The Role of Human Papillomaviruses in the Development of Non-Melanoma Skin Cancers

Melanom Dışı Deri Kanserleri Gelişiminde Human Papillomavirüsün Rolü

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ABSTRACT Objective: Non-melanoma skin cancers (NMSC) represent the most frequent cancers among the Caucasian population worldwide. The possibility of the involvement of human papillomavirus (HPV) in the development of these cancers is suggested by several studies. The purpose of this study is to evaluate the presence of various types of HPV in NMSC lesions of immunocompetent patients. **Material and Methods:** Biopsies taken from NMSC and non-sun exposed healthy skin from each of 32 immunocompetent patients were analysed for presence of HPV DNA by a polymerase chain reaction (PCR) using degenerate primers which can detect a broad spectrum of HPV types. **Results:** HPV DNA was detected in one of 21 basal cell carcinomas, from one of two actinic keratosis and from none of 8 squamous cell carcinomas, and none of the control biopsy specimens. It was thought that in addition to factors associated with Turkish population, the specimens which were found to be HPV negative did not harbor HPV DNA, or although tissues harbor HPV DNA, it was not detected with the technique we used because of low viral load. **Conclusion:** We could not show an association between HPV infection and NMSC by using real-time PCR technique. Our data suggest that the use of MY09/MY11 primer pairs alone in PCR technique is not sufficient to evaluate the HPV prevalence in NMSC. We thought that studies with more patients and using more sensitive detection techniques which will be developed in the future are needed to show a significant association between HPV and NMSC development.

Key Words: Skin neoplasms; betapapillomavirus; polymerase chain reaction

ÖZET Amaç: Melanom dışı deri kanserleri (MDDK) dünya çapında beyaz ırkta en sık görülen kanserlerdir. Human papillomavirüslerin (HPV) bu kanserlerin gelişimine katkıda bulunuyor olabileceği birçok çalışmada öne sürülmüştür. Bu çalışmanın amacı, bağışıklık sistemi normal hastalardaki MDDK lezyonlarında farklı HPV tiplerinin mevcudiyetini değerlendirmektir. **Gereç ve Yöntemler:** Bağışıklık sistemi normal 32 hastanın her birinin MDDK ve güneş görmeyen sağlıklı derilerinden alınan biyopsiler, HPV tiplerinin geniş bir spektrumunu saptayabilen dejenere primerlerin kullanıldığı polimeraz zincir reaksiyonu (PZR) ile incelendi. **Bulgular:** HPV DNA, 21 bazal hücreli karsinomun 1 tanesi ve 2 aktinik keratozun 1 tanesinde saptanırken, 8 yassı hücreli karsinom ve kontrol deri örneklerinin hiçbirinde HPV DNA saptanmadı. Bu durumun, Türk toplumuyla ilişkili faktörlere ek olarak, HPV negatif olarak bulunan numunelerin HPV DNA içermemesine veya bu dokular HPV DNA içeriyor olmasına rağmen, viral yükün düşük olması nedeniyle bizim kullandığımız yöntemle saptanamamış olmasına bağlı olabileceği düşünüldü. **Sonuç:** Biz çalışmamızda real-time PZR tekniği kullanarak MDDK ve HPV enfeksiyonu arasında ilişki gösteremedik. MDDK'de HPV prevalansını değerlendirmek için PZR tekniğinde MY09/MY11 primer çiftinin tek başına kullanılması yeterli olmadığı kanısındayız. HPV ve MDDK gelişimi arasında anlamlı bir ilişki gösterebilmek için daha çok sayıda hastayla ve gelecekte geliştirilecek daha duyarlı saptama metodları kullanılarak yapılacak çalışmalara ihtiyaç bulunduğunu düşünmekteyiz.

Anahtar Kelimeler: Deri neoplazmları; betapapillomavirüs; polimeraz zincir reaksiyonu

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Non-melanoma skin cancers (NMSC), which include squamous cell carcinoma (SCC) and basal cell carcinoma (BCC), are the most common malignancies in the Caucasian population.[1] Exposure to ultraviolet radiation (UVR) plays a major role in the development of NMSC.[1,2] Although several viruses have been claimed as additional risk factors in the development of NMSC, most convincing evidence to date is that for human papillomaviruses (HPVs).[3]

Human papillomaviruses are double-stranded DNA viruses which infect the epithelial cells of skin and mucosa.[4] One hundred twenty HPV types have been described to date, with the probability that many more exist and they are characterized into five phylogenetic genera: alpha, beta, gamma, mu and nu.[5,6]

