EJGO European Journal of Gynaecological Oncology

HPV16 E6 mutations and p53 codon72 polymorphism among women with cervical intraepithelial neoplasia 2 and 3 in China

J. Piao, K. You, Y. Guo, Y. Zhang, Z. Li, L. Geng

Department of Obstetrics and Gynecology, Peking University, Third Hospital, Beijing (China)

Summary

Objective: To study the distribution of HPV16 E6 gene mutations and p53 codon72 polymorphism among women with HPV16+ cervical precancerous lesions and explore their relationship with the risk of cervical intraepithelial neoplasia (CIN) 2, 3. *Materials and Methods:* This study analyzed a total of 112 cases of exfoliated HPV16+ cervical cell specimens which were divided into group 1 (normal and CIN1, 55 cases) and group2 (CIN2, 3, 57 cases). Among the 112 specimens, 85 cases were successfully amplified for HPV E6 gene by PCR and the PCR products were sequenced directly. P53 codon72 region was also amplified from the 112 specimens and the PCR products were sequenced directly and compared with the standard sequence. *Results:* Among the 85 amplified HPV sequences, point mutations such as T178G, T350G, G132A, A442C, T310G, G94T, C551A, etc. were found, among which, T178G showed the highest rate (51.76%). The rate of HPV16 E6 mutation T178G in CIN2, 3 group was significantly higher than that in normal and CIN1 group, i.e., in the 112 amplified p53 codon72 sequences, the distribution of Pro/Pro genotype in normal, and CIN1 group was significantly different from that in CIN2, 3 groups, and the disease risk of Pro/Pro genotype was much higher than that of Arg/Arg and Arg/Pro genotypes. *Conclusion:* HPV16 E6 T178G mutation increases the disease risk of CIN2, 3. Meanwhile, compared with Arg/Arg and Arg/Pro genotypes, p53 codon72 Pro/Pro genotype more associated with the disease risk of CIN2, 3.

Key words: Cervical intraepithelial neoplasia; HPV16 E6; P53 codon72.

Introduction

Cervical cancer is one of the common gynecological malignancies and the second most common malignancy in women worldwide. The incidence of cervical cancer accounts for more than 13% of all female cancers globally, only second to breast cancer. A large amount of epidemiological data showed that more than 85% cervical cancer cases occur in developing countries [1]. In consistence, in China, cervical cancer also ranks second only to breast cancer among all female malignancies [2]. International Agency for Research on Cancer (IARC) clearly indicated that HPV infection is the major risk factor and initiator for the occurrence of cervical cancer [3]. According to the survey results, 99.7% patients with cervical cancer have HPV infection. In the tissues of CIN and cervical carcinoma, HPV16, 18, 31, 33, 35, 39, 45, 56, 58, 59, and 68 accounted for more than 90% of HPV subtypes, among which, HPV16 is the most common HPV subtype infecting cervical cells and causing cervical squamous carcinoma [4].

Recent studies have shown that factors such as HPV genetic variations, persistent and repeated HPV infections, host immune status, etc., are closely related to the occurrence of cervical cancer. There is also report indicating that persistent HPV infection may be caused by gene mutations [5]. HPV16 genotype changes play an important role in the epidemic of cervical cancer and the distribution of HPV16 E6 mutations exhibits regional and racial characteristics. According to the global changes in genotype, HPV16 has been classified into subtypes as the following: European type (E), Asian type (As), Asian American type (AA), African type I, African type II, and North American type (NA1), etc. [6]. Among type E, the most common mutant is L83V (i.e., T350G), followed by D25E (i.e., T178G); there are also rare mutants such as E113D, Q14H, etc. In Africa, the most common mutants are R10G, Q14D, D64E, H78Y, A61G, etc. [7]. Currently, studies on HPV-16 E6 region mutations in Asian populations are not only much less than those in European populations, but also with controversial results.

