of the E2 gene using PCR.

			Metastases						
Patients	HPV type	Primary tumor	Lymph node	Liver	Large intestine	Small intestine	Peritoneum	Bladder	Vagina
A.A.	16	F-----	F-----						
B. A.	16	F-----	F-----		F-----	F-----	F-----		
M.L.	16	F-----						F-----	
M.A.	16	F-----		F-----					
O.M.	52	F-----					F-----		
B.M.	16	Fabcde					Fa----		
	18	Fa----					Fa----		
Q.L.	31	Fabcde							F-----
	54	F-----							F-----
T.M.A.	16	Fabc-e		F-bc--		F-----			
	53	F-----		F-----		F-----			
R.A.	31	Fab---	Fa----					F----e	Fa----
	54	F-----	F-----					F-----	F-----

Results

This study was performed on sections of paraffin-embedded histological samples of SCC. Cervical samples were collected from 102 patients undergoing biopsy, colposcopy or surgery. HPV positivity was established by PCR analysis performed using consensus and specific primers for the L1 and E6/E7 region, respectively. Eighty-seven samples were positive for the L1 gene: 32 for MY09/11 + SPF + GP5$^+$/6$^+$, 27 for SPF + GP5$^+$/6$^+$, 17 for SPF, and 11 for GP5$^+$/6$^+$. Another 5 samples,

re negative for the L1 gene, were positive for E6/E7 ng primers specific for HPV-16, -18, and -31 (data not shown). Overall, 92 samples contained segments of HPV-DNA (90.2%). Innolipa and sequence analysis showed that 12/92 samples contained two genotypes (13%), while 80 contained one genotype (87%).

Genotyping of single and double infections is reported in Table II. HPV-16 was detected in 52 cases as a single genotype (56.5%) and in 4 cases as associated with other genotypes. The frequencies of other types detected in single infections (HPV-33, -18, -58, -45, -56, -31, -52, -51, -35, and -73) varied from 6.5% of HPV-33 to 1.1% of HPV-51, -35, and -73.

All genotypes identified in single infections, except HPV-73, belonged to the high-risk HPV group. Among multiple infections, the HPV 31+54 couple was the most frequent.

Eleven patients who died during this study were further investigated to compare the HPV type found in the primary tumors with that of its associated metastases. The autopsy samples were handled carefully to avoid contamination between primary tumors, adjacent tissues and metastases. As shown in Table III, 7 patients showed a single genotype in the primary tumor, while others had a double genotype. Seven patients with either a single or double genotype showed the same HPV pattern in both the primary tumor and metastases (A.A., B.A., M.A., B.M., T.M.A., Q.L. and R.A.), even when the metastases of each subject were multiple. In a second group of patients (S.L. and L.M.), the metastases were HPV-negative. Finally, in a third group of patients (O.M. and M.L.) with multiple metastases, HPV was present in one metastatic site, but not the others.

Since 9 out of 11 patients showed the same HPV pattern in their primary tumors and associated HPV-positive metastases, a question arises about the physical state of viruses in the two compartments. We tried to answer this question by amplifying short segments of the E2 gene, as shown in Fig. 2. In all primary tumors except two (B.M. and Q.L.), we found a total or partial loss of the E2 gene (Table IV), indicating that the virus is integrated. The primary tumor of patient B.M. contained the genotypes HPV-16 and -18. Short fragment analysis of the E2 gene showed that HPV-18 was integrated. In contrast, the E2 gene of the coinfecting HPV-16 was intact, suggesting that the virus was present in episomal form. A similar picture was found in the primary tumor of patient Q.L. where HPV-54 was integrated, while HPV-31 was present in episomal form. Interestingly, both the single or multiple viruses identified in metastases showed a partial or total loss of the E2 gene, suggesting that all viruses present in metastatic tissues were integrated.