There is growing evidence that HPV is an important human carcinogen. It is suggested that HPV acts as a carcinogen especially in cervical cancer and in other several epithelial malignancies, including cancers of skin, oral cavity, tongue, hypopharynx, larynx, esophagus, conjunctiva, bladder, urethra.[7-10] Although the association between high risk alpha HPV types (previously designated 'mucosal' types), such as HPV 16 and 18, and cervical cancer is well established, the role of infection with HPV in the development of NMSC is still unclear.[7-9] The first evidence for the involvement of specific HPV types in NMSC oncogenesis comes from studies in patients with epidermodysplasia verruciformis (EV).[11]

EV is a rare, autosomal recessive disease, which is characterized by abnormal susceptibility to infection with beta HPV types.[9,12] There is a high risk of developing SCC on sun-exposed sites of affected patients later in life, and predominantly HPV 5 and 8 are found in majority of these tumors.[12,13]

Renal transplant recipients are at increased risk of developing viral warts and skin cancers, especially SCC.[14,15] The frequent detection of HPV DNA in NMSC of renal transplant recipients, ranging from 46.7 to 91% for SCC, 35.7-83% for BCC and 23.5-93% for premalignant lesions, supports the causative role of HPV infection in the development of NMSC in these patients.[15-22] Despite no specific HPV type recognized as consistently associated with NMSC in these group of patients, beta HPV types has been detected predominantly in the lesions.[15,19,22]

There have been few studies investigating the presence of HPV in NMSC from immunocompetent individuals, and many of them are conducted with a limited number of patient. The prevalence of HPV DNA reported to be lower ranging from 27.2 to 59.7 % for SCC, 21-48 % for BCC and 36-70 % for premalignant lesions in these individuals.[3,20,21,23,24] Despite no HPV type has emerged specifically to be associated with skin cancer, beta HPV types have been detected predominantly in the lesions.[3,13,18,25-27]

The establishment of the relationship between the NMSC and HPV provide a basis for not only the preventive measures like vaccination but also improvement of antiviral/antitumor treatment. Characterization of HPV types that are significantly related with skin cancer is important especially for development of vaccine against these tumors. In this study, we investigate the presence of HPV in NMSC from immunocompetent patients.

MATERIAL AND METHODS

I. MATERIAL

A total of 37 nonimmunosuppresed patients, who were referred to Dermatology and Plastic, Reconstructive and Esthetic Surgery Department of University of Gazi, Faculty of Medicine between November 2005 and April 2008, for the removal of suspected cutaneous SCC and BCC, were enrolled in the study. While patients over the age of 18 years who had a clinical diagnosis SCC and BCC and who were not immunosuppressed were included in the study, patients who were immunosuppressed due to organ transplantation, immunosuppressive treatment or cancer, and had a history of genetic sydromes that increase susceptibility UVR induced damage were excluded from the study. Before the study, the medical ethical committee approved the protocol which is compatible with principles of Helsinki Declaration and all patients gave informed consent.

Skin samples were taken from the excisional biopsy material with 3 mm diameter punch biopsy during the operation procedure. As a control group, 3 mm diameter punch biopsy specimens were taken from non-sun exposed skin region (gluteal region) of same patients. Different punch biopsy equipment and plastic storage tubes were used for each region to prevent carry-over contamination in subsequent DNA isolation and PCR amplification. The specimens were snap-frozen within the sterile phosphate buffer solution (PBS [pH 8.0]) and stored at -86°C until study time.

Histopathological examination were done to the excisional biopsy specimens of patients, but clinically normal skin biopsies taken from gluteal region were not assessed for histology. After histopathological examination, while 32 patients whose diagnoses confirmed as SCC (8 patients), BCC (21 patients), actinic keratosis (AK) (2 patients) and Bowen disease (BD) (one patient) were included in the study, a total of 5 patients (one melanoma, one sebaceous adenoma, 3 seborheic keratoses) with confirmed diagnosis of other than NMSC excluded from the study.

II. METHODS

DNA Extraction

DNA extraction was accomplished according to kit's manual (High Pure Viral Nucleic Acid Extraction Kit (Roche, Germany).

DNA Amplification

L1 region of the samples were replicated by using MY09/MY11 primers. Nested real-time PCR method was used for the analysis of HPV DNA and HPV16 positivity. PCR amplifications were done by MY09/11 primer set after extraction of the DNA. Real time nested amplifications of MY09/11 products were done by GP5+/GP6+ primers and Cyanine-5 labeled HPV 16 DNA specific probe. Real time PCR product analysis was done by melting curve analysis on LightCycler Software version 3.5.3 (LC 2.0 Roche Diagnostics, Germany). Melting peaks of 78-82°C showed the detection of HPV DNA in the sample. Probe melting peaks of positive samples has been analyzed in the same run and HPV16 positive samples gave peaks around 68°C.

