# Study of NFKB Polymorphism with HPV Infection in Women of Reproductive Age Group

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**Abstract**— Human Papilloma Virus (HPV) is highly prevalent, with around 80% of women thought to be infected at some point. They are thought to produce proteins which inhibit the tumour suppressor protein p53 in cervical epithelial cells, allowing for uncontrolled cell division. The persistent HPV infection changes a premalignant lesion to cancerous state, and this seems to be accompanied by a progressive loss of responsiveness to the NFκB mediated growth inhibitory signal. This study was undertaken to provide a more precise evaluation of association of NFκB -94ins/del ATTG promoter polymorphism and cancer risk. The study included 75 cases (unhealthy cervical pathology) and 75 controls (healthy normal cervix) belonging to reproductive age (15-44years) group. Cervical swab for HPV genotyping was performed in cobas x 480 and cobas z 480 analysers. DNA was extracted from EDTA blood sample using cobas DNA sample preparation kit. NFκB gene polymorphism was seen by PCR-RFLP method in gel electrophoresis. Statistical analysis was done using SPSS 20. Among the healthy normal cervical cytology, HPV infection rate was found to be 6.6%. In cases, maximum HPV positivity was for HPV 16. Among the study population, insertion allele was seen more in both the study groups compared to deletion allele. We found del/del genotype contribution maximum among the cases with cervical pathology. Ins/del genotype was found in cases group. In unhealthy cervix, we found that ins/ins genotype was prevalent among all the HPV strain except HPV 18 followed by del/del genotype. But, among HPV 18 strain, del/del was seen more common. The study provided information about genotype distribution among women with and without cervical cancer which would help planning an appropriate strategy for disease monitoring.

**Index Terms**— Cancer, Cervical pathology, DNA, Genotype, HPV, NFκB, Reproductive age

## 1 INTRODUCTION

Human Papilloma Virus (HPV), belongs to papillomaviridae family, is one of the commonest causes of carcinoma cervix, cancers of the vulva, vagina, penis, anus, and oropharynx [1]. The etiological role of infection with high-risk papilloma viruses in cervical carcinoma is well established. But over few decades only limited progress has been made in the systemic treatment of patients with advanced or recurrent cervical cancer [2].

The HPV viral oncogenes E7 and E6 are the main suppliers to the development of HPV-induced cancers. Inactivation of tumour suppressors p53 and pRb is a mutual event in the carcinogenesis of human cells. HPV E6 and E7 oncogenes are key regulatory proteins inside host cells and are associated with the transcriptional activity of NF-κB. Therefore, activation of NF-κB by viral oncogenes may be the mechanism of tumour formation in cervical cancer [3].

Worldwide, an estimated 5,70,000 new cases of cervical cancer representing 7.5% of all female cancer deaths [4]. Every year in India, 122,844 women were diagnosed with cervical cancer and 67,477 succumb to the disease. India has a population of 432.2 million women aged 15 years and older who are at risk of developing cancer [5].

The transcription factor NF-κB was discovered in 1986 as a nuclear factor that binds the enhancer element of the immunoglobulin kappa light-chain of activated B cells [6]. Five members have been seen in mammals; p65 (RelA), RelB, c-Rel, NF-κB1 and NF-κB2. The NF-κB family play an essential role in innate immunity, inflammation, viral replication and the initiation and progression of cancer [7]. Activation of NF-κB occur within minutes by release from IκB or by cleavage of the inhibitory ankyrin repeat domains of p100 and p105. This process is catalysed by an enzyme complex containing IκB kinases (IKK1 and IKK2) and at least one non-catalytic accessory protein (NF-κB Essential Modulator: NEMO) [8]. The non-canonical or atypical pathway is an alternative pathway of NF-κB activation and is independent of the activity of IKK2 and NEMO. It has functions like tumour suppressing and facilitating apoptosis [9].

The NF-κB pathway is an important player in the development of cervical cancer [10]. The persistent HPV infection and mutational changes a tumour may emerge from a premalignant lesion, and this seems to be accompanied by a progressive loss of responsiveness to the NF-κB mediated growth inhibitory signal. As cervical cancer progresses the anti-proliferative functions of the NF-κB network are decreased. Further studies are to be done to clarify if members of these pathways are of clinical interest as biomarkers or therapeutic targets [11].

Hence, this case-control study is designed to know the HPV incidence rate among the women belonging to reproductive age group, and to detect the association of NF-κB gene polymorphism with the persistence of HPV infection in the study group.

