The relationship among human papilloma virus infection, survivin, and p53 gene in lung squamous carcinoma tissue

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ABSTRACT

الأهداف: دراسة العلاقة بين التهاب فيروسي البابيلوما من النوع 16 و18، والعلاقة بينهما وطفرة الجين p53، وكذلك العلاقة بين بروتين سورفايفين (survivin) وطفرة هذا الجين في الأنسجة الرئوية المصابة بسرطان الخلايا الحرشفية وذلك من أجل دراسة أسباب سرطان الرئة.

الطريقة: أُجريت هذه الدراسة في معامل بيولوجيا الجزيئات بمستشفى شانغفان المركزي، هيوبي، الصين وذلك خلال الفترة من سبتمبر 2008م إلى مايو 2010م. لقد قمنا بجمع 45 عينة من الأنسجة المصابة بسرطان الخلايا الحرشفية وتشريحها بعد أخذها من قسم الجراحة الصدرية بالمستشفى. تم تعيين الأنسجة الطبيعية القريبة من الأنسجة السرطانية على أنها مجموعة التحكم وذلك من أجل تحليل طفرة الجين p53، فيما تم اختيار 16 عينة مأخوذة من أنسجة أمراض الرئة الحميدة لتكون مجموعة التحكم في الأمراض الغير سرطانية. ولقد قمنا بتحليل الحمض النووي لفيروس البابيلوما البشري بواسطة التفاعل التسلسلي المبلمر (PCR)، وتم تعيين طفرة الجين p53 بواسطة التفاعل التسلسلي المبلمر المتعدد الأشكال لخيط مُفرد من الحمض النووي وباستخدام تصبغ الإيثيديوم بروميد (PCR–SSCP–EB). وتم الكشف عن بروتين سورفايفين باستخدام الاختبار المناعي لكيمياء الأنسجة (immunohistochemistry).

النتائج: لقد ظهرت طفرة الجين p53 في حوالي 68.9% من أصل 45 نسيجاً مُصاباً بسرطان الخلايا الحرشفية الرئوية، ولقد وصلت عدد المرات التي حصلت فيها طفرات الجين إلى: 15.6%، 17.8%، 15.6%، 20%. وكانت نتيجة حوالي 42.2% من عينات الأنسجة السرطانية إيجابية فيما يخص ظهور الحمض النووي لفيروس البابيلوما البشري، و62.2% من العينات كانت نتيجتها أيضاً إيجابية فيما يخص ظهور بروتين سورفايفين. لقد كان هناك علاقة عكسية بين فيروس البابيلوما البشري وطفرة الجين p53، فيما كانت العلاقة طردية بين طفرة الجين p53 وبروتين سورفايفين.

خاتمة: أشارت الدراسة إلى أن كلاً من طفرة الجين p53 والتهاب فيروس البابيلوما البشري قد يدعمون بعضهما البعض في عملية تكاثر الخلايا السرطانية الحرشفية الرئوية، كما أن خلل إنتاج بروتين سورفافين قد يساهم في بداية نمو سرطان الخلايا الحرشفية الرئوية.

Objectives: To study the relationship between the infection of human papillomavirus (HPV) type 16, type 18, the expression of survivin, and the mutation of p53 gene in lung squamous carcinoma tissue for the research of pathogenesis of lung carcinoma.

Methods: This study was carried out at the Laboratory of Molecular Biology, Xiangfan Central Hospital of Hubei Province, China from September 2008 to May 2010. Forty-five specimens of lung squamous carcinoma tissue confirmed by histopathology were the excisional specimens taken by the Thoracic Surgery of Xiangfan Central Hospital. Normal tissue, closely adjacent to the fresh carcinoma specimens, was used as the control group for p53 gene mutation analysis. Sixteen surgical excisional specimens of benign lung disease were used as a control group of non-carcinomatous diseases. Human papillomavirus DNA were detected by polymerase chain reaction (PCR), and we used the PCR-single-strand conformation polymorphism-ethidium bromide (PCR-SSCP-EB) method to detect the mutations of the p53 gene. The expression of the survivin gene was detected by immunohistochemistry methods.

