行政院國家科學委員會九十年度結果報告

計畫名稱:

中文: **p53 之多形性及其與子宮頸上皮內癌及鱗狀細胞癌之關係 (2/2)**

英文: **p53 Polymorphism and Its Association with Cervical intraepithelial Neoplasia or Squamous cell Cancer (2/2)**

計畫類別: ■個別型計畫

計畫編號: **NSC 90-2314-B-002-174**

執行期限: **90/08/01~91/07/31**

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Introduction

Certain human papillomavirus (HPV) genotypes are aetiological agents in the development of cervical carcinoma [Zur Hausen 1991] HPV16 is the most frequently detected genotype in invasive cervical carcinoma as well as in cervical intraepithelial neoplasia (CIN) I-III. HPV 18 is also reported to be related with these lesions. Persistent HPV infection is a risk factor for the progression of a preinvasive lesion to invasive cancer. However, most high-risk HPV infections do not progress to cancer. Epidemiological studies have identified additional risk factors that may contribute to the development of cervical carcinoma. These include age at infection, smoking, hormonal factors, genetic predisposition, and immune response. [Schiffman 1993]

Thus,the tumour biology of cervical intraepithelial neoplasia and cervical cancer is unusual. A large variety of individually distinct forms crudely divided into slight, moderate, severe dysplasia and carcinoma in situ exist. Virtually all contain genital human papillomavirus (HPV) either as infectious virions or as episomal or integrated DNA. A proportion of infected women develop condyloma, precancer and subsequently, in a minority, invasive cancer. Risk of precancer is statistically related to infection with genital HPV, but differences in risk between populations with high and low prevalence of HPV are larger than expected from a direct correlation. Findings fit with HPV as a major risk factor, but other factors must also be operative.

High risk HPV, typically 16 or 18, is preferentially associated with high grade dysplasia and in situ cancer either because it increases risk of clonal progression to these forms or induces them de novo. Severe dysplasia, in situ and invasive cancer always present as monoclonal lesions. Spontaneous mutation rate and physicochemical carcinogens seem insufficient for the creation of a malignant phenotype in cells of the transformation zone. Currently HPV is the only strong candidate for such a feat. Any or all of the following mechanisms may play a role: overexpression of viral E6 and E7 genes, often triggered by disruption of control elements upon integration of viral DNA into the cellular genome, activity of specific (E6?) configurations in certain HPV variants, inactivation of TP53 with decreased capacity for DNA repair and enhanced likelihood of accumulation of "transforming" mutations and viral integration at sites controlling function of cellular oncogenes and/or suppressor genes. Low risk types are almost always associated with squamous differentiation, HPV 16 usually also with squamous differentiation and HPV 18 with adenosquamous or adenomatous differentiation

Mutations of the tumor-suppressor gene p53 are common in tumors,

especially in epithelial tumors.The evidence that such mutation is an important step in the sequence of genetic changes which underly tumor progression is outlined. Such evidence includes the study of human tumors, animal tumor models and cell culture. In several instances, p53 mutation seems to be a late event. This is by no means a universal finding, and important reservations are stated concerning the role of p53 mutation in tumor progression.

It has recently been reported that the extent of p53 dysfunction caused by HPVs depends on the status of a polymorphism at codon 72 of p53, Pro or Arg. In that study, it was demonstrated that a patient homozygous for the Arg allele had about a seven times higher risk of developing cervical cancer than a patient homozygous for Pro [Storey 1998]. They showed that polymorphisms in codon 72 of p53 could determine the efficiency of HPV 16 or HPV 18 E6 in degrading p53 in vitro. These data were further supported by testing cervical cancer biopsy specimens from UK women, which showed a seven-fold enrichment of the arginine allele over the proline allele. Several groups have failed to confirm this result [Rosenthal 1998, Lanham 1998, Hayes 1998, Josefsson 1998]. In the latter reports no association of the p53 codon 72 arginine with cervical cancer was found. Moreover,the proportion with codon 72 arginine in the healthy controls was considerably higher in these reports than in the study of Storey and colleagues [Storey 1998]. However, in a recent article,Zahbe et al. [zahbe 1999] found that the p53 arginine polymorphism represents a potential risk for cervical cancer development, consistent with the concept proposed by Storey and colleagues.

