Analysis of p53 codon 72 polymorphism and HPV 5,8 E6 oncoprotein expression in Basal cell carcinoma in Basrah.

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ABSTRACT

Background: **Two polymorphic forms of the p53 gene that codes either for Arginine or proline at codon 72 were identified, However, this individual might have one of the three genotypes: Arginine/Arginine, Proline/Proline or Arginine /Proline. Previous studies suggested that Arginine form of the p53 codon 72 polymorphism is a risk factor for HPV associated with cervical cancer and has been explored as a possible risk factor for the development of skin cancer in general. This study was aimed to clarify the association of this polymorphism in relation to beta Human Papilloma virus (HPV) 5,8 E6 oncoprotein expression in basal cell carcinoma in Basra, Iraq.**

Patients & Methods: **Blood samples and tissue biopsy from 29 histologically confirmed BCC cases and 31 normal controls were analyzed using polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) method to determine the genotype of p53 codon 72 and conventional PCR for HPV 5 and 8 E6 oncoprotein expression in BCC biopsies.**

Results: **the frequency of Arg/Arg, Pro/Pro or Arg/Pro were 10.3%, 24.1% and 65.5% respectively among patients group and 6.5%, 58.1% and 35.5% among the control group. This result showed a significant increase in the frequency of p53-72 Arginine/Proline heterozygous among BCC cases as compared with controls according to the dominant genetic model (P value =0.007, the Odds ratio = 4.3, 95% CI = (1.4340-13.2059). There was negative expression of HPV 5 and 8 E6 oncoprotein in all the biopsies tested.**

Conclusion: **These finding indicates that the heterozygosity of the p53 codon 72 could be an immunogentic risk factors in the development of BCC, but there was no association detected with HPV5,8 E6 oncoprotein from the examined cases.**

Key words: p53 codon 72 polymorphism, HPV 5,8

تحليل تعدد الاشكال لجين p53 كودون ٧٢ وتعبير البروتين المسرطن E6 لفيروس الورم الحليمي بيتا ٥،٨ في سرطان الخلايا القاعدية للجلد في البصرة

المقدمة: لقد تم تحديد شكلين من الجين p53 في الموقع ٧٢ التي تشفر إما الارجينين او البرولين فيكون الفرد حاملا واحد من المورثات الثلاثة: الارجنين / الارجنين، برولين / برولين أو الارجنتين / برولين. وأشارت دراسات سابقة أن شكل الارجينين من البروتين p53 كودون ٧٢ المتعدد الأشكال هو أحد عوامل الخطر لفيروس الورم الحليمي البشري المرتبط بسرطان عنق الرحم، وقد تم استكشافه كعامل خطر محتمل في تطور سرطان الجلد بشكل عام.

الاهداف: تهدف الدراسة إلى توضيح علاقة هذا التعدد فيما يتعلق بتعبير البروتين المسرطن (E6 oncoprotein) لفيروس الورم الحليمي بيتا نوع ٥، ٨ في سرطان الخلايا القاعدية في البصرة.

طريقة البحث: تم تحليل ما مجموعه ٢٩ حالة من سرطان الخلايا القاعدية للجلد (BCC) مؤكده تشريحيا و ٣١ ضوابط وتم استخدام أسلوب تفاعل البلمره المتسلسل – تعدد الأشكال لطول الشضايا المقيده (PCR–RFLP) لجين p53 كودون ٧٢ المتعدد الأشكال وطريقة تفاعل البلمره المتسلسل التقليدي (PCR) للكشف عن تعبير البروتين المسرطن (E6 oncoprotein) لفيروس الورم الحليمي البشري بيتا نوع ٥ و ٨ في خزعات BCC.