Sequencing

HPV DNA positive and HPV 16 negative MY09/11 amplicon were sequenced by OpenGene® automated DNA sequencing system and similarity percentage of sequences were calculated by GeneObjects® software (Visible Genetics, Canada). Cycle sequencing reactions were done by using Cy5.5 dye terminator sequencing kit (Amersham Biosciences, USA).

RESULTS

A total of 32 patients (25 males, 7 females) with confirmed diagnosis of SCC (8 patients), BCC (21 patients), AK (two patients) and BD (one patient) were included in this study. Actinic keratosis and BD were classified as premalignant lesions. The average of the patients' ages was 66.6 ± 9.2 years (range 49-82). The distribution of histopathological diagnosis according to age and sex of the patients was summarized in Table 1.

Sixty-four biopsy specimens (32 lesional and 32 healthy control) from 32 immunocompetent patients were analyzed for presence of HPV DNA with MY09/11 degenerate PCR assay. HPV DNA

TABLE 1: The distribution of histopathological diagnosis according to age and sex of the patients.

		Sex				
		Female		Male		Total
		Number	Percentage (%)	Number	Percentage (%)	Number
Diagnosis	Premalignant lesion	0	0.0	3	100.0	3
	Basal cell carcinoma	4	19.0	17	81.0	21
	Squamous cell carcinoma	3	37.5	5	62.5	8
Total		7	21.9	25	78.1	32

was detected from one of 21 BCC, from one of two AK lesions and from none of eight SCC, and none of the control biopsy specimens which was taken from gluteal regions of the same individuals. While HPV type 5 was identified in AK specimen, the type of HPV detected in the BCC specimen could not be identified. The characteristic of patients were summarized in Table 2.

TABLE 2: The characteristics of the patients, localisation of lesions and HPV types detected.

Patient No	Age	Sex	Lesion	Localisation	HPV Type
3	63	M	BCC	Forehead	-
4	60	M	BCC	Retroauricular region	-
5	64	M	BCC	Nose	-
7	64	M	BCC	Shoulder	-
9	56	M	BCC	Scalp	-
15	78	M	BCC	Preauricular region	-
16	68	M	BCC	Nose	-
18	55	F	BCC	Nose	+ (Untyped)
19	75	M	BCC	Nose	-
20	80	M	BCC	Forehead	-
21	53	M	BCC	Retroauricular region	-
22	50	F	BCC	Eyelid	-
23	72	M	BCC	Preauricular region	-
25	55	M	BCC	Scalp	-
26	49	M	BCC	Trunk	-
29	67	M	BCC	Eyelid	-
32	80	M	BCC	Forehead	-
33	75	F	BCC	Nose	-
34	65	F	BCC	Scalp	-
35	67	M	BCC	Back of the neck	-
36	73	M	BCC	Temple	-
8	72	F	SCC	Cheek	-
10	72	M	SCC	Scalp	-
11	62	F	SCC	Cheek	-
17	82	M	SCC	Dorsum of the hand	-
27	70	M	SCC	Cheek	-
30	79	F	SCC	Nose	-
31	67	M	SCC	Auricula	-
37	56	M	SCC	Cheek	-
6	62	M	AK	Shoulder	HPV Type 5
24	76	M	AK	Eyelid	-
2	65	M	BD	Retroauricular region	-

M: Male; F: Female; BCC: Basal cell carcinoma; SCC: Squamous cell carcinoma; AK: Actinic keratosis; BD: Bowen disease.

DISCUSSION

In the current study, we have detected HPV DNA from one (untyped HPV) of 21 BCC, one (HPV type 5) of two AK and none of eight SCC samples taken from immunocompetent patients. This result was not concordant with previous studies in which frequency of HPV DNA was found to be 27.2 to 59.7% for SCC, 21-48% for BCC and 36-70% for premalignant lesions.[3,15,20,21,23,24] One possible explanation for this result might be that other thirty specimens, in which we could not detect HPV DNA, did not harbor HPV. The other possibility is that some of the negative samples might contain HPV types that cannot be detected efficiently by the PCR method we used.