The discrepancy between these studies may be due to several factors. The tumour specimens were previously not always specified as adenocarcinomas or squamous-cell carcinomas. Secondly,none of the studies was precise about HPV typing. The most common high-risk types were either grouped [Lanham 1998] or the HPV status was unknown [Rosenthal 1998, Hayes 1998] .In one investigation, only a subgroup of precursor lesions was known to be HPV 16-positive [Josefsson 1998]. This problem is compounded by the use, in most studies, of allele-specific PCR for the classification of p53 polymorphisms, which is more open to misinterpretation by using formalin-fixed tissue than fresh tissue. Moreover, there are geographical differences in the distribution of p53 genotypic distribution [Zehbe 1998]. The frequency of arginine allele was found to be increased with distance from the equator [Beckman 1994]. In women of Japanese ethnicity, p53 polymorphism at residue 72 does not seem to be involved in the development of HPV-associated cervical carcinomas [Minaguchi 1998, Yamashita 1999]. In Taiwan, there are only scarce reports about p53 and cervical

neoplasms and without study on the p53 polymorphism yet.

In this project. In an attempt to confirm this result and elucidate whether this allelic deviation of the Arg genotype seen in invasive cervical cancer occurs in the pre-malignant lesion SIL in Taiwan,we will determine the distribution of p53 polymorphisms in preinvasive and invasive cervical cancer. To simplify the confounding factors, we will only include the cases of intraepithelial neoplasia and squamous cell carcinoma. The samples used will be fresh instead of archival tissue. Adenocarcinoma will not be included in this study. We will also check HPV status, especially HPV16 and HPV18. Samples positive for HPV 16 or HPV18, consisting of any grade of cervical intraepithelial neoplasia and invasive squamous cell carcinomas will be studied. We will detect the distribution of p53 polymorphisms in preinvasive and invasive cervical cancer by restriction fragment length polymorphis.

Materials and Methods

Tissue samples

Tumor specimens will include both cervical intraepithelial neoplasia and invasive cervical cancer. All lesions will be histologically confirmed.Frozen specimens will be collected at the time of biopsy or surgical excision, snap frozen and stored at –70°C. Samples for the control group consisted of DNA extracted from a). normal cervix, which were operated for benign conditions such as myoma or benign ovarian cysts, and b). whole blood collected from healthy person.

DNA extraction

DNA from frozen tissue will be prepared by digestion with protein K and phenol-chloroform extraction. Idendification of the relevant tissue for microdissection will be aided using an adjacent attained section. DNA will be extracted from whole blood form control individuals using a Nucleon DNA-extraction kit (Scotlab).

HPV detection and typing

All tumor specimens will tested for the presence and type of HPV DNA by using a degenrate PCR-based method with a panel of oligonuleotide primers located within the highly conserved L1 open reading frame of the HPV genome. Ampliphied PCR products will then be sequenced.Basically, two different consensus primer sets A and B will be used for PCR amplification to detect HPV DNA. These primer sets are at least able to amplify sequences of HPV types 6, 11, 18, 31, 33, 39, 45, 51, and 56 [Toshikawa 1991; Fujinaga 1991].

Each 25 μl of PCR mixture contains 2 μl of sample DNA solution containing 25-250 ng of genomic DNA fragment, 0.35 μM primers, 2.5mM MgCl2, 0.25 mN dNTP, 2.5 μl of suppled PCR buffer and 0.5 U of Tag polymerase (Boehringer Mannheim). PCR was carried out as follows. The PCR product will be electrophoresed on 2% agarose gels stained with ehidium bromide and viewed under UV light. Samples identified as HPV-positive with these sconsensus primers were then HPV genotyped with type 16 and 18 specific primers [De Roda Husman 1995].