Results: Approximately 68.9% of 45 lung squamous carcinoma tissue had p53 gene mutations. The mutation rate of exon 5-8 p53 were 15.6%, 17.8%, 15.6% and 20%. Approximately 42.2% of lung squamous cell carcinoma samples were shown to be positive for HPV DNA expression and 62.2% were positive for survivin expression. There was an inverse correlation between the presence of HPV infections and mutations of p53 gene; and the mutations of p53 gene and expression of survivin had a positive relationship.

Conclusions: Mutation of p53 gene and HPV infection may facilitate each other in the generation of lung squamous cell carcinoma. Abnormal expression of the survivin gene may take part in the onset and progression of lung squamous cell carcinoma.

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Apoptosis is an initiative cell death under the regulation of genes. Cells in tissues are in a dynamic balance of continuous proliferation and apoptosis.[1] Once the cell regulation is in disorder or there is an imbalance between cell proliferation and apoptosis, tumors are likely to be caused. Survivin is an inhibitor of apoptosis protein (IAP). The gene of survivin does not express in normal tissues, but the over-expression of it can be found in tumor tissues.[2] Human papillomavirus (HPV) has a highly affinity with squamous epithelial cells of the human body, and it often reproduces itself in well-differentiated squamous epithelial cells.[3] More and more research[4-6] have demonstrated that HPV infection correlates with tumorigenesis in multiple parts of the human body. This indicates that HPV may be involved in the tumorigenic process in multiple parts of the human body in terms of etiology, including lung cancer, especially lung squamous carcinoma. In the early 1980's, Syrjänen[7] suggested that HPV was closely associated with invasive bronchial squamous cell carcinomas. A recently meta-analysis of the literature also suggested that HPV was associated with 20-25% of non-small cell lung carcinomas.[8] Ciotti et al[9] suggested that the mechanism of HPV-induced squamous cell carcinoma is related to the transforming activity of HPV. According to the literature,[10] "high-risk" HPV, namely the E6 or/and E7 of HPV16 and HPV18 are transforming genes, expressed in HPV-related tumors. Therefore, it has been proposed that E6 or/and E7 are potential oncogenes.[11] The E6 or/and E7 genes have synergic effects with activated ras and fos oncogenes. Meanwhile, the E6 protein can combine with p53, which is the production of an antioncogene, and the E7 protein can combine with retinoblastoma gene product (PRB), in the production of another anti-oncogene Rb.[12] The latest research showed that the carcinogenesis of HPV correlates with the survivin gene and p53 tumor suppressor gene.[13] Based on these theories, and in order to explore the relationships between HPV infection, survivin, and p53 genes in lung squamous carcinoma tissue, we selected the fragment of HPV16 E6 and HPV18 E6/E7 genes as the primer for PCR amplification to detect the HPV-DNA. Survivin gene and p53 tumor suppressor genes were detected simultaneously.

Methods. The experiment was performed with informed consent and under ethical approval of the Ethical Committee of Xiangfan Central Hospital, Hubei Province, China. This study was carried out at the Laboratory of Molecular Biology, Xiangfan Central Hospital of Hubei Province, Xiangfan city, China between September 2008 to May 2010.

Deoxyribonucleic acid polymerase was purchased from Promega Company, USA. The DNA Marker was purchased from TaKaRa Company, Kyoto, Japan. The immunohistochemical kit was purchased from the Beijing Zhongshan Biological Technology Co, Ltd, Beijing, China. Other reagents were domestically produced biochemical analytical reagents.

The primer of p53 exon 5-8 gene and the primer of HPV16 E6 and HPV18 E6/E7 genes were synthesized by Shanghai Biochemistry and Cell Biology Institute affiliated by the Chinese Academy of Sciences. The primers necessary for detecting HPV16 E6 and HPV18 E6/E7 and exon 5-8 of p53 gene are shown in **Table 1**.