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النتائج: أن وتيرة الارجنين / الارجنين، برولين / برولين و الارجنين / برولين هي ١٠.٣٪ و ٢٤.١٪ و ٦٥.٥٪ على التوالي بين مجموعة المرضى و ٦.٥٪، ٥٨.١٪ و ٣٥.٥٪ من بين المجموعة الضابطة التي تشير إلى وجود زيادة كبيرة في وتيرة البروتين (**p53–72R/P**) المتخالف بين الحالات بالمقارنة مع الضوابط وفقا للنموذج الجيني المهيمن (قيمة **P** = ٠.٠٠٧، نسبة الأرجحية = ٤.٣، ٩٥٪ **CI** = (١،٤٣٤٠–١٣،٢٠٥٩)) والتي تدعم ان هذا التغاير في البروتين **p53** الموقع ٧٢ هو من عوامل الخطر المحتملة لسرطان الخلايا القاعدية للجلد.كما كان هناك تعبير سلبي للبروتين المسرطن (**E6 oncoprotein**) لفيروس الورم الحليمي البشري بيتا نوع ٥ و ٨ في جميع الخزعات التي تم اختبارها.

هذه النتائج ربما تدعم ان (**p53–72R/P**) المتخالف من عوامل الخطر المحتملة لسرطان الخلايا القاعدية للجلد (**BCC**). ولكن لم يتم الكشف عن أي ارتباط مع التعبير المسرطن لفيروس الورم الحليمي البشري بيتا ٥،٨ في الأورام المفحوصة.

INTRODUCTION

Codon 72 belongs to the exon 4 and has either the sequence CCC, which encodes proline, or CGC, which encodes arginine. Giving three allelic variants Pro/Pro, Arg/Arg and the heterozygous Pro/Arg[1]. This amino acid change results in structural changes with distinct biochemical and functional properties of the p53 protein with difference in its ability to signal apoptosis following ultra violet induced damage and hence influencing the cancer risk.[2] p53 codon 72 polymorphisms was reported to be affected with geographic distribution and the ethnic factors change. The frequency of p53-72 Arginine variant also reported to be increased with geographic distance from the equator and fair skin populations have a higher frequency of this allele.[3] The role of p53 codon 72 polymorphisms in non-melanocytic skin carcinomas (NMSCs) is still not clear. Dokianakis et al [4] suggested that p53 Arg homozygosity could represent a potential risk factor for high risk HPV related skin cancer in immunocompetent patients. Whereas McGregor et al[1] found that the arginine form of p53 is a risk factor to sun burn and NMSC in immunosuppressed individuals only. Furthermore, De Oliveira et al[5] indicated from a study on Brazilian Epidermodysplasia Verruciforms (EV) patients that all malignant form of EV patients were arginine homozygous in contrast with the benign form. However, Bendesky, et al[6] results revealed no significant association between p53 genotype and basal cell carcinoma(BCC), squamous cell carcinoma (SCC) or both combined and showed no differences regarding the basal level of DNA damage or DNA repair capacity among the different allelic forms. A similar finding was reported by O`conner, et al[7] which concluded there was no correlation between the presence of HPV, the p53 codon 72 arginine polymorphism, and the development of skin cancer. On the other hand Pezeshki, et al[8] were studied the association between BCC development and the p53 codon 72 polymorphism in Iranian population. No significant changes were reported from this study. However, there was an apparent increase in the Arg/Arg genotype among sun exposed patients compared to controls which suggested that Arg allele genotype might affect the risk of ultraviolet induced basal cell carcinoma. There are few conflicting studies on the role of Human papilloma virus in BCC ranging from serological to molecular studies. Most of these studies conclude a negative association.[9,10] Therefore, this study was conducted to evaluate the association of p53 codon 72 polymorphism and HPV 5,8 E6 oncoprotein expression in basal cell carcinoma of the skin.

MATERIALS AND METHODS

This study was conducted in Al Sader Teaching Hospital for the period from November 2013 to May 2015 including 29 patients of histologically confirmed basal cell carcinoma of the skin and 31 normal controls. DNA was

extracted from blood samples for all patients samples as well as the controls using Blood Mini DNA extraction kit (Qiagen, Germany) according to the manufacturer's instructions; a small part of the excised tumor biopsy was taken and emersed immediately in RNA stabilization solutions (RNA later solution Qiagen) and kept frozen for RNA extraction of the HPV 5,8 E6 oncoprotein using RNeasy fibrous tissue kit (Qiagen) according to the manufacturer's instructions. PCR amplification of the polymorphic codon 72 region was performed using the primers sequence p53 fw-5′-ATCTACAGTCCCCCTTGCCG-3′and p53 rev-5′-GCAACTGACCGTGCAAGTCA-3′.