HPV consensus A (forward) 5'-TTTTCACCCATCTACAGTCCCCCTTG-3'
HPV consensus A (reversed) 5'-TACCCTAAATACTCTGTATTG-3'
HPV consensus B (forward) 5'-TGTCAAAAACCGTTGTGTCC-3'

HPV consensus B (reversed) 5'-TCTGAGTCGCTTAATTGCTC-3'
HPV 16 specific (forward) 5'-TACACGCAGTACAAATATGT-3'
HPV 16 specific (reversed) 5'-ATTCCTCCCCATGTCGTAGG-3'
HPV 18 specific (forward) 5'-CACTCGCAGTACCAATTTAA-3'
HPV 18 specific (reversed) 5'-ATTCCTCAACATGTCTGCTA-3'

PCR amplification of p53 polymorphic sequences

To detect p53 polymorphism at condom 72, we will use allele-specific PCR and PCR-restriciton fragment length polymorphism. The plasmid pBR322 digested with MspI will be used as size marker. Fifteen microliters of extracted DNA will be used in a 50 μl PCR containing 50 mM KCL, 10mM Tris-HCl(pH 8.3)m 200 μM of each dNTP, 1.5 mM MgCl2, 1U Tag DNA polymerase (Gibco BRL) and 50 pmol of each primer.The following primer pairs will be used based on those described by Storey et al., using p53/Arg- amplifying a 137-bp fragment from the arginine allele; and Pro+/p53- amplifying a 206-bp fragment from the proline allele.

P53+, 5'-GTCCCCCTTGCCGTCCCA-3'
Arg-, 5'-CTGGTGCAGGGGCCACGC-3'
Pro+, 5'-GCCAGAGGCTGCTCCCCC-3'
P53-, 5'-GGAAGCCAGCCCCTCAGG-3'

Samples will be subjected to an initial denaturation step at 95oC for 2 min, followed by 40 cycles of denaturation at 94oC for 45 sec, annealing at 60oC for 45 sed, extension at 72oC for 30 sec and a final period of extension at 72oC for 5 min. Both amplicons (10 μl) will undergon electrophoresis in a 9% polyacrylamide gel containing 5% glycerol and a 35-75% denaturing fradient (7M urea/40% deionised formamide) at a 150 V for 7.5 hours at 59°C and will

be stained with ethidium bromide.

The polymorphism will be confirmed in each case by amplification across the polymorphic site using primers p53+ and p53-, followed by restriction enzyme digestion with 20 U of the enzyme Bsh1236I (Stratagne, Cambridge).The recognition site is present only in the arginine-encoding allele [De La Calle-Martin 1990]. From this step, as a result, the arginine allele will be identified by the presence of 2 fragments of 120 and 187 bp and the proline allele by a single frgment of 307 bp. Heterzygous samples will show all 3 fragments. CaSki cells, which are heterozygous for the polymorphism [Storey 1998], will be used as a positive control in PCR for digestion.

Statistics

Odds ratio for Arg/Arg with 95% confidence interval and its P value will be evaluated for control (whole blood and normal cervix respectively), CIN (total cases), high-grade SIL (HPV-positive), invasive cancer (HPV-positive) and invasive cancer (types 16- or 18- positive).

Results

HPV analysis

Figure 1. PCR product by the consensus primers for the L1 region to check the presence of HPV. Lane 1: molecular weight standard. Lane 1: positive for HPV with a PCR product length of 262 bp; Lane 2: positive for HPV with a PCR product length of 253 bp; Lane 3: negative positive for HPV; Lane 4: negative for HPV; Lane 5: positive for HPV with a PCR product length of 244 bp; Lane 6: positive for HPV with a PCR product length of 253 bp.

Figure 2. Restriction mapping for L1-PCR product. a). Six cases positively cloned HPV types were subjected to the following restriction fragment length polymorphism (RFLP) analysis. b). The PCR-product was digested with HaeIII. Lane 1: molecular weight standard, Lane 2: HPV type 52 (two bands of 184 and 78 bp); Lane 3& 4: HPV type 33 (two bands of 112 and 91 bp); Lane 5: HPV 31 (two bands of 122 and 90 bp); Lane 6: HPV type 18 (210 bp). c). The PCR product was digested with RSAI. Lane 1: molecular weight standard, Lane 2: HPV type 16 (253 bp); Lane 3, HPV 11 (204bp); Lanes 4 & 5: HPV 31 (216 bp). d). The PCR product was digested with DdeI. Lane 1: molecular weight standard; Lane 2: HPV 11 (244 bp); Lane 3 & 4: HPV 31 (256 bp); Lane 5: HPV 52 (178 bp).