All specimens were provided by Xiangfan Central Hospital of Hubei province, China. Patients who had any other malignant tumor or involved in any other virus infection were excluded. Forty-five specimens of lung squamous carcinoma tissue confirmed by histopathology were the excisional specimens taken by the Thoracic Surgery Department of Xiangfan Central Hospital. Meanwhile, normal tissues closely adjacent to the fresh carcinoma specimens were used as the control group for p53 gene mutation analysis. Sixteen surgicalexcisional specimens of benign lung disease were used as a control group of non-carcinomatous diseases. Benign lung disease included 6 specimens of pneumatocele, 2 specimens of inflammatory pseudotumor, 5 specimens of bronchiectasis, and 3 specimens of pulmonary tuberculoma.

Fresh tissue was digested by sodium dodecyl sulphate (SDS) and proteinase after being homogenated, then extracted by phenol/chloroform. The DNA was precipitated by dehydrated alcohol, and double distilled water was added, and stored in the refrigerator at -20^{0}C. The plasmid DNA was extracted by alkaline denaturation after the amplification of HPV16 and HPV18 clones. The reaction system for PCR was carried out by 10

Table 1 - The primers of polymerase chain reaction.

P	Primers	Target
P1	TCAAAAGCCACTGTGTCCTG	120 bp of HPV16 E6
P2	CGTGTTCTTGATGATCTGCA	120 bp of HPV16 E6
P3	TGCCAGAAACCGTTGAATCC	268 bp of HPV18 E6/E7
P4	TGTGAGTCGCTTAATTGCTG	268 bp of HPV18 E6/E7
P5	TTCCTCTTCCTGCAGTACTCC	Exon 5 of p53 gene
P6	GCCCCAGCTCTGCACCTCG	Exon 5 of p53 gene
P7	CACTGATTGCTCTTAGGTCT	Exon 6 of p53 gene
P8	AGTTGCAAACCAGACCTCAGG	Exon 6 of p53 gene
P9	TCTCCTAGGTTGGCTCTGAC	Exon 7 of p53 gene
P10	CAAGTGGCTCCTGACCTGGA	Exon 7 of p53 gene
P11	CCTATCCTGAGTAGTGGTAA	Exon 8 of p53 gene
P12	GTCCTGCTTGCTTACCTCG	Exon 8 of p53 gene

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× PCR buffer, deoxyribonucleotide triphosphate, primers, template DNA, and sterile distilled water. After 5 minutes at 95^{0}C for degeneration, the Taq DNA polymerase was added and covered by paraffin oil, and centrifuged for delamination. The PCR procedure was one minute at 55^{0}C, one minute at 93^{0}C, for 35 cycles, and 5 minutes at 72^{0}C for extention.

Agarose gel electrophoresis (3%) was carried out for analysis of PCR products. Then, stained by ethidium bromide, and observed with an ultraviolet analyzer. The plasmid DNA of HPV16 and HPV18 was used as a positive control for PCR products of HPV-DNA, and as a negative control mutually. The PCR products of white blood cells from normal persons were used as another negative control.

The main procedure of single-strand conformation polymorphism-ethidium bromide (SSCP-EB) was performed as follows: 20μl of p53 gene PCR products were selected, and 50μl of cold absolute alcohol was added for DNA precipitation, and centrifuged for 10 minutes at 12000 rotates/minute. The alcohol was removed and 20μl of buffer was added. The solution was denaturalized for 5 minutes at 95^{0}C, and was immediately cooled in ice ethyl alcohol. The 6% polyacrylamide gel electrophoresis containing 5% glycerin was carried out, and stained by ethidium bromide.