## 2 MATERIALS AND METHODS

This study was conducted according to the standards of the Declaration of Helsinki and has been approved by the 36th Institutional Ethics Committee. The study was conducted at Molecular Genomics and Research Laboratory, Post Graduate Department of Biochemistry, in collaboration with Department

of Obstetrics and Gynaecology, SCB Medical college and Hospital, Cuttack. Study was conducted for a period of 2 years. According to the examination findings on the Outpatient Department (OPD) case sheets, we have selected age matched study groups as following: Group with normal healthy cervix (N = 75) were taken as controls; Group with unhealthy cervical pathology (N = 75) as cases.

Inclusion criteria: Women in reproductive age group (15-44 years). Patients with unhealthy cervix who were having erosion, chronic cervicitis, and hypertrophied cervix, bleeding on touch, suspicious growth on the cervix. Women with cervical cancer who were having history of post coital, or postmenopausal bleeding along with dyspareunia. Exclusion criteria: Diabetic patients, smokers, HIV patients, previous history of Sexually Transmitted Diseases (STDs).

After obtaining the written consent of patients, cervical swab was taken with the help of a cervical brush and Roche cell collection media for HPV Genotyping. 2 ml of venous blood was collected in EDTA vials for molecular techniques. Biochemical parameters like Random Blood Sugar (RBS), Renal Function Test (RFT), and Lipid Profile values were taken from the OPD case sheets of the study subjects. HPV genotyping was performed on cobas x 480 and cobas z 480 analysers from cervical swab specimen [12]. DNA was extracted from EDTA blood sample using QIAGEN DNA Mini kit and DNA quality/quantity was checked in Nanodrop spectrophotometer [13].

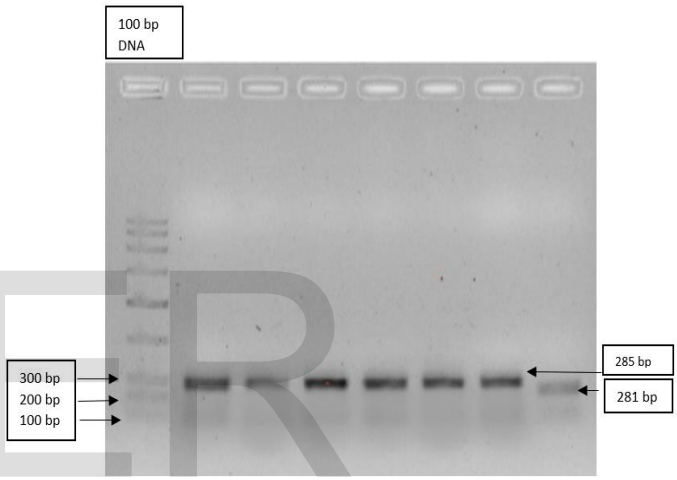
Elute DNA were amplified using the PCR primer sets. PCR was performed with conditions: 1 min at 95$^{o}$C, 30 sec at 95$^{o}$C, 30 sec annealing at 60$^{o}$C, 1 min at 72$^{o}$C, and finally 5 min at 72$^{o}$C, for 35 cycles. Post PCR products were subjected to electrophoresis and visualised with Bio-Rad Gel Documentation system [14].

Polymorphism was assessed by Restriction Fragment Length Polymorphism (RFLP). For detection of the -94ins/del ATTG polymorphism in NFκB-1, a PCR product of 281 base pair (del allele) and 285 base pair (ins allele) were digested with restriction enzyme PfIMI (10 U/ µL, BioLabs), which has a recognition site in this region. The ins allele was cleaved into two fragments of 45 bp and 240 bp after restriction digestion. However, there was no cleavage at the deletion allele (del). Ideally heterozygotes showed three fragments, since the 45 base pair is a very small fragment only two fragments were visualised. The digested products were subjected to electrophoresis and the bands were visualised with the help of a Bio-Rad Gel Doc system [15].

For quantitative data, statistical analysis was done using SPSS 20 (IBM Inc, Chicago, Illinois). Results were expressed as mean ± standard deviation for continuous variables and as percentages for categorical variables. A 'p' value of ≤ 0.05 was considered statistically significant.