Figure 3. DNA sequencing of the L1-PCR product showing the type was HPV 52.

Of the 43 cases of CIN examined, 5 contained low-risk HPVs, 28 contained high-risk HPV, and in 7 cases there were no HPV can be identified for cases with single type infection. Multiple type infections were found in 3 cases in which HPV 11/18, 31/18 and 11/16 were the infected viral types. Totally, the case number with single type of high-risk viral infections by HPV16 was n = 4; for HPV18, n = 16; for HPV31, n = 5; for HPV33, n = 0, for HPV52b, n = 1; for HPV58, n = 2. For low-risk viral infections, case number for HPV6 was n = 3; for HPV11, it was n = 2.

Analysis of p53 polyphorphism

Figure 4. Detection of *p*53 polymorphism at codon 72 by allele-specific PCR. The PCR product of 162 bp containing a Pro or Arg allele.

Figure 5. RFLP of PCR product containing a Pro or Arg allele digested by restriction enzymes. Two polymorphic forms amplified with the primers containing a nuleotide substitution. Sma is only to cut the product (162 bp) with Pro allele into a 135 and a 27 bp fragment. Ksp can cut the product with the Arg allele into fragments of the same size. a). Lane 1: molecular standard; Lane 2: no digestion in SmaI, indicating the homozygous Arg allele; Lane 3 & 4: Partial digestion resulting two bands of 135 and 162 bp, indicating the heterozygous, containing both the Pro and Arg alleles; Lane 5: completely digested in SmaI, indicating the homozygous Pro allele; Lane 6: molecular standard; Lane 2: no digestion in SmaI, indicating the homozygous Arg allele. b). Lane 1: molecular standard; Lane 2: completely digested resulting a band of 135 bp, indicating the homozygous Arg allele; Lane 3, 4, 6, 8, & 10: partial digestion resulting two bands of 135 and 162 bp, indicating the heterozygous, containing both the Pro and Arg alleles; Lane 4 & undigested resulting a band of 162 bp indicating indicating the homozygous Arg allele.

Discussion

At present, the distributions of the different alleles of codon 72 (Pro/Pro, Pro/Arg and Arg/Arg) seems not show significant differences between either control and SIL groups. Also, no difference in the frequency of Arg/Arg genotype seems not detected even between the control and HSIL groups or control and invasive cancer infected with high risk HPVs groups. In conclusion, there seems no obvious relationship between the Arg genotype at codon 72 of p53 and predisposition to HPV-associated cervical neoplasia. The risk of cervical cancer may not be increased with the allele of TP53 encoding arginine at codon 72. Although the time for the study project is over,

in the following period of this project for P53 genotype and HPV, we will study more cases. Thus the relationship between the Arg genotype at condon 72 or p53 and HPV typing and the predisposition to HPV-associated cervical neoplasia will be further evaluated.