Lyophalised mastermix from Bioneer (20ul) was used with 10 ul of template DNA, 2ul of each primer and the volume was completed with 6ul of water. The PCR program include 94 Cº initial denaturation step and 30 cycles of 94 Cº for 40 seconds denaturation step, 56 Cº for 30 seconds annealing step and 72 Cº for 30 seconds extension step and final extension step with 72 Cº for 10 minutes. The PCR product of p53 codon 72 PCR reaction (a 296 bp fragment) was digested by restriction enzyme BstUI (New England BioLabs) for one hour at 60°C. The reaction components include 1X CutSmart® Buffer (5ul), PCR product (10 ul), 10 units of restriction enzyme BstU1(1ul) and Nuclease free water (34 ul), Total volume 50 ul.

The product of the restriction enzyme digestion was resolved on 2.5% agarose gel (promega) stained with ethidium bromide and photographed under U.V light. The p53 72Pro allele, which lack the BstUI restriction site, had only a single 296 bp band, whereas p53 72Arg, which had the BstUI restriction site, produced 169 and 127 bp bands. More than 50% of the samples were retested randomly, and incubated overnight and similar results were obtained. Samples yielding 169 bp and127 bp fragments were scored as Arg/Arg, those with single 296 bp fragments as Pro/Pro, and 296, 169 and 127 bp as Arg/Pro.

RT–PCR of HPV 5, 8 E6 oncoprotein

Reverse Transcription PCR of HPV 5 E6 oncoprotein and the internal control beta actin was performed using the primers sequence HPV 5 E6 FW 5#TGAATTCGACTACAAAAGGCTTA-3#, HPV 5 E6 Rev 5#-GGTTAAATTCATAAGTTGCAGTGG-3#, beta actin FW 5#-CCAGACAGCACTGTGTTGGC-3#, beta actin rev 5#-GAGAAGCTGTGCTACGTCGC-3#.

The QIAGEN One-step RT-PCR Enzyme Mix (containing the Omni script and Sensiscript Reverse Transcriptases and HotStarTaq DNA Polymerase) 2 ul was used with 1.5 ul of each primer, 2ul dNTP Mix, 10 ul of 5x QIAGEN One-step RT-PCR Buffer, 10 ul RNA template and 20 ul RNAase free water to complete the volume to 50 ul. The Thermal cycler conditions include:30 min reverse transcription at 50 Cº followed by initial PCR activation step for 15 min at 95Cº, then 25 cycles of 0.5 min denaturation step at 95Cº, 0.5 min annealing step at 59Cºand 0.5 min extension step at 72Cº and finally 10 min extension step at 72Cº. Similarly, RT- PCR component was used for detection of HPV 8 E6 oncoprotein (HPV 8 E6 FW 5#-CCGCAACGTTTGAATTTAATG-3#, HPV 8 E6 Rev 5#-ATTGAACGTCCTGTAGCTAATTCA-3# with change of annealing condition in the thermal cycling program to (57Cº). Agarose gel (2.5%) was used to analyse the RT - PCR product. The estimated product size for the internal control (beta globin) is 270bp and for HPV 5E6 transcript is 77 bp and 55 bp for HPV 8 E6 as calculated from PavE papilloma virus episteme , at papilloma virus specific blast (the amplicon size equals the lower sequence number of the forward strand from the lower sequence number of the reverse strand).

Statistical analyses, were performed using (SPSS) for Windows (version 20). Pearson Chi-square was used to determine any significant difference in the distribution of demographic characters and genotypes frequency between cases and controls.

RESULTS

The age of the 29 patients ranged from (40-85 years) with a mean 59.6 while the age of the 31 controls ranged from (36-85 years) with a mean of (55.8). The male sex was predominate in both groups presenting 58.6% for cases and 54.8% for controls.

