Acta Oncologica, 2005; 44: 694–699

Taylor & Francis
Taylor & Francis

ORIGINAL ARTICLE

Human papilloma virus (HPV) is rarely detected in malignant melanomas of sun sheltered mucosal membranes

LISELOTTE DAHLGREN[1], KJELL SCHEDVINS[2], LENA KANTER-LEWENSOHN[3], TINA DALIANIS[1] & BOEL K. RAGNARSSON-OLDING[1,4]*

[1]*Department of Oncology-Pathology, Karolinska Institute, Cancer Center Karolinska, Karolinska University Hospital, 171 76 Stockholm, Sweden,* [2]*Department of Gynecology, Karolinska University Hospital, 171 76 Stockholm, Sweden,* [3]*Department of Pathology, Karolinska University Hospital, 171 76 Stockholm, Sweden and* [4]*Department of Oncology, Radiumhemmet, Karolinska University Hospital, 171 76 Stockholm, Sweden*

Abstract

Human papillomavirus (HPV) has been associated with some types of human cancer. The aim of this study was to investigate if HPV could be associated with human primary malignant melanoma in non sun-exposed body areas like mucous membranes. Through the Swedish National Cancer Registry, in compliance with the rules of the Human Ethical Committee, histopathological specimens were collected from different pathological laboratories throughout Sweden. The histopathological diagnosis was reviewed, and from 45 primary melanomas, tumour tissue was micro-dissected and analysed further. A protocol for detection of HPV DNA using general HPV primers GP5+/GP6+ or CPI/IIG, which together identify 36 different HPV subtypes, was developed. This protocol could detect presence of HPV DNA in less than 10 ng of DNA of a control cell that contained 1- 2 copies of HPV type 16/cell. Before HPV testing the melanoma samples were examined for amplifiable DNA by a β-microglobulin PCR and 39 were positive. Thirty-five of these could be evaluated for HPV DNA and no samples were positive according to all five defined criteria for HPV positivity although two were positive according to 4/5 criteria. In conclusion, HPV is rarely detected in primary malignant melanomas of non-sun exposed body areas.

Malignant melanomas emerging in sun-protected areas of the body, like in the mucous membranes, and in the border zone between skin and mucous membrane are often named extracutaneous melanomas. Melanomas in tissues shielded from sunlight, particularly those in mucosal membranes e.g. in the vulva, and melanomas in chronically sun exposed skin have revealed significant epidemiological, clinical, histopathological, and molecular-genetical differences [1,2]. Interestingly, however, significant histopathological differences also remain between the hairy and the glabrous skin (mucosal) compartments of the vulva, i.e. in the same sun-shielded anatomical site [1]. Presumably, since ultraviolet radiation alone cannot account for such a range of differences, other etiological factors or co-factors have to be searched for. Viruses might be such factors.

Furthermore, although UV radiation has been identified as a main risk factor for cutaneous melanomas of the superficial malignant melanoma (SMM), and lentigo malignant melanoma (LMM) types it does not account as etiologic factor for all melanomas. UV radiation is questionable for the development of the nodular melanoma (NM) type and has no association with the acral lentiginous (ALM) and mucosal lentiginous melanoma (MLM) types (reviewed in [3]). Thus, there are subgroups of melanomas not caused by UV radiation. With the increasing incidence of cutaneous melanomas there is an urgent need to search for etiological and pathogenic factors other than, or additional to, UV radiation. In the search of UV independent factors in melanoma genesis, factors involved in the development of extracutaneous melanomas may be of considerable interest. As mentioned above, such a

Correspondence: Boel K. Ragnarsson-Olding, Radiumhemmet, Karolinska University Hospital, SE-171 76 Stockholm, Sweden. Fax; +46 8 5177 6290 Tel: +46 8 5177 2733 e-Mail: Boel.Ragnarsson-Olding@cck.ki.se

(Received 21 December 2004; accepted 30 May 2005)

ISSN 0284-186X print/ISSN 1651-226X online
DOI: 10.1080/02841860500247461

factor could be a virus, and a candidate of interest could be human papillomavirus (HPV) previously described in skin cancer [12].