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Fig. 1

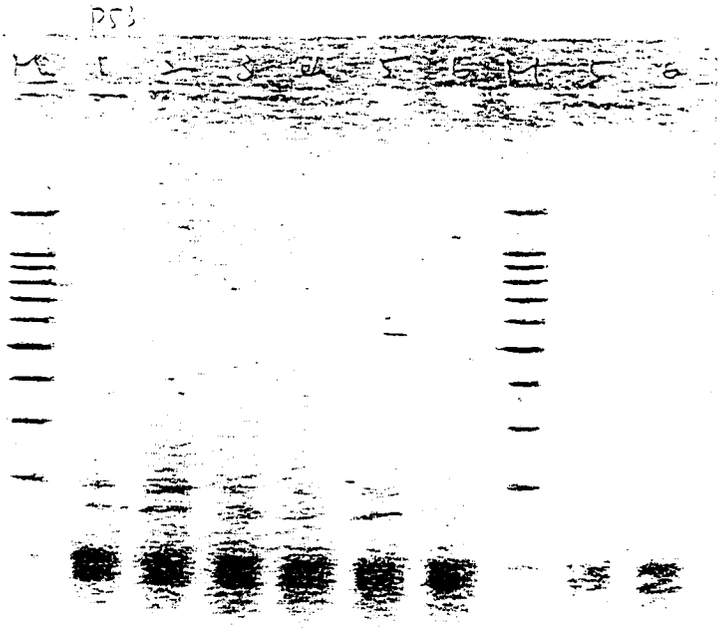
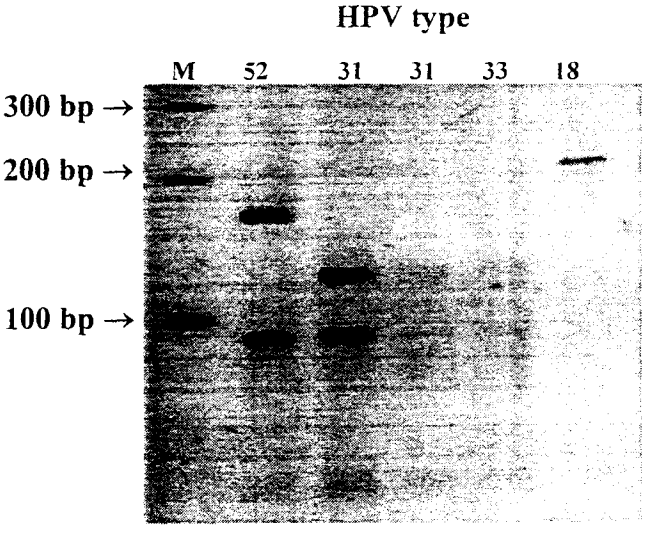


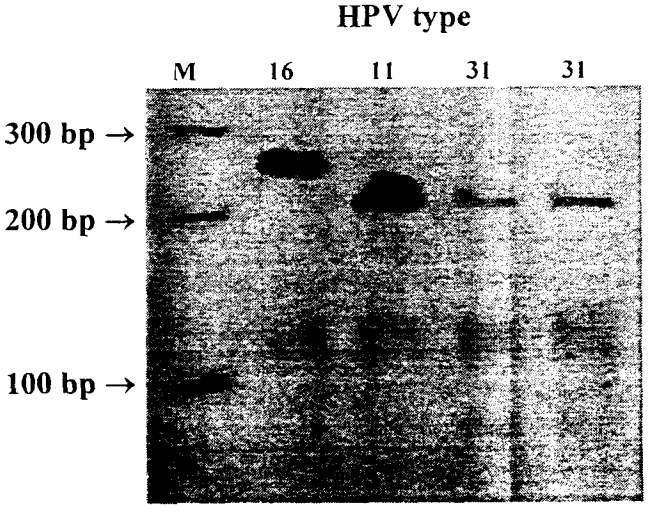
Fig. 2.

a) PCR product of HPV

b) Hae III RFLP of HPV



c). Rsa I RFLP of HPV



d) Dde I RFLP of HPV

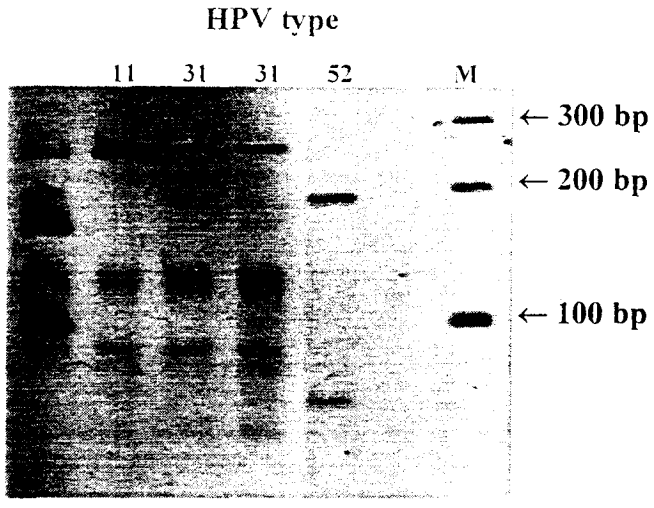


Fig 3.