The Polymerase Chain Reaction - Restriction Fragment Length polymorphism (PCR-RFLP) products were analysed on 2.5% ethidium bromide stained agarose gel as shown in Figure 1.

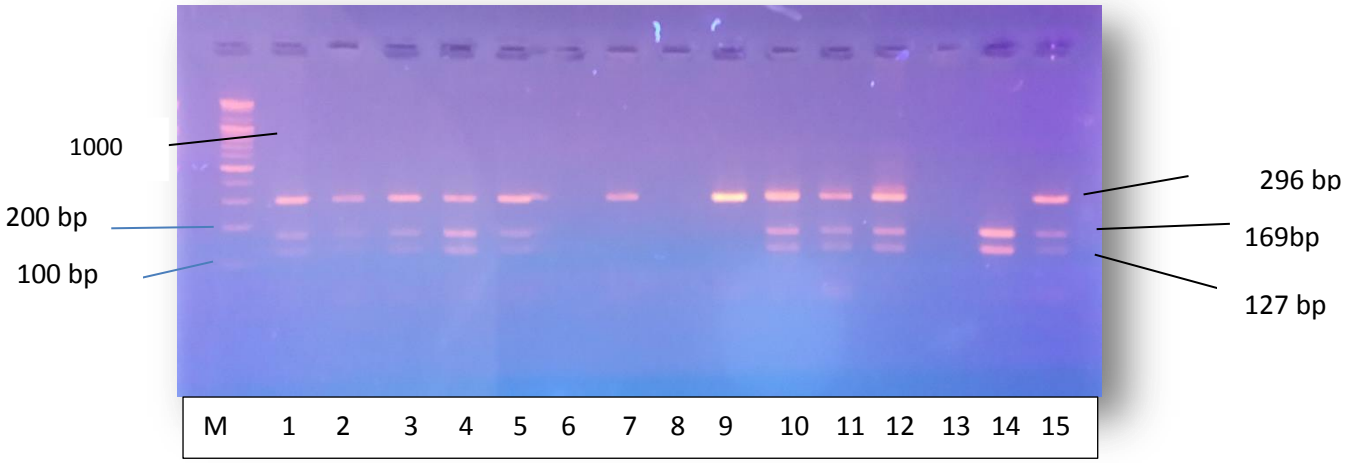

Fig 1. Agarose gel picture showing PCR-RFLP analysis of p53 codon 72 genotypes with restriction endonuclease enzyme BstUI.

M: 100-bp DNA ladder, Lanes 1, 2,3,4,5,10,11,12 and 15 Arg/Pro heterozygous (296, 169 and127 bp), lane 7,9 Pro/Pro homozygous (296 bp), lanes 13 Arg/Arg homozygous (169 and127 bp).

The frequency of Arg/Arg, Pro/Pro or Arg /Pro were 10.3%, 24.1% and 65.5% respectively among patients group and 6.5%, 58.1% and 35.5% among the control group, (Table-1). Statistical analysis showed that there were significant differences in the distribution of the various alleles between the BCC cases and control groups (P value = 0.026) and a significant increase in the frequency of Arg/Pro heterozygous among cases as compared with controls according to the dominant genetic model where Arg /Arg + Arg/Pro are considered versus Pro/Pro genotype (P value = 0.007,the Odds ratio = 4.3, 95% CI = (1.4340-13.2059), but according to the recessive model where Arg/Arg allele group are considered versus Arg/pro + Pro/Pro, there was no significant difference in the alleles distribution between the BCC cases and control group (Table-1).

Table 1. The distribution of p53 codon 72 polymorphism among BCC cases and controls.