The detection of HPV in tissues has some difficulties, because a few cells of the skin, hair follicle or dermatological lesion is infected with HPV due to latent infection.[28] The finding that viral load in cutaneous tumors was 1 HPV DNA/ 20-5000 tumor cells, pointed out that not every tumor cell harbours HPV genome.[11,25,29] This finding explains that why we need highly sensitive detection techniques to show HPV DNA in the lesions. It was reported that HPV could not be detected with degenerate PCR and other techniques in up to 40% of cutaneous warts which were known to develop as a consequence of active infection with HPV.[30] Due to the low viral load in tumor cells, the frequency and spectrum of HPV detected by different techniques with different sensitivities show a great diversity.[25] In PCR technique, while degenerate primers enable detection of a broad range of HPV types with limited sensitivity for different HPV types, nondegenerate primers allow detection of only small subset of HPV types but with high sensitivity.[25] To detect all HPV-positive cases, several degenerate PCR primers should be combined with multiple nondegenerate PCR primers, each of which is specific for different HPV types. Probably, frequency of HPV infection in skin tumors could be found higher than current data, even 100%, by this way. However, due to diverse spectrum of HPV, it is difficult to perform this strategy. One of the limitations of our study is, as we intended to investigate the presence of HPV in NMSC and HPV types associated with NMSC, we prefered

to use MY09/MY11 primer pair, that allows the detection of broad range of HPV types, rather than type-specific primers. However, observed sensitivity of MY09/MY11 degenerate PCR method is high for mucosal HPV types and low for cutaneous HPV types.[30,31] It is possible that some of the negative samples, in our study, contained very low copy number of HPV genomes which could not be detected by our degenerate PCR method with low sensitivity for cutaneous HPV types. In the studies using techniques combining degenerate and/or nested PCR, the prevalence of HPV DNA appears to be higher.[18,25,31]

Although no particular HPV type recognized as significantly associated with NMSC in immunocompetent patients to date, beta HPV types has been detected predominantly in the malignant and premalignant lesions of these patients.[3,13,18,25,26] Consistent with the literature, we detected HPV Type 5 in one AK specimen, but the HPV detected in BCC specimen could not be typed.

The detection of HPV DNA in only a few samples in our study may not signify that these viruses do not play a role in the NMSC carcinogenesis. As we mentioned above, only a few tumor cells were shown to harbor HPV DNA in NMSC.[11,25] Existing data suggest that cutaneous HPVs are possibly important for initiation and progression of carcinogenesis but not for the maintenance of malignant phenotype ('hit-and-run' mechanisms of carcinogenesis).[11] Human papillomavirus may act as an indirect carcinogen in such a way that virus causes some genetic disturbances, which in turn results in the development of transformed cells that do not require the HPV genome to conserve neoplastic phenotype.[32]

It was reported that HPV DNA was detected frequently not only in NMSC but also in healthy skin, hair follicle and benign hyperproliferative skin disorders in several studies, and these findings complicate the interpretation of presence of HPV in NMSC.[31,33-39]

Some recent studies have suggested that the prevalence of HPV DNA was higher on sun-exposed skin regions. In a study, Antonsson et al. evaluated the presence of HPV DNA in skin swabs taken from five different skin regions of each patient by PCR method, and HPV prevalence was found to be higher on forehead compared to arm or thigh.[39] The relatively high prevalence of HPV at sun-exposed skin regions might be the result of local immunosuppression due to sun exposure, enhancing HPV replication or direct activation of functions of HPV by sunlight.[40] In addition, the relatively high exposure of this regions to HPV might be the other reason for high detection rate of HPV. We thought that, in our study, no detection of HPV DNA in control skin samples taken from gluteal region, might be result of unexposure of this region to the sunlight.

Despite there are lots of studies that demonstrated HPV DNA frequently in NMSC and healthy skin in the literature, detection of HPV DNA in only a few patients in our study may be associated with some traditional practices. Namely, going to Turkish bath is commonly seen and also cleaning the surface of skin using bath glove is a common habit in Turkish population. If we think that HPV infects epithelial cells of skin and mucosa, as a result of using bath glove in the bath, cells infected with HPV might be thrown out of body together with epithelial cells of skin.

In addition to this, due to high prevalence of dark skin phenotype among Turkish population, the level of immun suppression induced by UVR might not be enough for inducing HPV proliferation. All at once might decrease frequency of HPV infection and viral load in case of a infection in Turkish population.

As a result, we could not show a significant relationship between NMSC and HPV using a degenerate PCR method. The reason for us to find a positive result for only 2 of the cases might be due to the fact that the others really do not contain the virus or we could not detect the virus with the PCR method we used as a result of low viral load. We suggest that, among the Turkish population the prevalence of HPV infection or viral load in case of a HPV infection might be low because of dark skin color or some traditional habits. Although HPV DNA can be detected in a number of NMSC, larger well designed studies are necessary to clarify the role of HPV infection in the pathogenesis of NMSC.

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