Discussion

HPV-DNA was detected in 92 out of 102 SCC (90.2%) using PCR analysis performed with different sets of primers. This choice was based on the finding that integration of HPV-DNA into the host genome can cause a partial loss of the L1 gene in cervical cancers (24). Furthermore, amplification of a relatively long fragment (My09/11) may be difficult in paraffin-embedded tissues. Thus, primers like $GP5^+/GP6^+$ and SPF, which amplify a short fragment of the L1 gene, have a higher degree of sensitivity for detection of HPV (7,17). Our results confirm the relationship between cervical cancer and HPV infection. HPV genotyping of our samples shows that the HPV-positive SCC harbored either a single (80/92, 87%) or double genotype (12/92, 13%). All genotypes detected as a single infection belonged to the high-risk HPV group. Only one case harbored the HPV-73 genotype that, based on phylogenetic analysis, was classified as low risk (1). Munoz *et al*, however, detected HPV-73 in cervical cancer (13).

In this study, 10 out of 12 tumors with a double infection contained high-risk genotypes. In the remaining 2 cases, the high- and low-risk genotypes of HPV-33/54 and HPV-16/53 were associated.

It is well known that a consistent number of dysplastic lesions of the cervix can contain multiple HPV types (7-9). Interestingly, the presence of multiple HPV types has also been found in cervical cancers (10-13). The contemporary presence of two different genotypes in a tumor raises two important issues: i) are they present in the same or different cells? and ii) are they integrated? We tried to answer these questions by comparing HPV patterns in primary tumors and metastases. It was particularly important to verify whether a double genotypic pattern was present in both the primary tumor and derived metastases. In the case of two genotypes integrated in a single cell of the primary tumor, we expected to also find both in metastases. In fact, metastases are clonal and originate from cells released from primary tumors (19-22), and the clonal origin of metastases has also been clearly shown in experimental models using tumor cells tagged with different markers (23).

We compared the HPV pattern of 11 patients with primary tumors containing one or two HPV genotypes. In 7 of these patients, 3 who were single-infected and 4 double-infected, the HPV patterns in primary tumors and metastases overlapped (A.A., B.A., M.A., B.M., Q.L., T.M.A. and R.A.). In the remaining 4 subjects, HPV in metastases was absent (S.L. and L.M.) or present in one metastatic site but not in others (M.L. and O.M.). Since the HPV-negative metastases concerned unusual sites of HPV carcinogenesis, we believe that there was a presence of HPV-unrelated primary tumors as reported by other authors (5).

The second question concerns the physical state of virus(es) in primary tumors and associated metastases. A study by Daniel *et al* reported that HPV was integrated in all examined cervical cancers (26). However, the physical state of HPV genotypes in multiple-infected SCC and metastases has not been reported. A study by Badaracco *et al* described that some double-infected cervical tumors contained one integrated HPV genotype, while the second was present in episomal form (14). This prompted us to further investigate the physical state of the E2 gene in 9 primary tumors and their HPV-positive related metastases. To this end, we investigated the physical state of the E2 gene in cervical cancer and metastases by amplifying short overlapping fragments of this gene.

This PCR strategy indicated that most of the genotypes detected in primary tumors contained cleaved or completely deleted E2 genes, suggesting the integration state of the virus in these tumors. Only in 2 cases with double infection,

B.M. (HPV 16+18) and Q.L. (HPV 31+54) did the physical state of the two viruses appear discordant. In fact, in patient B.M., we found that HPV-16 was in episomal form and HPV-18 was integrated, using PCR of the E2 gene. Similarly, in patient Q.L., HPV-31 was present in episomal form, while HPV-54 was integrated. However, considering that all viruses detected in the metastases of patients B.M. and Q.L. showed a disrupted E2 gene, we believe that integrated forms of HPV-16 and -31 were already present in the primary tumors.

The most significant finding of our study is that the same genotype pair of HPV was detected in both primary tumors and all related metastases in double infections. Considering that the integration of one virus is independent from that of a second, this was unexpected. Thus, we hypothesize that, in the emerging cervical tumor, cells containing two genotypes have a selective advantage both in growth and metastasis with a negative impact on tumor progression.

Acknowledgements

The project was in part supported by Progetto speciale Ministero della Sanità-Regione Abruzzo 2003 (Meccanismi di cancerogenesi da HPV. Problemi correlati al processo di integrazione virale e all'infezioni di multipli genotipi.

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