The immunohistochemical method was adopted to detect the expression of survivin. The main operating process was as follows: the antigen was boiled in citrate buffer solution, incubated by hydrogen peroxide, then blocked by normal serum. The polyclonal antibody of survivin was added, and kept overnight at 4^{0}C. The second antibody that was biotin-labeled was added, and then the horseradish-labeled streptavidin-peroxidase as the third antibody. The product was stained by diaminobenzidine (DAB) and re-stained by hematoxylin, and then differentiated by hydrochloric acid alcohol, and turned to blue by ammonia water.

The semi-quantitative method was used to detect the expression of survivin. Stained survivin was brown granules, were mainly located in the cytoplasm. The number of positive cells with survivin expression at middle level in every 1000 tumor cells was accounted under 5 high power lenses. Positive cells were divided into 5 levels: a) 0 = ≤5%; b) 1 = ≤5-25%; c) 2 = 25-50%; d) 3 = 50-75%; e) 4 = ≥75%. Positive cells were divided into 3 groups according to the stain intensity of immunohistochemical: (i) weak positive = 1, b) moderate density = 2 and, c) strong positive = 3. The score of each specimen was the score of positive cells multiplied by the score of the stain intensity. If the final score was zero, the expression of survivin was presumed negative, and if the final score was higher than one, the expression of survivin was presumed positive.

Chi-square criterion and 4-fold table exact probability were adopted for statistics. Statistical Package for Social Sciences Version 12.0 and Microsoft Excel software were used for the statistical analyses.

Results. After PCR amplification of the p53 gene exon 5-8 in specimens of lung squamous carcinoma tissue, the length of amplified fragments were 204 BP, 144 BP, 133 BP, and 168 BP. The relative molecular weight was consistent with the theoretical value, confirmed by agarose gel electrophoresis (**Figure 1a**). The size of HPV16 DNA fragment was 120 BP and HPV18 was 268 BP. The amplification of HPV16 and HPV18 DNA was also confirmed by agarose gel electrophoresis, the relative molecular weight of PCR products was consistent with theoretical value (**Figure 1b**). According to SSCP analysis, the total mutation rate of the p53 gene among 45 specimens of lung squamous carcinoma tissue was 68.9%, and the mutation rates of exon 5-8 were 15.6%, 17.8%, 15.6%, and 20%. There was no significant difference among each (p>0.05). Forty-five specimens were divided into smoker group and non-smoker group. The mutation rate of p53 gene in the smoker group was significantly higher than that of the non-smoker group (p=0.011, 95% confidence interval [CI] 85.35-90.06% versus 30.25-36.32%, **Table 2**). The positive rate of correlated series of HPV16 and HPV18 DNA in lung squamous carcinoma tissue was detected by PCR and agarose gel electrophoresis; the results show that it was obviously higher than that in the lung benign disease group (p=0.0002, the 95% CI 36.33-43.36% versus 0,

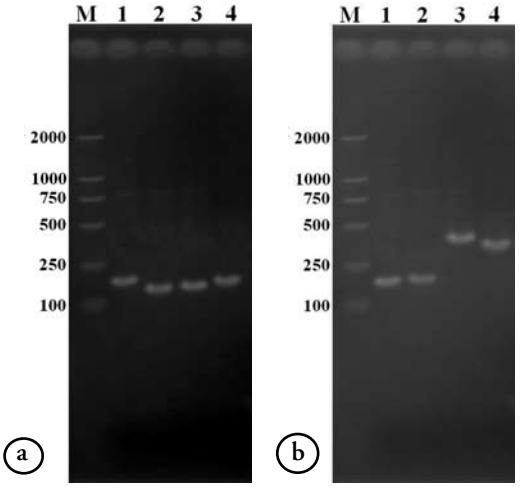

Figure 1 - Agarose gel electrophoresis of **a**) p53 gene exon 5-8 in lung squamous carcinoma tissue M: DNA marker DL2000, line 1: exon 5, line 2: exon 6, line 3: exon 7, and line 4: exon 8. **b**) HPV16 E6 and HPV18 E6/E7 in lung squamous carcinoma tissue M: DNA marker DL2000, line 1: HPV16 E6, line 2: positive control of HPV16 E6, line 3: HPV18 E6/E7, and line 4: positive control of HPV18 E6/E7.