## 3 RESULTS

Table 1 showed biochemical parameters in the study participants. All data were represented as Mean ± Standard Deviation (SD). The data was compared by one-way Anova and Post hoc Turkey HSD test. No significant findings were present within the study groups. In table 2, among the 58 HPV positive participants, 19(32.7%), 4(6.9%) and 35(60.3%) of them




were positive for HPV 16, 18 and other high-risk strain respectively. In normal healthy cervix, 4% (3) HPV16 and 2.6% (2) other high-risk strain positive. 21.3% (16), 5.3% (4) and 44% (33) were HPV 16,18 and other high-risk positive among unhealthy cervix study group.

**Figure 1: Agarose gel picture showing post PCR DNA bands of NFκB1 gene, LANE 1: 100 bp LADDER, LANE 2-7: 281 bp bands, LANE 8: 240 bp band.**

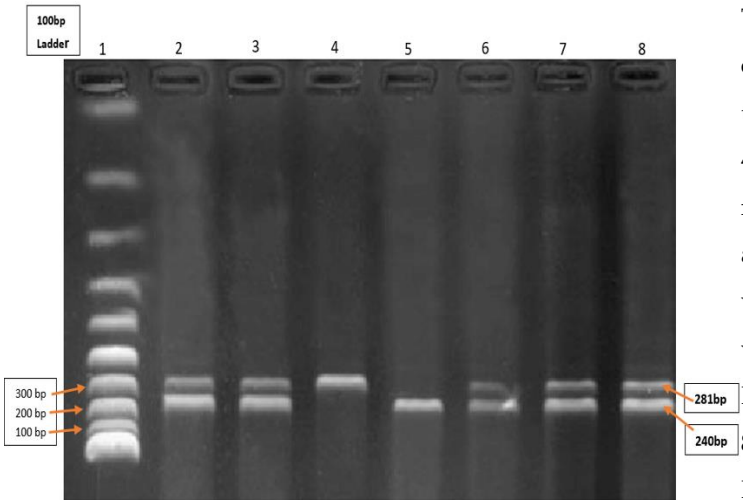



**Figure 2: Agarose gel picture showing post RFLP bands of NFκB1 -94 ATTG promoter region with rs28362491, LANE 1: 100 bp LADDER, LANE 2,6 and 7 are ins/del ATTG; LANE 3,4 and 5 are ins/ins ATTG; and LANE 8 is del/del ATTG genotype.**

| BIOCHEMICAL PARAMETERS (Total N = 150) | CONTROLS WITH HEALTHY CERVIX (N=75) | CASES WITH CERVICAL PATHOLOGY (N=75) |
|---|---|---|
| Random Blood Sugar (RBS) (mg/dl) | 60.85 ± 6.41 | 121.69 ± 6.96 |
| Serum Urea(mg/dl) | 13.07 ± 6.14 | 13.77 ± 3.34 |
| Serum Creatinine(mg/dl) | 0.44 ± 0.19 | 0.43 ± 0.08 |
| Serum Total Cholesterol (mg/dl) | 85.8 ± 8.69 | 81.28 ± 8.86 |
| Serum Triglyceride (mg/dl) | 63.61 ± 9.63 | 64.18 ± 7.47 |
| Serum High Density Lipoprotein (HDL) (mg/dl) | 24.59 ± 3.74 | 23.43 ± 3.84 |
| Serum Low Density Lipoprotein (LDL) (mg/dl) | 54.72 ± 3.81 | 55.41 ± 7.03 |
| Serum Very Low-Density Lipoprotein (VLDL) (mg/dl) | 12.58 ± 2.02 | 12.28 ± 2.49 |

**Table 1: Biochemical parameters in the study participants.**

| HPV GENOTYPES | CASES (N = 75) | CONTROLS (N = 75) |
|---|---|---|
| HPV 16 | 16(21.3%) | 3(4%) |
| HPV 18 | 4(5.3%) | 0 |
| OTHER HIGH-RISK STRAIN | 33(44%) | 2(2.6%) |

**Table 2: Distribution of HPV 16, 18 and OTHER HIGH-RISK STRAIN among the study groups.**

Table 3 depicted distribution of genotype and allele frequencies of *NFκB1* gene polymorphism in the study groups. Insertion allele was found to be more than deletion allele that is 42.7%, and 61% in healthy and unhealthy cervix study group respectively. Del/del genotypes was seen in healthy as 28.2% and unhealthy cervix as 71.8%. Ins/ins in healthy cervix group was 59.3% and unhealthy cervix was 40.7%. In carcinoma cervix, ins/del was seen in highest number as 100%.