The region of early genes in HPV genome, i.e., the E region, contains six open reading frames (ORFs) [6], among which E6 region encodes the most important oncoprotein of HPV16, the E6 protein. The most important mechanism of E6 protein causing carcinogenesis in cells is its binding to tumor suppressor gene p53 and leads to degradation of the latter, which causes the loss of p53 biological function, hence resulting in cell cycle disorder, DNA damage accumulation, and ultimately carcinogenesis. P53 is the tumor suppressor gene most closely related to cancer and presently the gene mostly mutated in human malignancies.

In 1998, Storey *et al.* firstly proposed that the polymorphism at the codon72 in the 4th exon of p53 gene may be an

Revised manuscript accepted for publication June 29, 2015

important genetic factor causing woman susceptible to cervical cancer. They also showed that the risk of cervical cancer among women with homozygous Arg/Arg at p53 codon72 was seven times higher than that with homozygous Pro/Pro [8]. Thereafter, many researchers investigated the relationship between p53 polymorphisms and cervical cancer, but the results remained controversial. The current prevailing view is that p53 codon72 has racial and regional differences. For instance, Beck *et al.* pointed out that the distribution of p53 codon72 allele exhibited significant racial differences and gradient north-south latitudinal changes among races. However, there were no recent studies focusing on the relationship of HPV-16 E6 mutations and p53 codon72 polymorphisms with the risk of CIN2, 3 among women in Beijing of China. Therefore, the present study investigated HPV-16 E6 mutations and p53 codon72 polymorphisms in women with cervical precancerous lesions in Beijing.

Materials and Methods

Sample collection

Residual exfoliated HPV16+ cervical cell specimens from 112 women (Han ethnicity) visited the Peking University Third Hospital, Beijing, China, during June, 2013-December, 2014 were used in this study. These cases were diagnosed normal or with CIN1-3; they were divided into group 1(normal and CIN1, n=55) and group 2 (CIN2 and 3, n=57) according to the pathological diagnosis. The ages of patients were 22-78 years (mean, 43 years). The samples were collected with informed consent obtained from each patient.

Methods

The full-length sequence of HPV16 E6 gene was amplified by PCR using specific primers primer express software as follows: P1, 5-CGTAACCGAAATCGG TTGAAC-3 and P2, 5-GCTCATAACAGTAGAGATC-3. A pair of primers to amplify the coding sequence of the codon72 of p53 gene were also designed as the following: P3, 5-GACCTGGTCCTCTGA-3 and P4, 5-GATACGGCCAGGCATTGAAG-3.

PCR amplification, sequencing, and analysis of HPV16 E6 gene

With genomic DNA extracted from SiHa human cervical cancer cells as positive control and H_2O as negative control, HPV16 E6 sequence was amplified by PCR with specific primers using the genomic DNA from HPV16+ cells as template. The PCR conditions were as follows: initial denaturation at 95°C five minutes; 94°C 30 seconds, 51°C 30 seconds, 72°C 60 seconds, 35 cycles; 72°C seven minutes. The PCR products were stored at 4°C and later analyzed by agarose gel electrophoresis.

After purification, the PCR products were directly sequenced with HPV16 E6 primers P1 and P2 from both sides. The PCR products purification and sequencing services were provided and the obtained sequences were analyzed by blasting GenBank database at NCBI and compared with the HPV16 E6 sequence of German standard strain (Accession number, NC_001526).

Meanwhile, sequencing and analysis of the PCR products of p53 codon72 with genomic DNA extracted from SiHa human cervical cancer cells as positive control and H_2O as negative control, p53 codon72 region was amplified by PCR with specific primers under the conditions as follows: initial denaturation at 95°C five minutes; 94°C 30 seconds, 51°C 30 seconds, 72°C 60 seconds, 35 cycles; 72°C seven minutes. The PCR products were stored at 4°C and analyzed by agarose gel electrophoresis later. The PCR products purification and sequencing services were provided and the obtained sequences were analyzed by blasting GenBank database at NCBI and compared with the sequence of wild type p53 (Accession number, NC_000017.11).