Table 2 - The mutation of p53 gene in smoker and non-smoker groups.

Group	Case number *	Positive number	Ratio of positive (%)
Smoker group	27	24	88.89†
Non-smoker group	12	4	33.33

*6 cases with unknown smoking status, who were not accounted; †compared with non-smoker group, p=0.011.

Table 3 - The HPV16 and HPV18 DNA sequence detection in lung squamous carcinoma group and lung benign disease group.

Group	Case number	HPV16(+)	HPV18(+)	Ratio of positive (%)
Squamous carcinoma	45	13†	6†	42.2*
Benign disease	16	0	0	0

†One case in lung squamous carcinoma group had positive HPV 16 and HPV 18 DNA sequence simultaneously; *compared with lung benign disease group, p=0.0002

Table 4 - Correlation between the mutation of p53 gene and HPV 16, HPV 18 DNA sequence detection

HPV detection	p53 mutation (+)	p53 mutation (-)	Total
HPV (+)	9	10	19
HPV (-)	20	6	26
Total	29	16	45

x^2=4.37, p=0.05, γ=0.42

Table 5 - The relationship between the expression of surviving gene and p53 gene in lung squamous carcinoma tissues.

Group	n	Expression of survivin			x^2	*P*-value
		Positive	Negative	Ratio of positive (%)		
Squamous carcinoma	45	28	17	62.2		
p53 (+)	31	25	6	80.6		
p53 (-)	14	3	11	21.4	17.3	0.0003

Table 3). All the positive correlated series of HPV16 and HPV18 DNA in lung squamous carcinoma tissue were that of heavy smokers. Correlation analysis shows that there was a significant negative correlation between the p53 gene mutation and the positive correlated series of HPV16 and HPV18 DNA in lung squamous carcinoma tissue (p=0.05, **Table 4**). The positive staining of survivin expression mainly could be found in the cytoplasm of tumor cells, appearing as brown granules of different sizes. Few of them can also be found in the nucleus. Of all the 45 specimens of lung squamous carcinoma tissue, 28 specimens found in positive survivin expression, accounted for 62.2% of the total amount, while no survivin expression could be found in benign lung tissues. Of all 45 specimens of lung squamous carcinoma tissue, the mutation rate of the p53 gene was 68.9%. Meanwhile, the mutation of the p53 gene was closely related to the expression of survivin in lung squamous carcinoma tissue. The score of survivin expression in lung squamous carcinoma tissue with positive p53 gene expression was obviously higher than that of tissue with negative p53 gene expression, and there was a significant difference between them. The positive rate of survivin expression in the p53 positive group was 80.6%; the positive rate of survivin expression in the p53 negative group was 21.4%, and there was a significant difference between them (x^2=17.3, p=0.0003, 95% CI: 78.37-83.43% versus 18.72-23.68%, **Table 5**).

Discussion. Branca et al[14] analyzed survivin as a marker of cervical intraepithelial neoplasia and high-risk human papillomavirus (HR-HPV) and a predictor of HPV clearance and disease outcome in cervical cancer (squamous cell carcinomas), the results showed that up-regulated survivin expression was an independent predictor of HR-HPV in cervical lesions, most plausibly explained by its normal transcriptional repression by wild-type p53 being eliminated by HR-HPV E6 oncoprotein. But up to date, no research demonstrated whether the same mechanism existed in lung squamous carcinoma. A recent meta-analysis of 37 studies with 2435 cases of primary lung cancer showed that HPV might have a certain relationship with the development of lung squamous carcinoma.[15] However, the positive rates of HPV16 and HPV18 DNA sequence in lung squamous carcinoma tissue showed significant difference with different regions as well as different histological types, ranging from 0-78.3%.[16] The highest detectable rate of HPV16 and HPV18 was found in Asia (with an average rate of 50%), especially in squamous carcinoma.[16] In this study, the correlative sequence of HPV16 and HPV18 DNA in 45 specimens of lung squamous carcinoma tissue was detected; the result showed that the positive was 42.2%, which was in accordance with previous data, while no HPV-DNA was found in the benign lung disease group. The results also showed that all patients with lung squamous carcinoma and positive HPV-DNA were heavy smokers. This result indicates that smoking might have a certain relationship with HPV infection. This is because minor injury of the bronchial mucosa is common in smokers, and the minor injury is likely to become one of the pathologic bases of HPV's proliferation in basal epithelial cells.[17] Moreover, smoking could lead to a decrease of Langerhans cells in justo endepidermis, which has the function of antigen presentation, and then prompted HPV infection and the continuous existence of it.[18]