The HPV family includes a multitude of different genotypes and so far close to 100 HPV types have been identified based on isolation of complete genomes [4]. Furthermore, several sub genomic sequences have been found, suggesting additional putative novel HPV types [4]. Most HPVs are epitheliotropic and have been divided into "high risk" types, which are mainly associated to malignant tumours and "low risk" types, which are mainly associated with benign papillomas and warts. The genome of HPV codes for at least six different early and two late proteins. The early proteins are involved in viral replication and the late proteins build up the viral capsid and are involved in the maturation and release of viral particles. In the oncogenic HPVs three early proteins (E5, E6, and E7) are regarded to have growth-stimulating properties and two of these (E6 and E7) are classified as oncoproteins based on their ability to transform cells in vitro [5]. E6 expression has been shown to down regulate cellular p53 levels by binding to the E6 associated protein and the formed complex then interacts with p53 leading to its degradation [6]. The E7 protein exerts its proliferation stimulating properties by binding to pRb, which leads to liberation of the E2F transcription factor and promotion of the cell cycle [7]. These interactions are suggested to lead to chromosomal instability in infected cells since cell-cycle checkpoints are over run.

The aim of the present report was to examine if HPV plays a role in the aetiology of human primary melanomas in non sun-exposed body areas. For this purpose, archive materials of formalin fixed and paraffin embedded extracutaneous melanomas, were investigated for the presence of HPV DNA by PCR.

Material and method

Subjects and tumour characterisation

With permission from the Swedish Data Inspection Authority, and with approval by the Human Ethical Committee at the Karolinska Institute, we collected clinical records, pathology reports and histopathological specimens from 64 patients with primary melanomas in mucosal membranes. All histological slides of mucosal melanomas were reviewed and only primary melanomas, not metastases, were used for further analysis. For technical reasons only 45 cases could be analysed for presence of HPV DNA; cases with a shortage of tumour tissues in the paraffin blocks were excluded. Similarly, cases with a heavy infiltration of lymphoid cells were omitted in order to diminish the risk for contamination by HPV DNA in those cells. The sites of these 45 melanomas were; anus 15, rectum 4, sinonasal 9, vulva 7, vagina-cervix 4, tongue 1, penis 1, subungual 2, and on the skin 2 (see Table I).

Preparation of the samples and DNA extraction

From each paraffin block of formalin fixed tissue, one 4 μm and two 20 μm thick consecutive sections were cut. Cryostat knives and scalpels were washed in 70% ethanol between each preparation. Furthermore, each cryostat-cut section was stretched on a drop of sterile, distilled water placed on each slide instead of a regular water bath. The 4 μm sections were stained with haematoxylin and eosin to guide the dissection of tumour tissue to be used for DNA analysis. A dissection of tumour tissues, resulting in histologically about 90% pure tumour samples for analysis, was performed in each case in order to avoid potential contamination of HPV from surrounding tissues. In 9 cases the whole biopsy contained tumour tissue and any further dissection was not necessary.

DNA was extracted using the "High Pure RNA extraction kit" (Roche, Diagnostics GmbH, Mannheim, Germany) according to the manufacturers protocol but with exclusion of DNase treatment. In short; the paraffin was removed by treatment with xylene and ethanol. The tissue pellet was disrupted by overnight incubation at 55°C in tissue lysis buffer with addition of SDS and Proteinase K. On day two, the DNA was bound to a membrane in collection tubes, washed and eluted by elution buffer. The Proteinase K treatment was repeated once (incubation for 1 hour at 55°C) for removal of trace proteins. The DNA was bound to the membrane in a fresh collection tube, washed, eluted and finally the DNA amount and purity was measured by NanoDrop technology (NanoDrop Technology Inc., Wilmington, DE). To avoid and check for HPV carry over between the melanoma samples tubes with a slice from an empty paraffin block was placed inbetween the samples and treated in the same way as the melanoma samples through out the experimental procedures.

PCR protocol for detection of HPV

To detect possible presence of HPV DNA in the melanoma samples, a PCR protocol for the iCycler (BioRad, Hercules, CA) was developed based on general HPV primers and SYBR Green fluorescence. The SYBR Green dye binds to double stranded DNA and emits a fluorescent light at 490 nm that is proportional to the amount of bound

Table I. Extracutaneous melanomas are rarely HPV positive when assayed by general HPV PCR, type specific HPV PCR and sequencing