In healthy cervix group, among the HPV negative individuals, 88.6% were having ins/ins and 11.4% were del/del genotype. In HPV 16 positive group, 66.7% were ins/ins and 33.3% were del/del genotype. Other high-risk strain has only del/del genotype (100%).

Table 5 showed distribution of HPV strains among the different *NFκB1* genotypes in the group with unhealthy cervix. Within control study group, in HPV 16 positive category, 56.3%, 31.2% and 12.5% were having ins/ins, del/del and ins/del genotypes. In HPV 18, 25% and 75% were having ins/ins and del/del variety. 42.5% were del/del type, 54.5% were ins/ins type and 3% were ins/del among the other high-risk strain positive individuals. In HPV negative individuals, we got 72.7% ins/ins and 27.3% del/del genotype.

| GENOTYPES | CONTROLS (%) (N = 75) | CASES (%) (N = 75) |
|---|---|---|
| ins allele | 128(42.7) | 90(61) |
| del allele | 22(7.4) | 60(39) |
| del/del (N = 39) | 11(28.2) | 28(71.8) |
| ins/ins (N = 108) | 64(59.3) | 44(40.7) |
| ins/del (N = 3) | 0(0) | 3(100) |

**Table 3: Distribution of genotype and allele frequencies of NFκB1 gene polymorphism in the study groups.**

| GENOTYPES | NEGATIVE | HPV 16 | OTHER HIGH-RISK STRAIN |
|---|---|---|---|
| del/del | 8(11.4%) | 1(33.3%) | 2(100%) |

| | | | |
|---|---|---|---|
| **ins/ins** | 62(88.6%) | 2(66.7%) | 0 |

**Table 4: Frequency of *NFκB1* genotypes among HPV strains in healthy cervix group.**

| HPV GENO-TYPES | del/del (N = 28) | ins/del (N = 3) | ins/ins (N = 44) |
|---|---|---|---|
| **NEGATIVE (N = 22)** | 6(27.3%) | 0 | 16(72.7%) |
| **HPV 16 (N = 16)** | 5(31.2%) | 2(12.5%) | 9(56.3%) |
| **HPV 18 (N = 4)** | 3(75%) | 0 | 1(25%) |
| **OTHER HIGH-RISK STRAIN (N = 33)** | 14(42.5%) | 1(3%) | 18(54.5%) |

**Table 5: Distribution of HPV strains among the different *NFκB1* genotypes in the group with unhealthy cervical pathology.**

## 4 DISCUSSIONS

According to WHO, HPV infection is the most common viral infections of the reproductive tract worldwide [16]. In India, the infection of HPV type 16 is found to be exclusively high followed by type 18 in cervical cancer cases [17]. Cervical cancer is the most common cancer in women in India after breast cancer in women living in rural area [18]. The knowledge about the distribution of HPV types in cervical cancers and HPV types circulating in the communities in different regions of India would be useful in planning the optimum strategy for vaccination in India as well as for application in the National Cancer Prevention Strategies [19].

**Table 2** was evident that study showed out of 150 women, 58 were HPV positive. Among the positives, we found 32.7% HPV16, 6.9% of HPV18 and 60.3% of other high-risk strain. But contradictory to above data, Nunes et al. in 2014 found that global distribution pattern of HPV appears to be 60-65% positivity for HPV16; 4-20% for HPV18; and a low prevalence of other HPV types [20]. In our study might be a larger sample size is required for better conclusion of our study. In **table 2**, among the healthy cervical cytology, HPV infection rate was found to be 6.7%, of which 4% was for HPV 16 and 2.6% for other high-risk strain. Similar finding was found according to recent studies, in women with Normal Cervical Cytology (NCC), HPV prevalence is 9.9% in present scenario [21].

Among the unhealthy cervix group, 21.3% positivity for HPV 16, 5.3% for HPV 18 and 44% for other high-risk strain as explained in **table 2**. The rate of single genotype infection especially HPV 16, was very high in the abnormal histopathological group as compared to the normal group which was in accordance with Baloch et al. Single HPV16 increases with the disease severity and highest in cervical carcinoma cases [22]. Apart from carcinoma cervix study group, our result did not match with the previous studies mainly due to regional variation in HPV genotypes distribution in different geographical regions of India. Presence of other high-risk strain mostly in unhealthy cervical pathology might have its role in development of cervical cancer by initiating inflammation and persistent infection.