Genotypes models	Cases N (%)	Control N (%)	P value	OR (95% CI)
p53 Arg 72 Pro genotype			0.026	
Arginine/ Arginine	3 (10.3)	2 (6.5)		
Proline/ Proline	7 (24.1)	18 (58.1)		
Arginine/Proline	19 (65.5)	11 (35.5)		
Dominant genetic model			0.007	4.3(1.4340-13.2059
Arg/Arg+ Arg/Pro	22 (75.8)	13 (41.9)		
Pro /Pro	7 (24.13)	18 (58.06)		
Recessive genetic model			0.588	1.673 (0.2589-10.8109)
Arg/Arg	3 (10.3)	2 (6.5)		
Arg /Pro +Pro/Pro	26 (89.6)	29 (93.6)		
Total	29 (100)	31 (100)		

Reverse transcription PCR for HPV 5,8 E6 oncoproteins

Amplified products were loaded to 2.5% agarose gel for detection of E6 oncoprotein of HPV 5 (77 bp) and HPV 8 (55bp) which were not visualized in all tested samples although the internal control (beta actin) bands was clear at 270 bp position and amplified in most of the samples (Figure 2).

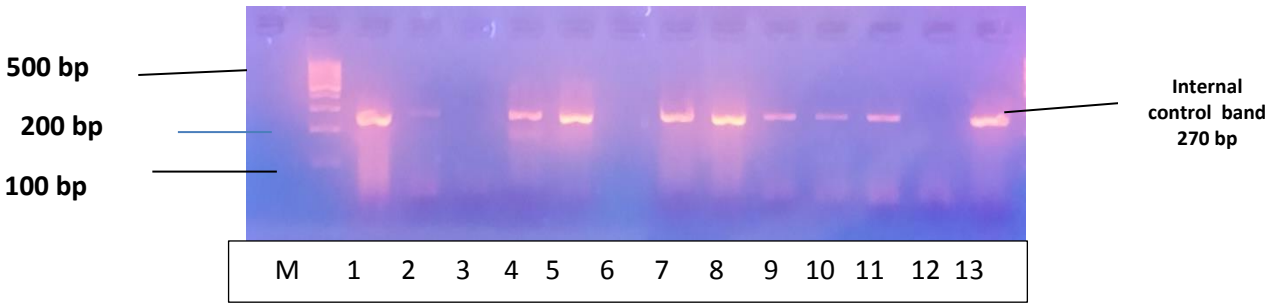

Fig 2. Ethidium bromide stained agarose electrophoresis 2.5% of RT- PCR product of HPV 5 E6 and beta actin internal control. Beta actin bands were visualised in 1,2,4,5,7,8,9,10,11,13 . No positive band (77 bp) detected in all wells.

DISCUSSION

The main finding of the current study is that patients carrying the p53 codon 72 Arg/pro genotype have more risk of getting BCC as compared with Pro/Pro variant (OR = 4.44, 95% CI = 1.4118 to 13.9733) and according to the dominant model (Arg/Arg + Arg/Pro versus Pro/Pro) the association is significant ($P < 0.05$) with odds ratio (OR) = 4.3, 95% confidence interval (CI): 1.4340-13.2059 and the P-value 0.0077. The dominance of the heterozygous variant (Arg/Pro) in the BCC cases group in our population may reflect functional changes as stated by Levine et al.[11] However, the change in this amino acid may results in structural changes with distinct biochemical and functional properties of the p53 proteins with differences in the ability to signal apoptosis, hence influencing the cancer risk.[1,8] The finding of negative HPV 5,8 E6 RNA expression in all studied BCC biopsies may reflect the negative association of these viruses and BCC aetiology. Due to high prevalence of beta HPV in the skin of healthy individuals, we tried to analysed the E6 oncoprotein expression of the most commonly studied types HPV5 and 8 other than studying the HPV DNA only. In a