		General HPV PCR				
Code[1]	Site	Ct-value (>300 RFU)"	Melting point (>250 RFU)	Size (bp)	HPV-type specific PCR	Sequencing of PCR fragment
Mm5	vulva	neg	neg	neg	nd	nd
Mm6	cervix	neg	neg	neg	nd	nd
Mm7	rectum	neg	neg	neg	nd	HPV 33
Mm10	rectum	neg	neg	neg	nd	nd
Mm11	anus	neg	neg	neg	nd	nd
Mm13	subungual	neg	neg	neg	nd	nd
Mm14	nasal cavity	neg	neg	neg	nd	nd
Mm19	anus	neg	neg	neg	nd	nd
Mm28	subungual	neg	neg	neg	nd	nd
Mm32	vagina	neg	neg	neg	nd	nd
Mm41	vulva	neg	neg	neg	nd	nd
Mm44	penis	neg	neg	neg	nd	nd
Mm47	anus	neg	neg	neg	nd	nd
Mm48	vulva	neg	neg	neg	nd	nd
Mm50	rectum	neg	neg	neg	nd	nd
Mm51	anus	neg	neg	neg	nd	nd
Mm52	anus	neg	neg	neg	nd	nd
Mm53	anus	neg	neg	190	nd	nd
Mm55	anus	neg	neg	neg	nd	nd
Mm56	anus	neg	neg	neg	nd	nd
Mm58	anus	neg	neg	neg	nd	nd
Mm59	anus	neg	neg	190	nd	nd
Mm63	cutaneous	neg	neg	neg	nd	nd
Mm1	sinus	37.8	<250	neg	neg	nd
Mm8	anus	38.2	<250	neg	neg	nd
Mm33	nasal cavity	38.3	<250	nd	nd	nd
Mm35	nasal cavity	36.8	<250	nd	nd	nd
Mm40	vagina	37	<250	nd	nd	nd
Mm61	nasal cavity	37.4	<250	190	nd	nd
Mm62	nasal cavity	36.8	<250	nd	nd	nd
Mm9	vulva	35.7	81°	neg	neg	nd
Mm42	vagina	36	80.5°	neg	nd	nd
Mm18	tongue	38.5	81.5°	190	neg	nd
Mm15	rectum	34.6	80.5° & 85°	190	neg	HPV-16
Mm21	nasal cavity	36.9	81.5°	190	HPV-16 & -33	no HPV resemblance

[1]Four of the analyzed samples (Mm 4, 17, 22 and 37) were not possible to evaluate due to background reactions in the blanks.
[2]neg; negative result, nd; analysis not done.

DNA. First a gradient ranging from 40–60°C for the GP5+/6+ primer set [8] and 48–60°C for the CPI/IIG primer set [9] was run to determine the optimal annealing temperature for the primers. To determine the specific melting points for various HPV types a total of approximately 100 pg HPV plasmid per well was run with each primer set for HPV types -6, -11, -16, -18 and -33 alone, or in the combinations; HPV-6 and -16, HPV-6 and -11, HPV-16 and -18, HPV-16 and -33, HPV-6, -16 and -33, HPV-6, -11, -16, -18 and -33. This protocol had hence the capacity to test 36 of the most commonly found HPV subtypes. GP5+/6+ detects HPV-6, -11, -13, -16, -18, -30-33, -35, -39-40, -43, -45, -51-52, -54-56, -58-59, -66, while CPI/IIG detects HPV-1-8, -10-12, -14, -16, -18-22, -24-25, -31, -33, -36, -46. Finally a detection limit experiment was run with paraffin embedded SiHa cells, known to harbour around 1–2 HPV type 16 copies/cell [10], ranging from 500–0.01 ng per well to determine the sensitivity of the protocol.

HPV DNA detection by PCR

Ten melanoma samples were run in the GP5+/6+ SYBR Green protocol. The reaction mixture consisted of 12.5 μl iQ™ SYBR® Green mix (BIO RAD Laboratories, Hercules, CA), 1.25 μl forward primer (10 pmol/μl), 1.25 μl reverse primer (10 pmol/μl), 10 μl sample (100 ng). The PCR started with the initial steps; 50°C for 2 minutes and 95°C for 10 minutes, than 40 cycles were run with 95°C for 15 seconds, 50°C for 30 seconds and 71°C for 30 seconds followed by 40°C for 30 seconds. Finally a melt curve was run starting at 40°C and increasing by 0.5°C every 10th second until 120°C was reached.

All PCR products were then run on a 3% agarose gel containing ethidium bromide and visualised under UV illumination.