The P53 gene is a cancer suppressor gene located on human chromosome 17P13.1. Many tumors correlate with the p53 gene mutation.[19] Many studies have been performed concerning the relationship between the p53 gene and tumors. Based on the analysis of p53 gene mutation spectrum in various tumor tissues, 86% of mutation were found in 5-8 exon of p53 gene evolutional conservative regions,[20] most mutations were missense mutations, which can change the protein function.[21] In this study, the p53 gene exon 5~8 in 45 specimens of lung squamous carcinoma tissue were detected, the results showed that the mutation rate of p53 gene was 68.89%, and the mutation rates of exon 5~8 were 15.6%, 17.8%, 15.6% and 20.0%, showing no significant difference. Correlation analysis has shown that there was a significant negative correlation between the p53 gene mutation and HPV16 and HPV18 infection. The results suggested that the p53 gene dysfunction in lung squamous carcinoma correlates with dysfunction of p53 protein caused by HPV infection. Human papillomavirus infection and p53 gene mutation may have a synergistic effect. Survivin is an inhibitor of the apoptosis protein located on human chromosome 17q25, whose several residues can combine with caspase and directly restrain the activity of caspase,[22] and thereby play a major part in cell apoptosis. It is the strongest inhibitor of apoptosis protein ever found.[23] Liu et al[24] reported that survivin exists in all the tumor cells of the body, including non-small-cell-lung cancer. Our study had also shown that survivin existed in 62.2% of lung squamous carcinoma tissue, while no survivin expression was found in benign lung disease tissues. This result indicates that the expression of survivin gene had an up-regulation expression in lung cancer and also get involved in the development of lung cancer by inhibiting the apoptosis of lung cancer cells. Meanwhile, we have also found that the expression of the survivin gene has significant relationship with the expression of the p53 gene. The positive rate of survivin expression in the p53 positive group was obviously higher than that of the p53 negative group, and the scores of survivin expression in p53 positive group was much higher than that of p53 negative group. This result further indicatesd that the wild-type p53 gene might play the role of negative feedback regulation in the expression of the survivin gene. Taken together, our results indicate that survivin expression, HPV infection, and p53 gene mutation may have a delicate relationship.

It is interesting that survivin expression is related significantly to the mutation of p53 gene, while there is significant negative correlation between p53 gene mutation and the positive correlated series of HPV16 and HPV18. Review of the data from the studies on the molecular basis of survivin overexpression in human cancer reveals that there is something that may provide a plausible explanation for such a molecular link among the infection of HPV, the expression of survivin and the mutation of p53 gene. Research showed that survivin was one of the genes that was repressed transcriptionally by wild type p53[25] and the transcriptional repression of survivin contributed to p53-dependent apoptosis.[26] Perhaps the well-established function of HR-HPV oncoprotein mediated the degradation of p53 gene, which results in the dysfunction of the p53 protein, then the expression of survivin was up-regulated. This would be a plausible explanation, as it was not confined exclusively to lesions with HR-HPV types, and survivin up-regulation in lung squamous carcinoma probably has other triggering mechanisms, this is a practically

unexplored field and the exact mechanism of this process still requires further study. The next step of this study will focus on how the expression of survivin, mutation of p53 gene, and infection of HPV interact.

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Related topics

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