study by Dang et al[12] one case of BCC was included to study the HPV 5,8 E6/E7 RNA expression in tumor biopsies of NMSC using real time RT PCR. The study of Dang et al[12] revealed an increase in the transcriptional activity in actinic carcinoma and squamous cell carcinoma biopsy only. The negative expression of HPV 5,8 E6 oncoprotein in BCC samples may further coincides with the negative relationship of beta HPV and BCC obtained by other studies.[10,13,14] A study conducted by Mokhtari, et al[13] which were using immunohistochemistry, no HPV DNA was detected in all BCC biopsies. However, lannacome et al[10] carried out the first and largest case control study that focused on BCC rather than SCC or NMSC using molecular and serological methods and the researchers were suggested that HPV is not involved in BCC carcinogensis. In addition Nahidi etal [4] in an investigation on Iranian population concluded that HPV infection which was assessed by PCR using GP+5/GP+6 primers for L1 region of HPV DNA had no significant role in BCC pathogenesis. It has been reported that heterozygous variant Arg/Pro decreases the susceptibility to HPV E6 oncoprotein degradation which is increased in homozygous of Arg allele. [15] On the other hand, studies demonstrated that the Arg form is a potent inducer of apoptosis than the proline form, in contrast p53 proline form is a strong inducer of transcription than p53 Arg form 2 which may clarify the reason for the negative expression of the viral oncoprotein in this study due to the dominance of hetrozygosity of p53 genotypes in our population where individuals with Arg/Arg homozygous variant are more susceptible to HPV associated carcinogenesis, thus Arg/Pro heterozygous or Pro/Pro homozygous variant of p53 codon 72 decreases this susceptibility. [16,17]
Several studies have shown inconsistent and conflicting results on the genetic susceptibility role of the p53 codon 72 polymorphism in BCC that have been attributed to different studied ethnic population, different geographic areas, different sample size and genotyping methods used. Recently Tian, et al[18] presented meta-analysis with the largest dataset to date that did not show a statistically significant association of p53 codon 72 polymorphism and the overall BCC risk. Even subgroup analysis by ethnicity with different genetic models did not show any significant association in Caucasians as well as in Asians population.[8] The association of the p53 codon 72 polymorphism and skin cancer in general was also explored in different meta-analysis studies 19,20 and indicated that p53 codon 72 polymorphism may have a little association with skin cancer. The low frequency p53 Arginine allele in the present study (10.3% for cases and 6.5% for controls) could be explained by the fact that frequency of p53 codon 72 Arginine allele increases with geographic distance from the equator and that fair skin population have higher frequency of p53 Arg allele than dark skin population.[21,22] Most of these studies were conducted on Caucasian population and only one involves Asian population in Iran 8 which included the BCC and revealed no significant difference in the p53 genotypes between patients and controls but indicated an increase in the frequency of arginine allele among sun exposed compared to control which may affect the risk of UV induced BCC. However, a positive association of the p53 codon 72 and BCC was found in transplant patient by McGregor, et al[1] but not in immunocompetent group.

In conclusion, the study suggested that p53 codon 72 Arginine / proline heterozygous tend to confer a genetic susceptability in BCC development. The negative expression of the HPV 5,8 E6 oncoprotein should be confirmed by larger sample size and more reliable real time PCR.

REFERENCES

1. **McGregor JM, Harwood CA, Brooks L, Fisher SA, Kelly DA, O'nions J, et al. Relationship between p53 codon 72 polymorphism and**