Thirty-nine melanoma samples were run in the CPI/IIG SYBR Green protocol. The reaction mixture consisted of 12.5 μl iQ™ SYBR® Green mix (BIO RAD Laboratories, Hercules, CA), 0.85 μl forward primer (10 pmol/μl), 1.3 μl reverse primer (10 pmol/μl), 0.35 μl water and 10 μl (100 ng) sample. The PCR started with the initial steps of 50°C for 2 minutes and 95°C for 10 minutes, than 40 cycles were run with 95°C for 15 seconds, 55°C for 30 seconds and 71°C for 30 seconds followed by 40°C for 30 seconds. Finally a melt curve was run starting at 40°C and increasing by 0.5°C every 10th second until 120°C was reached.

All PCR products were then run on a 3% agarose gel containing ethidium bromide and visualised under UV illumination.

Typing of HPV

To verify presence of HPV all amplicons with a length between 130–150 bp (GP5+/6+) or 190 bp (CPI/IIG) were sequenced as previously described [11] or run in a type specific PCR protocol as previously described [11].

Verification of amplifiable DNA

To avoid false negative results due to DNA unsuitable for PCR analysis a control PCR with β-microglobulin primers was run in 100–120 ng DNA. Primers for the human β-microglobulin gene were designed using Clone Manager Professional Suite, Primer Designer 4, version 4.20. The sequence for the forward primer was GTG GAG CAT TCA GAC TTG TC and for the reverse primer ACG CGA GGC ATA CTC ATC TT. The PCR mixture, in a total of 50 μl, consisted of 5 μl PCR buffer II (Applied Biosystems, manufactured by Roche Molecular Systems Inc. Branchburg, New Jersey), 8 μl dNTP (1.25 mM/dNTP, Invitrogen™, life technologies, Carlsbad, CA), 3 μl $MgCl_2$ (25 mM), 2 μl of each primer (10 pmol/μl) and 1 U of Taq Polymerase (Applied Biosystems). Amplification was run in an automated thermocycler (GeneAmp PCR system 9700, Applied Biosystems). The PCR program was initiated with 94°C for 1 min followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 60°C for 30 seconds and elongation at 72°C for 45 seconds, the program ended with a 5 minute hold at 72°C. All products were then run on a 3% agarose gel containing ethidium bromide and visualised under UV illumination.

Results

Details of the PCR protocol for detection of HPV

The optimal temperature for the GP5+/6+ primers in the SYBR Green iCycler system was 50°C and annealing was hence performed at 50°C. The GP5+/6+ protocol was most sensitive for HPV-11 followed by HPV-18, -33, -6 and -16. HPV-16 could be detected in all cases of paraffin embedded SiHa, down to 10 ng/well and in most cases down to 0.01 ng/well.

The optimal temperature for the CPI/IIG primers in the SYBR Green iCycler system was 55°C and annealing was hence performed at 55°C. The CPI/IIG protocol was equally sensitive for HPV-11 and -18 followed by -33, -6 and -16. HPV-16 could, with the CPI/IIG protocol, be detected in all cases of paraffin embedded SiHa cells down to 10 ng/well and in most cases down to 0.01 ng/well, which is similar to the Gp5+/6+ protocol.

For samples to be considered as HPV positive, irrespective of protocol, five criteria had to be fulfilled. Firstly, the fluorescence value had to reach a threshold of at least 300 RFU. Secondly, the melt point for the amplicon had to be specific and have a 250 RFU drop in fluorescence at the melting point. Thirdly, the amplicon should have the approximate length of 130–150 bp when run in the GP5+/6+ protocol, or 190 bp when run in the CPI/IIG protocol. Fourthly, when sequenced the PCR product should result in an HPV sequence. Finally, presence of HPV had to be possible to confirm by type specific PCR.

In summary, this method had a sensitivity, which allowed for the detection of 0.1 HPV copies/cell in paraffin embedded material.

Amplifiable DNA was detected by PCR in mucosal melanoma samples

Thirty-nine of 45 tested melanoma samples had amplifiable DNA, since they were positive for β-microglobulin by PCR and these were tested further for presence of HPV.

HPV DNA was rarely detected by PCR in mucosal melanoma samples

Ten melanoma samples were tested for presence of HPV DNA by the GP5+/6+ protocol and none of these 10 samples harboured any detectable HPV DNA using this method (Table I).