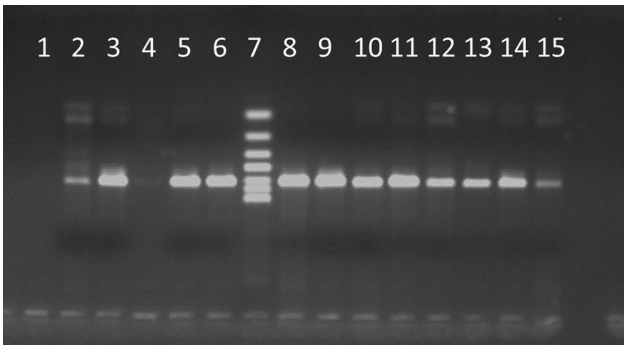

Figure 1. — PCR amplification of HPV16 E6 with samples No.1-14. Lanes 3, 4, and 6 are positive control (Siha cells), negative control (H_2O), and marker, respectively.

Statistical analysis

The data were analyzed using SPSS statistical software. The mutation rates and the p53 codon72 polymorphic loci were compared using c^2 test.

Results

The relationship between HPV16 E6 mutations and the risk of CIN2, 3.

In this study, HPV16 E6 sequences were successfully amplified from 85 out of the 112 HPV16+ cervical cytology specimens; the positive PCR products appeared on agarose gel as bright 550-bp bands (Figure 1), which were further purified and sequenced. Among the 85 amplified E6 sequences, 15 (17.6%) were identical to the wild-type sequence, i.e., without any mutations; the other 82.3% sequences were with at least one point mutation, and there was no insertion or deletion mutations observed among these sequences. in the 85 sequences, T178G (D25E) showed the highest rate (51.76%), while other mutations such as A442C, T350G, G132A, C551A, G94T, and T310G accounted for 14.11 %, 8.2%, 3.5%, 2.35%, and 1.17%, respectively. There were eight cases with T178G and A442C double mutations, and four cases were with T350G and A442C double mutations; however, no double mutation of T178G and T350G and single mutation of A442C was observed. Among these point mutations, T178G, T350G, and A442C caused amino acid changes in E6 protein.

The present authors also found that in normal and CIN1 group, there were 38.46% (15/39) cases with T178G (D25E) mutation, while the same mutation was observed in 64.44% (29/45) cases in CIN2, 3 group; c^2 test indicated that the difference in T178G mutation rate between the two groups was

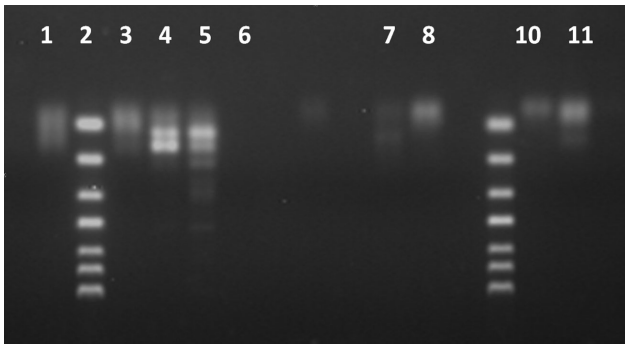

Figure 2. — PCR amplification of p53 condon72 fragment with samples No. 1-11. Lanes 1, 8, and 10 are Pro/Pro genotype, lane 2 is marker, lanes 4 and 5 are Arg/Pro genotype, lane 11 is Arg/Arg genotype, and lane 6 is negative control (H_2O).

statistically significant ($c^2 = 4.661$; OR = 2.9; $p < 0.05$).

The relationship between p53 codon72 polymorphism and the risk of CIN2, 3.

This study amplified the p53 codon72 polymorphic region by PCR from 112 cases of HPV16+ cervical cytology specimen. The positive PCR products appeared respectively on agarose gel as bright bands at 117 and 140 bp (Figure 2), which were sequenced subsequently. The distribution of the three genotypes of p53 codon72 (Arg/Arg, Arg/Pro, and Pro/Pro) are shown in Table 1, which indicates significant genotypic differences between the two groups ($\chi^2 = 6.834$; $p = 0.033$. According to the calculated OR value, the risk of cervical cancer in patients with either Arg/Arg or Arg/Pro was ten-times lower than that with Pro/Pro.