ABI PRISM™

Model 310
Version 3.3
ABI-CE1
Version 3.2

B2•7

7
Lane 6

Signal G:1293 A:1972 T:1684 C:1195
DT POP6(BD Set-Any Primer)
MATRIX E
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Fri, May 25, 2001 5:01 PM
Tue, May 22, 2001 9:51 PM
Spacing: 11.51(11.51)

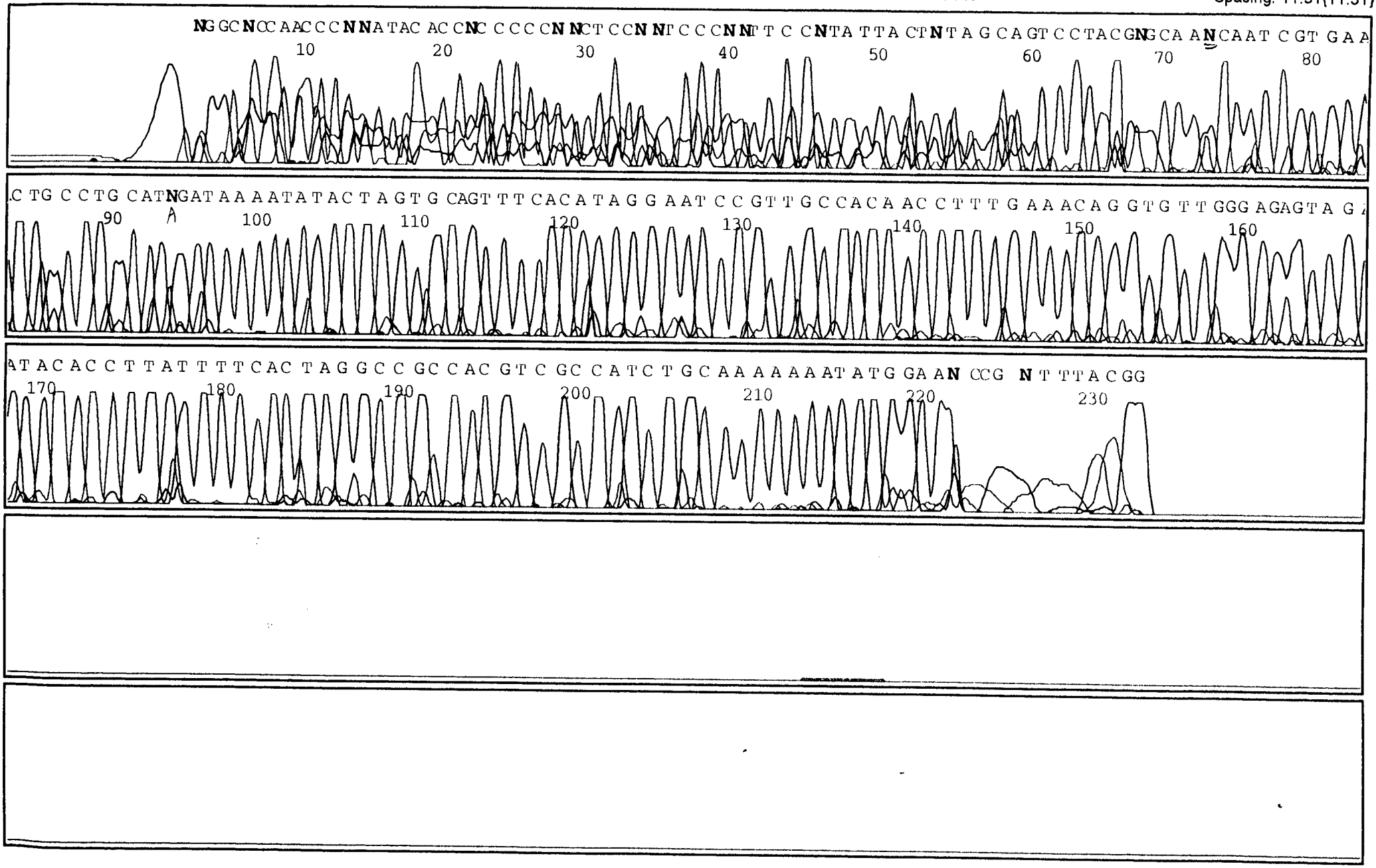


Fig. 4

PCR product of p53