Thirty-nine melanoma samples were tested for presence of HPV DNA by the CPI/IIG protocol. Twenty-three samples showed no signs of HPV DNA using this method (Table I). Four samples

could not be evaluated due to an unspecific background. Twelve melanoma samples gave indication of presence of HPV DNA, but were considered HPV negative since they did not fulfil the criteria above. Seven of the 12 samples did not have the right melting point and 2 samples since produced amplicons with the wrong size. Of the remaining 3 samples one was considered negative since it was not possible to sequence and it was negative in the type specific PCR. The final 2 samples were positive by 4 of the 5 criteria. One showed no resemblance to HPV when sequenced, even though it was positive for HPV-16 and HPV-33 in the type specific PCRs. The other sample was negative in the type specific PCRs for HPV-16 and HPV-33, although it showed resemblance with HPV-16 when sequenced (Table I).

:

Discussion

Several reasons compelled us to investigate the presence of HPV DNA in extracutaneous melanomas. Firstly, several types of HPV have been found to cause specific types of cancers [12,13], but, to our knowledge, only occasional investigations of HPV in cutaneous melanomas [14–17], and only one on HPV in extracutaneous melanoma i.e. in the vulva [18], have been published. Secondly, HPV can often be isolated from the vaginal/vulvar, ano/rectal, and sino/nasal mucosal membranes (reviewed in [12]). Thirdly, in previous studies we have shown that the frequency of TP53 mutations, assayed by sequencing [19], and p53 over-expression, assayed by immunohistochemistry [20], did not differ significantly between melanomas from chronically sun-exposed skin and extracutaneous melanomas. However, the extracutaneous melanomas showed a higher frequency of TP53 silent mutations than the cutaneous ones, which may speak for a genomic instability [21]. Furthermore, the presence of HPV in melanomas with e.g. degradation of the p53 protein would be a possible way to explain genomic instability with ensuing unlimited cell growth and progression into malignancy. This led us to look for an alternative agent in melanomas on non sun-exposed body areas.

However, 33/36 of the melanomas of non-sun-exposed body areas possible to evaluate were not HPV positive by most criteria ($\leq$2/5 criteria fulfilled). One fulfilled 3/5 criteria and only 2 melanomas fulfilled 4/5 criteria.

This outcome was despite a PCR protocol detecting around 0.1 HPV copies/cell. In fact, HPV-16 was detected in 10 ng cellular SiHa DNA (corresponding to around 2000 SiHa cells) and since SiHa cell harbours 1–2 copies of HPV-16 per cell [10] the method used could detect down to 4000 copies of HPV. To be well above the detection limit, 100 ng of total DNA was included in each well potentially allowing the detection of one single HPV copy per 10 cells, a level which is well above a reasonable range to assess a functional virus.

The fact that HPV is generally not detected in malignant melanomas could be due to that the melanocyte is of neuroepithelial origin and all identified HPV types appear to be strictly epitheliotropic [22,23].

Although there is no evidence of HPV DNA in most of the melanoma samples in this study, some previous data suggest that cutaneous HPV might be important for tumour initiation and progression – by means of a "hit-and-run" mechanism of carcinogenesis [24]. Evidence for such a mechanism has been proposed with regard to bovine papillomavirus type 4 infections of the alimentary tract in cattle [25], however, if this is the case here it can only be speculated upon.

In summary, the present investigation clearly indicates that the 36 HPV subtypes tested do not play a major role in the development of extracutaneous melanomas. The search for agents other than UV radiation has to be continued.

Acknowledgements

This investigation was supported by the Swedish Research Council (VR), The Swedish Cancer Foundation, The Stockholm City Council, The Karolinska Institute, and Radiumhemmets Research Funds; King Gustav V′s Jubilee Fund and The Stockholm Cancer Society.

References

[1] Ragnarsson-Olding BK, Kanter-Lewensohn LR, Lagerlöf B, Nilsson BR, Ringborg UK. Malignant melanoma of the vulva in a nationwide, 25-year study of 219 Swedish female: clinical observations and histopathological features. Cancer 1999;86(7):1273–84.

[2] Jiveskog S, Ragnarsson-Olding B, Platz A, Ringborg U. N-ras mutations are common in melanomas from sun-exposed skin of humans but rare in mucosal membranes or unexposed skin. J Invest Dermatol 1998;111:757–61.

[3] Ragnarsson-Olding BK. Primary malignant melanoma of the vulva - An aggressive tumor for modeling the genesis of non-UV light-associated melanomas. Acta Oncol 2004;43(5): 421–35.

[4] de Villiers E-M, Fauquet C, Broker TR, Bernard H-U, zur Hausen H. Classification of papillomaviruses. Virology 2004; 324(1):17–27.