Zhou B et al. found that the frequency of ATTG polymorphism in nasopharyngeal carcinoma patients was significantly higher than that in control subjects, indicating that the functional *NFκB1* promoter polymorphism was associated with increased risk for nasopharyngeal carcinoma [23]. In **table 3**, healthy cervical pathology got 59.3% ins/ins and 28.2% del/del genotype. While in unhealthy cervix we got 71.8% of del/del, 40.7% ins/ins and 100% ins/del. In the present study from **table 3**, insertion allele was predominant as 42.7% in healthy and 61% in unhealthy cervix group. Similarly, B. Zhou et al. identified a significant association between the -94 insertion/deletion ATTG polymorphism in *NFκB1* promoter and the risk of cervical carcinoma. But they indicated that individuals homozygous for insertion allele have a 2.560-fold risk to develop cervical cancer compared with those homozygous for deletion [24]. An increase in IκB messenger RNA expression in cervical cancer has been observed, which was probably the consequence of functional activation of *NF-κB*. The activation of *NF-κB* was known to cause strong transcriptional up-regulation of IκB as a response mechanism found effective in cancers [25].

In healthy cervix group, we found homozygous ins/ins genotype maximal in 88.6% of HPV negative and 66.7% of HPV 16 positive individuals according to **table 4**. While other high-risk strain genotype has 100% homozygous del/del genotype. Previous studies demonstrated that the frequency of insertion allele in carcinoma cervix patients was significantly higher than that in control subjects, indicating that this polymorphism might contribute to the constitutive *NF-ƙB* activity in carcinoma cervix. Deletion allele results in comparatively decreased *NFƙB1* message and hence decreased p50/p105 NF-ƙB protein production [24].

From **table 5**, in unhealthy cervix, we found ins/ins genotype in majority that is 72.7% in negative, 56.3% in HPV 16, and 54.5% in other high risk. Followed by homozygous del/del genotype as 27.3% in negative, 31.2% in HPV 16, and 42.5% other high-risk genotype group. While in HPV 18 strain 75% was of del/del and 25% of ins/ins genotypes. Similar findings were found by Zhou et.al as precancer cases with cervicitis have 2.26-fold risk to develop cervical cancer in homozygous insertion genotype compared to those with homozygous deletion [24].

Possible explanation for above finding might be variant type of *NF-ƙB* genes are favourable target for HPV to modify host immune system for its survival and persistent infection. The deletion allele causes the loss of binding to nuclear proteins, which leads to reduced promoter activity. The promoter sequence with -94 deletion allele results in lower transcriptional activity leading to decreased levels of p50/p105 in the cytoplasm. In addition, the p50 in deletion allele can form lesser heterodimers with p65 to facilitate the inflammatory pathway compared to the insertion allele. Therefore, the deletion allele may play a protective role in cancer risk [26].

## 5 CONCLUSIONS

We found del/del genotype contribution maximum in the group with unhealthy cervix pathology. Ins/del genotype prevalent more with cases. In unhealthy cervix, we found that ins/ins genotype was prevalent among all the HPV strains except for HPV 18. HPV 18 positive groups were associated more with del/del genotype. The limitations of our study are small sample size and different stages of cervical cancer patients is not evaluated, so further study should be done with a larger sample size and by comparing all stages of CIN with our study parameters. Based on the association of staging of cancer, HPV infection and NFƙB polymorphism, we can look for the possibility of NFƙB as a potential marker for progression of invasive cervical cancer in near future.

## ACKNOWLEDGMENT

The authors wish to thank Dr M K Mandal, Dr U K Das, Dr Lucy Das, Dr S Jena and Dr A Lamare. This work was supported in part by a Multidisciplinary Research Unit, SCB Medical College and Hospital.