Table 1. — *The distribution of p53 gene polymorphic allele and genotype in the two groups.*

Genotype	Normal and CIN1 N (%)	CIN2 and 3 N (%)	OR	*p*
Arg/Arg	20 (36.3)	18 (31.6)	0.808	0.037
Arg/Pro	34 (61.8)	30 (52.6)	0.686	0.032
Pro/Pro	1 (1.8)	9 (15.8)	10.8	

Discussion

In this study, the authors analyzed the risk of CIN2, three in women of Beijing area and found that most HPV16+ cases (51.76%, 44/85) had the European type mutation T178G (D25E); further statistical analysis showed that the rate of HPV16 D25E mutation in CIN2, 3 group was significantly higher than that in normal and CIN1 group, which means mutation type of T178G relates with the risk of CIN2, 3. Statistical analysis indicated OR = 2.6 when HPV16 wild type served as control, i.e., the risk of cervical cancer in women with D25E HPV16 mutant was higher than that in women with wild type HPV16, suggesting that HPV16 E6 D25E mutation increased the probability of progression of the cervical lesions in HPV16+ women, which is in agreement with a few reports from other areas of China. For example, detections of HPV16 E6 mutations in Liaoning, Jiangxi, Hubei, and Guangdong provinces of China have shown that D25E is the most common type of HPV16 E6 mutations, the mutation rate of which shows a trend of increased risk of cervical cancer [9-11]. However, there were recent studies reporting inconsistent results that in Xinjiang of China, HPV16 E6 T350G (L83V) was a risk factor of cervical cancer for local Uyghur women but not for the local women of Han ethnicity [12-14], suggesting that this mutation may be related to ethnicity and region. Meanwhile, common HPV16 E6 mutations and their relationship with cervical cancer in women of other Asian countries remains controversial. For example, D25E was a common mutation among women in Korea, but showed no significant correlation with the progression of cervical lesions [15, 16]. Moreover, study on European women suggested that HPV-16 E6 T350G (L83V) was a common mutation in European population and was believed to be one of the risk factors for cervical cancer [17]. In addition, European type L83V as the most common HPV16 E6 mutation among women in the Morocco region of Africa was associated with the progression of cervical cancer [18].

In this study, the authors found that among the HPV16 E6 mutants, another European type mutation L83V (T350G) accounted for 14.11% of the total mutations (only second to D25E) was not associated with the risk of CIN2, 3. In addition, they also detected a few rare mutations such as G132A, A442C, C551A, G94T, and T310G, at the rates of 8.2%, 5.29%, 3.5%, 2.35%, and 1.17%, respectively. These mutations were rarely seen in previous reports.

In 1998, Storey *et al.* [8] firstly proposed that the polymorphism at codon72 in the 4th exon of p53 gene may be an important genetic factor causing greater susceptibility to cervical cancer. They also believed that the risk of cervical cancer among women with homozygous Arg/Arg at p53 codon72 was seven times higher than that with homozygous Pro/Pro [10], p53 codon72 polymorphism may change the E6 protein activity, and Arg/Arg genotype p53 is more easily degraded by the E6 protein of high-risk HPV [8]. The present study detected p53 codon72 polymorphism in 112 cases of exfoliated HPV16+ cervical cell specimen, and statistical analysis indicated that the risk of cervical cancer in women with p53 codon72 Arg/Arg or Arg/Pro genotype was lower than that with Pro/Pro, i.e., homozygous Arg/Arg and heterozygous Arg/Pro might serve as relatively protective factors against cervical lesions, whereas Pro/Pro genotype might be the cervical cancer promoting risk factor. However, most previous studies including that of Storey *et al.* [8] considered p53 codon72 genotype Arg/Arg as a risk factor causing cervical cancer, but Pro/Pro genotype as a possible protective factor against the progression of cervical lesions, which is

inconsistent with the present findings. Nevertheless, some recent studies on patients from other regions, such as women of Han ethnicity in China's Guangdong and Xinjiang, have shown that Pro/Pro genotype may be a risk factor of cervical cancer [19-22], which is in agreement with our results. The above discrepancies may be related with the differences in factors such as sample size, region, ethnicity, etc., which may be solved in future studies with expanded sample size and groups.