[5] Syrjanen S, Syrjanen K. New concepts on the role of human papillomavirus in cell cycle regulation. Ann Med 1999; 31(3):175–87.

[6] Scheffner M, Werness BA, Huibregtse JM, Levine AJ, Howley PM. The E6 oncoprotein encoded by human

papillomavirus types 16 and 18 promotes the degradation of p53. Cell 1990;63(6):1129–36.

[7] Dyson N, Howley P, Munger K, Harlow E. The human papilloma virus-16 E7 oncoprotein is able to bind to the retinoblastoma gene product. Science 1989;243(4893): 934–7.

[8] de Roda Husman A, Walboomers J, van den Brule A, Meijer C, Snijders P. 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 1995;76(4):1057–62.

[9] Tieben L, ter Schegget J, Minnaar R, Bouwes Bavinck J, Berkhout R, Vermeer B, et al. Detection of cutaneous and genital HPV types in clinical samples by PCR using consensus primers. J Virol Methods 1993;42(2-3): 265–79.

[10] Baker C, Phelps W, Lindgren V, Braun M, Gonda M, Howley P. Structural and transcriptional analysis of human papillomavirus type 16 sequences in cervical carcinoma cell lines. J Virol 1987;61(4):962–71.

[11] Mellin H, Dahlgren L, Munck-Wikland E, Lindholm J, Rabbani H, Kalantari M, et al. Human papillomavirus type 16 is episomal and a high viral load may be correlated to better prognosis in tonsillar cancer. Int J Cancer 2002; 102(2):152–8.

[12] zur Hausen H. Papillomavirus infections - a major cause of human cancers. Biochimica et Biophysica Acta 1996;1288: F55–78.

[13] zur Hausen H. Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. J Natl Cancer Inst 2000;92(9):690–8.

[14] Dreau D, Culberson C, Wyatt S, Holder WJ. Human papilloma virus in melanoma biopsy specimens and in relation to melanoma progression. Ann Surg 2000;231(5): 664–71.

[15] Miracco C, Lavergne D, Nyongo A, Tosi P. Malignant melanomas: Search for human papillomaviruses. Arch Dermatol 2001;137(6).

[16] Scheurlen W, Gissman L, Gross G, zur Hausen H. Molecular cloning of two new HPV types (HPV 37 and HPV 38) from a kerato-acanthoma and a melanoma. Int J Cancer 1986;37:505–10.

[17] Takamiyagi A, Asato T, Nakashima Y, Nonaka S. Association of human papillomavirus type 16 with malignant melanoma. Am J Dermatopathol 1998;20(1):69–73.

[18] Rohwedder A, Philips B, Malfetano J, Kredentser D, Carlson AJ. Vulvar malignant melanoma associated with human papillomavirus DNA: Report of two cases and review of literature. Am J Pathol 2002;24(3):230–40.

[19] Ragnarsson-Olding BK, Karsberg S, Platz A, Ringborg UK. Mutations in the TP53 gene in human malignant melanomas derived from sun-exposed skin and unexposed mucosal membranes. Melanoma Res 2002;12:453–63.

[20] Ragnarsson-Olding B, Platz A, Olding L, Ringborg U. p53protein expression and TP53 mutations in malignant melanomas of sun-sheltered mucosal membranes vs. chronically sun-exposed skin. Melanoma Res 2004;14(5):395–401.

[21] Strauss BS. Silent and multiple mutations in p53 and the question of the hypermutability of tumors. Carcinogenesis 1997;18:1445–52.

[22] Le Poole IC vdBF, van den Wijngaard RM, Galloway DA, van Amstel PJ, Buffing AA, Smits HL, et al. Generation of a human melanocyte cell line by introduction of HPV16 E6 and E7 genes. In Vitro Cell Dev Biol Anim 1997;33(1):42–9.

[23] Howley PM. Papilloma viridae and their replication. In: Field BN, Knipe DM, et al, editors. Virology, 2nd ed. New York: Ravenpress ltd; 1990. p. 1625–50.

[24] Pfister H. Human papillomavirus and skin cancer. J Natl Cancer Inst Monogr 2003(31):52–6.

[25] Campo M, Moar M, Sartirana M, Kennedy I, Jarrett W. The presence of bovine papillomavirus type 4 DNA is not required for the progression to, or maintenance of, the malignant state in cancers of the alimentary canal in cattle. EMBO J 1985(4):1819–25.