susceptibility to sunburn and skin cancer. J Invest Dermatology. 2002; 119(1): 84-90.
2. Pim D, Banks L. p53 polymorphic variants at codon 72 exert different effects on cell cycle progression. Int J Cancer. 2004; 108(2): 196-9.
3. Själander A, Birgander R, Saha N, Beckman L, Beckman G. p53 polymorphisms and haplotypes show distinct differences between major ethnic groups. Hum Hered. 1996; 46(1): 41-48.
4. Dokianakis DN, Koumantaki E, Billiri K, Spandidos DA. P53 codon 72 polymorphism as a risk factor in the development of HPV-associated non-melanoma skin cancers in immunocompetent hosts. Int J Mol Med. 2000; 5(4): 405-414.
5. De Oliveira WR, Rady PL, Grady J, Hughes TK, Neto CF, Rivitti EA, et al. Association of p53 arginine polymorphism with skin cancer. Int J Dermatol. 2004; 43(7): 489-493.
6. Bendesky A, Rosales A, Salazar AM, Sordo M, Peniche J, Ostrosky-Wegman P. p53 codon 72 polymorphism, DNA damage and repair, and risk of non-melanoma skin cancer. Mutat Res Fund Mol Mech Mut. 2007; 619(1): 38-44.
7. O'Connor DP, Kay EW, Leader M, Atkins GJ, Murphy GM, Mabruk MJ. p53 codon 72 polymorphism and human papillomavirus associated skin cancer. J Clinic Path. 2001; 54(7): 539-542.
8. Pezeshki A, Sari-Aslanl F, Ghaderi A, Doroudchi M. p53 codon 72 polymorphism in basal cell carcinoma of the skin. Path Oncol Res. 2006; 12(1): 29-33.
9. Zakrzewska K, Regalbuto E, Pierucci F, Arvia R, Mazzoli S, Gori A, et al. Pattern of HPV infection in basal cell carcinoma and in perilesional skin biopsies from immunocompetent patients. Virology Journal. 2012; 9(1):1.
10. Iannacone MR, Gheit T, Waterboer T, Giuliano AR, Messina JL, Fenske NA, et al. Case–control study of cutaneous human papillomavirus infection in Basal cell carcinoma of the skin. J Invest Dermatol. 2013; 133(6): 1512-1520.
11. Levine AJ, Finlay CA, Hinds PW. P53 is a tumor suppressor gene. Cell. 2004; 116: S67-70.
12. Dang C, Koehler A, Forschner T, Sehr P, Michael K, Pawlita M, et al. E6/E7 expression of human papillomavirus types in cutaneous squamous cell dysplasia and carcinoma in immunosuppressed organ transplant recipients. B J Dermatol. 2006; 155(1): 129-136.
13. Mokhtari M, Mesbah A, Rajabi P, Rajabi MA, Chehrei A, Mougouei K. Determination of the relationship between basal cell carcinoma and human papilloma virus, based on immunohistochemistry staining method. Ind J Dermatol. 2009; 54(3): 225.
14. Nahidi Y, Meibodi NT, Meshkat Z, Esmaili H, Jahanfakhr S. No evidence of human papilloma virus infection in basal cell carcinoma. Ind J Dermatol. 2015; 60(4): 356.
15. Thomas M, Kalita A, Labrecque S, Pim D, Banks L, Matlashewski G. Two polymorphic variants of wild-type p53 differ biochemically and biologically. Mol Cell Biol. 1999; 19(2): 1092-100.
16. Habbous S, Pang V, Eng L, Xu W, Kurtz G, Liu FF, et al. p53 Arg72Pro polymorphism, HPV status and initiation, progression, and development of cervical cancer: a systematic review and meta-analysis. Clin Cancer Res. 2012; 18(23): 6407-6415.
17. Burroni E, Bisanzi S, Sani C, Puliti D, Carozzi F. Codon 72 polymorphism of p53 and HPV type 16 E6 variants as risk factors for patients with squamous epithelial lesion of the uterine cervix. J Med Vir. 2013; 85(1): 83-90.
18. Tian Y, Li L, Yang R. p53 Arg72Pro polymorphism and risk of basal cell carcinoma: a meta analysis. Int J Clin Exp Med. 2015; 8(2): 2350.
19. Ye J, Li XF, Wang YD, Yuan Y. Arg72Pro polymorphism of TP53 gene and the risk of skin cancer: a meta-analysis. PloS One. 2013 Nov 8; 8(11): e79983.
20. Jiang DK, Wang WZ, Ren WH, Yao L, Peng B, Yu L. TP53 Arg 72 Pro polymorphism and skin cancer risk: a meta-analysis. J Investigative Dermatol. 2011; 131(1): 220-228.
21. Loeb KR, Asgari MM, Hawes SE, Feng Q, Stern JE, Jiang M, et al. Analysis of Tp53 codon 72 polymorphisms, Tp53 mutations, and HPV infection in cutaneous squamous cell carcinomas. PloS One. 2012; 7(4): e34422.
22. Thurow HS, Haack R, Hartwig FP, de Oliveira IO, Dellagostin OA, Gigante DP, et al. TP53 gene polymorphism: importance to cancer, ethnicity and birth weight in a Brazilian cohort. J Bioscien. 2011; 36(5): 823-831.