Meanwhile, the present authors also analyzed the existing reports and found that previous studies on HPV16 E6 mutations and p53 codon72 polymorphism were with pathological paraffin-embedded specimens from patients, but in the preset study, the authors used residual cervical cytology specimens. Compared with paraffin-embedded specimen, cervical cytology specimen has advantages, i.e., the present method is non-invasive and relatively simple to perform.

Conclusions

This study analyzed DNA samples extracted from cervical exfoliated cells of 112 women in Beijing of China. However, certain aspects of the present results are inconsistent with some previous studies. The present data showed that D25E was the most common mutation of HPV16 E6 among women in Beijing area, and its rate in CIN2, 3 group was significantly higher than that in normal and CIN1 group, suggesting that HPV16 E6 D25E may be a risk factor of CIN2, 3. The authors also showed that the rate of p53 codon72 Pro/Pro genotype in CIN2, 3 group was significantly higher than that in normal and CIN1 group, i.e., this polymorphic genotype may be a risk factor of cervical cancer for women in Beijing. To conclude, the above two types of mutations may help determine not only normal and CIN1-3 lesions but also the probability and risk of cervical cancer progression from normal and CIN1 to CIN2, and 3. These methods are non-invasive, simple and cost-effective.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (No.81472429 and No.11072006)

References

[1] Zhang J.G., Liu S.Z., Chen Q., Quan P.L., Lu J.B., Sun X.B., *et al.*: "Analysis of cervical cancer incidence and death of Chinese in 2009". *Chinese Tumor*, 2013, *8*, 612.

[2] Zhou Q.Y., Pan X.L., LI Xinmin, Zheng Xz, Yang Aq, Li HA, Yan Lh, *et al.*: "Mechanism of p53Arg72Pro polymorphism and HPV related cervical cancer". *Chinese Tumor*, 2009, *2*, 136.

[3] Cantarella G., Liniger M., Zuniga A., Schiller J.T., Billeter M., Naim H.Y., *et al.*: "Recombinant measles virus-HPV vaccine candidates for prevention of cervical carcinoma". *Vaccine*, 2009, *27*, 3385.

[4] Hariri S.., Steinau M., Rinas A., Gargano J.W., Ludema C., Unger E.R., et al.: "HPV genotypes in high grade cervical lesions and invasive cervical carcinoma as detected by two commercial DNA assays, North Carolina, 2001-2006". *PLoS One*, 2012, *7*, e34044.

[5] Li Jh, Wang Wj, Gao Wl, Guo Lf, Feng Lm, *et al.*: "Effects of HPV18 E6 siRNA on the proliferation and chemotherapy of cervical cancer cell sensitivity". *Basic Clin. Med.*, 2010, *30*, 500.

[6] Derrien T., Johnson R., Bussotti G., Tanzer A., Djebali S., Tilgner H., Guernec G., *et al.*: "The GENCODE v7 catalog of human long noncoding RNAs: analysis of their gene structure, evolution, and expression". *Genome Res.*, 2012, *22*, 1775.

[7] Ho L., Chan S.Y., Burk R.D., Das B.C., Fujinaga K., Icenogle J.P., *et al.*: "The genetic drift of human papillomavirus type 16 is a means of reconstructing prehistoric viral spread and the movement of ancient human populations". *J. Virol.*, 1993, *67*, 6413.