## REFERENCES

[1] Bruni L, Albero G, Serrano B ICO/IARC Information Centre on HPV and Cancer (HPV Information Centre) 2019.
[2] Chauhan, S. C., Jaggi, M., Bell, M. C., Verma, M. & Kumar, D. 2009. Epidemiology of Human Papilloma Virus (HPV) in Cervical Mucosa. Methods Mol Biol, 471, 439-56.
[3] Y. Jing, T. Wang, Z. Chen et al., "Phylogeny and polymorphism in the long control regions E6, E7, and L1 of HPV 8 Journal of Oncology Type 56 in women from southwest China," Molecular Medicine Reports, vol. 17, no. 5, pp. 7131–7141, 2018.
[4] https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer.
[5] Zur Hausen, H. 2009. Papilloma viruses in the causation of human cancers - a brief historical account. Virology, 384, 260-5.
[6] Sreedevi A, Javed R, Dinesh A, ICO Information Centre on HPV and cancer. Human Papillomavirus and Related Diseases in India (Summary Report 2014-08-22); 2014.
[7] Sen, R. & Baltimore, D. 1986. Inducibility of kappa immunoglobulin enhancer-binding protein NF-kappa B by a posttranslational mechanism. Cell, 47, 921-8.
[8] Albert S. Baldwin, Series Introduction: The transcription factor NF-κB and human disease Jr. J Clin Invest. 2001;107(1):3-6.
[9] Andrea Oeckinghaus and Sankar Ghosh, The NF-kB Family of Transcription Factors and Its Regulation, Cold Spring Harb Perspect Biol 2009;1: a000034.
**[10]** Guttridge, D. C., Albanese, C., Reuther, J. Y., Pestell, R. G. & Baldwin, A. S., JR. 1999. NF-kappa controls cell growth and differentiation through transcriptional regulation of cyclin D1. Mol Cell Biol, 19, 5785-99.
**[11]** Sam Tilborghs, Jerome Corthouts, Yannick Verhoeven, David Arias, Christian Rolfo, Xuan Bich Trinh, Peter A van Dam, the role of Nuclear Factor-kappa B signaling in human cervical cancer,2017 Dec;120:141-150. doi: 10.1016/j.critrevonc.2017.11.001.
[12] cobas® HPV Test manual; for in vitro diagnostic use; Doc Rev 12.0.
[13] QIAamp® DNA Mini and Blood Mini Handbook; fifth edition, May 2016.
[14] GeNet Bio Prime Taq DNA Polymerase, G-1001, by ISO 9001 and 13485 system kit insert protocol.
[15] Protocol for optimizing restriction endonuclease reactions/NEB/2012/12/07

[16] https://www.who.int/news-room/fact-sheets/detail/human-papillomavirus-(hpv)-and-cervical-cancer.
[17] Basu P, Roychowdhury S, Bafna UD, Chaudhury S, Kothari S, Sekhon R, et al. Human papillomavirus genotype distribution in cervical cancer in India: Results from a multicenter study. Asian Pac J Cancer Prev 2009; 10:27-34.
[18] Kaarthigeyan K. Cervical cancer in India and HPV vaccination. Indian J Med Paediatr Oncol 2012; 33:7-12.
[19] Basu P, Roychowdhury S, Bafna UD, Chaudhury S, Kothari S, Sekhon R, et al. Human papillomavirus genotype distribution in cervical cancer in India: Results from a multicenter study. Asian Pac J Cancer Prev 2009; 10:27-34.
[20] Bruni L, Albero G, Serrano B, Mena M, Gómez D, Muñoz J, et al. ICO/IARC information centre on HPV and cancer (HPV Information Centre). Human Papillomavirus and Related Diseases in the World.
[21] Nunes JD, Vidal FC, Ferraro CT, Chein MB, Brito LM, Monteiro SC, et al. Molecular detection of human papillomavirus in Brazilian women with cervical intraepithelial neoplasia in a Northeast Brazilian city. Genet Mol Res 2014; 13:9077-85.
[22] Oliver J, Gomez-Garcia M, Paco L et al. A functional polymorphism of the NFKB1 promoter is not associated with ulcerative colitis in a Spanish population. Inflamm Bowel Dis 2005; 11: 576–579.
[23] Zhou B, Rao L, Li Y et al. A functional insertion/deletion polymorphism in the promoter region of NFKB1 gene increases susceptibility for nasopharyngeal carcinoma. Cancer Lett 2009; 275: 72–76.
[24] B. Zhou, M. Qie, Y. Wang, L. Yan, Z. Zhang, A. Liang, T. Wang, X. Wang, Y. Song & L. Zhang; Relationship between NFKB1 294 insertion/deletion ATTG polymorphism and susceptibility of cervical squamous cell carcinoma risk; Annals of Oncology 21: 506–511, 2010.
[25] Pacifico F, Leonardi A. NF-kappaB in solid tumors. Biochem Pharmacol 2006; 72: 1142–1152.
[26] DuanWang, TianhangXie, JinXu, HaoyangWang, WeinanZeng, Shuquan Rao, KaiZhou, Fuxing Pei & Zongke Zhou Genetic association between NFKB1 −94 ins/del ATTG Promoter Polymorphism and cancer risk: a meta-analysis of 42 case-control studies, Scientific Reports | 6:30220 | DOI: 10.1038/srep30220.