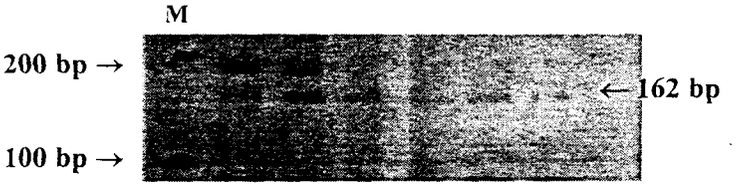
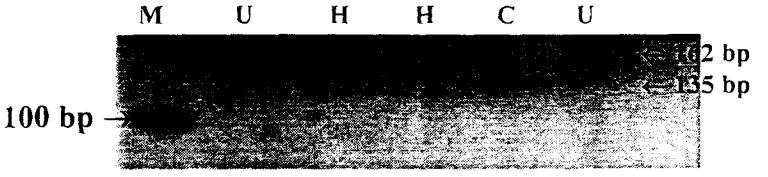


Fig.5

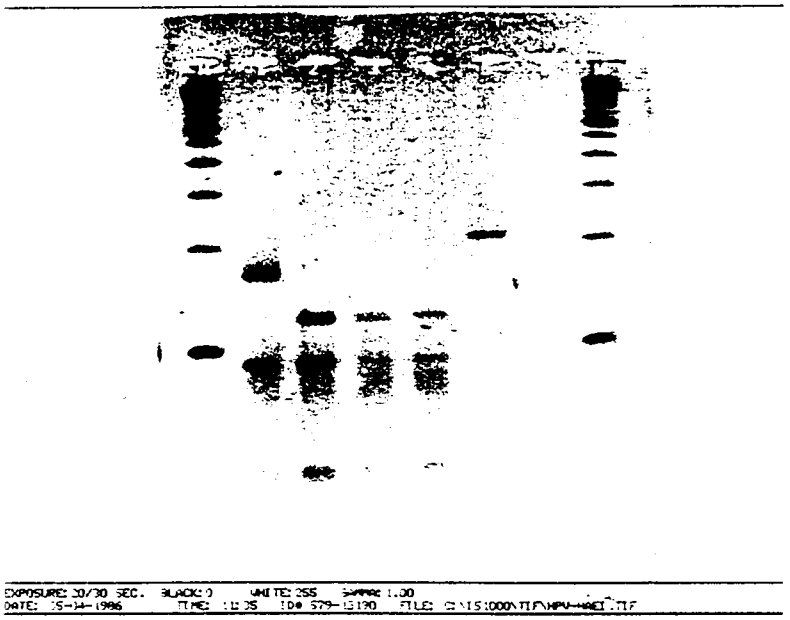
SmaI RFLP of p53



KspI RFLP of p53

- **C: cut**
- **H: heterozygous**
- **U: uncut**

Fig. 6



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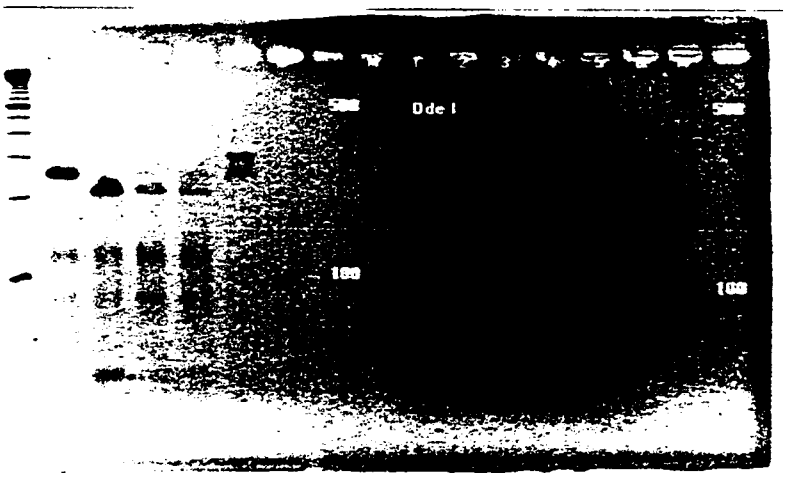

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