[8] Storey A., Thomas M., Kalita A., Harwood C., Gardiol D., Mantovani F., *et al.*: "Role of p53 polymorphism in the development of human papillomavirus-associated cancer". *Nature*, 1998, *93*, 229

[9] Sun Z., Lu Z., Liu J., Wang G., Zhou W., Yang L., *et al.*: "Genetic variations of E6 and long control region of human papillomavirus type 16 from patients with cervical lesion in Liaoning". *China BMC Cancer*, 2013, *13*, 459.

[10] Wu Y., Chen Y., Li L., Yu G., He Y., Zhang Y., *et al.*: "Analysis of mutations in the E6/E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China". *J. Gen. Virol.*, 2006, *87*, 1181.

[11] Cai H.B., Chen C.C., Ding X.H.: "Human papillomavirus type 16 E6 gene variations in Chinese population". *BMC Urol.*, 2005, *5*, 15.

[12] Liu F., Ma Y.T., Yang Y.N., Xie X., Li X.M., Huang Y., *et al.*: "Medicine and clinical relationship based in Xinjiang Han and Uygur HPV16E6 gene variants and uterine cervix cancer and cervical lesions". *Basic Clin. Med.*, 2012, *3*, 19.

[13] Jang M., Rhee J.E., Jang D.H., Kim S.S.: "Gene expression profiles are altered in human papillomavirus-16 E6 D25E-expressing cell lines". *J. Virol.*, 2011, *8*, 453.

[14] Choi B.S., Kim S.S., Yun H., Jang D.H., Lee J.S.: "Distinctive distribution of HPV16 E6 D25E and E7 N29S intratypic Asian variants in Korean commercial sex workers". *J. Med. Virol.*, 2007, *79*, 426.

[15] Wu Y., Chen Y., Li L., Yu G., He Y., Zhang Y., *et al.*: "Analysis of mutations in the E6/E7 oncogenes and L1 gene of human papillomavirus 16 cervical cancer isolates from China". *J. Gen. Virol.*, 2006, *87*, 1181.

[16] Qmichou Z., Khyatti M., Berraho M., Ennaji M.M., Benbacer L., Nejjari C., *et al.*: "Analysis of mutations in the E6 oncogene of human papillomavirus 16 in cervical cancer isolates from Moroccan women". *BMC Infect Dis.*, 2013, *13*, 378.

[17] Chen C.C.: "P53 gene codon72 polymorphism and HPV16 related cervical cancer in 2012". *Clin. Exp. Pathol.*, 2012, *19*, 9.

[18] Min-min H., Ming-rong X., Ze-yi C., Kai-xuan Y., Zhi-lin S., *et al.*: "The relationship between P53 gene codon72 polymorphism and HPV-16, 18 E6 and cervical cancer". *Journal of Sichuan University (Medical Edition)*, 2006, *37*, 404.

[19] Li J., Zheng Qy., *et al.*: "Meta analysis of P53 codon72 gene polymorphism and the risk for cervical cancer". *Journal of Xi'an Jiao Tong University (Medical Science Edition)*, 2005, *26*, 384.

[20] Leiros G.J., Galliano S.R., Sember M.E., Kahn T., Schwarz E., Eiguchi K., et al.: "Detection of human papillomavirus DNA and p53 codon 72 polymorphism in prostate carcinomas of patients from Argentina". *BMC Urol.*, 2005, *5*, 15.

[21] Wu Y., Chen Y., Li L., Yu G., Zhang Y., He Y., et al.: "Associations of high-risk HPV types and viral load with cervical cancer in China". *J. Clin. Virol.*, 2006, *35*, 264.

[22] Sun F., Ha X.Q., Lv T.D., Xing C.P., Liu B., Cao X.Z., *et al.*: "Human papillomavirus 16E6 gene mutation in cervical carcinoma tissues". *Chinese Journal of Cancer*, 2009, *28*, 478.

Address reprint requests to:
G. LI, M.D.
Department of Obstetrics and Gynecology
Peking University Third Hospital
No. 38 Xueyuan Road
Beijing 100191 (China)
e-mail: gengli1957@bjmu.edu.cn