MATERIALS AND METHODS
A. CYTOGENETIC ANALYSIS OF PATIENTS WITH CERVICAL CANCER

A.1 Materials
The reagents/chemicals viz. RPMI - 1640 medium (GIBCO, USA), Foetal Calf Serum (Hi-Sera, UK), Phytohaemagglutinin - M (Difco, USA), 5-Bromodeoxyuridine, Colcemid and Hoechst 33258 (Sigma, USA) and Giemsa Stain (Merck, Germany) were used.

A.2 Samples
Peripheral blood samples were collected from women with uterine cervical carcinoma from Radiotherapy Department of the Post Graduate Institute of Medical Education and Research (PGIMER), Chandigarh. The samples were drawn before the advent of radiotherapy in sterile heparinized disposable syringes. Blood samples from age matched healthy house-wives and relatives of the patients served as the controls.

A.3 Methods
A.3.1 Lymphocyte culture
The cultures were set with 0.5 ml of whole blood in 4.0 ml of RPMI - 1640 (pH 6.8 - 7.0) medium containing 0.1 ml of heat inactivated foetal calf serum, 0.1 ml of phytohaemagglutinin-M and bromodeoxyuridine in the final concentration of 10 µg/ml (Watt and Stephen, 1986). Lymphocytes were grown for 72 hr at 37°C in dark. Mitotic cells were arrested at metaphase by adding colcemid to give
a final concentration of 0.1 μg/ml after 69 hr of incubation. The cells were collected by centrifugation at 1000 rpm for 5-10 minutes, given hypotonic treatment with 0.075M KCl for 10-15 minutes and were fixed with chilled solution of methanol and acetic acid (3:1). Metaphase preparations were made by dropping the cells on the chilled slides and then flame dried.

A.3.2 Staining

The slides were processed for differential staining according to the method of Perry and Wolf (1974) with minor modifications. Slides were stained with Hoechst 33258 (5.0 μg/ml) for 10-15 minutes in dark, washed in distilled water and exposed to UV light (18W) from a distance of 10-15 cm for 2 hr while immersed in a solution of 2 X SSC [0.3 M NaCl + 0.03 M tri-sodium citrate, pH 7.0]. The slides were then washed in distilled water and stained with 2% Giemsa stain for 5 - 10 minutes.

A.3.3 Analysis

The slides were coded and subjected to the following evaluations:

A.3.3.1 Chromosomal aberrations (CAs)

Fifty uniformly stained first mitosis (M1) metaphase plates were scored for each subject and chromosomal aberrations were described as per the guidelines of Obe and Beek (1979).
A.3.3.2 Sister chromatid exchanges (SCEs)

Twenty five differentially stained second mitosis (M2) metaphase plates were scored for SCEs.

A.3.3.3 Cell cycle kinetics

Hundred metaphase plates were scored as M1, M2 and M3 depending upon the staining pattern of chromosomes. The M1 metaphases had both the chromatids darkly stained, M2s had one chromatid dark and the other light whereas M3s showed a mixture of chromosomes with differentially stained chromatids and those with both chromatids being lightly stained.

The data obtained from the patients with cervical cancer and the control group were subjected to statistical evaluation using student's t-test (Colton, 1980) and the association between various parameters was evaluated using Pearson Correlation Coefficient.

B. IMMUNOHISTOCHEMICAL DETECTION OF HUMAN PAPILLOMA VIRUS (HPV) IN PATIENTS WITH CERVICAL CANCER.

B.1 Materials

Formalin (10%) and Papilloma Virus - PAP kit (Dako, Denmark).

B.2 Samples

Punch biopsies were obtained from the patients in buffered formalin (pH 7.0) from the Departments of Radiotherapy and Obstetrics and Gynaecology of PGIMER, Chandigarh.
B.3 Methods

B.3.1 Peroxidase-antiperoxidase (PAP) staining for HPV antigen

The detection of antigen was carried out using the reagents provided in the PAP kit.

- Formalin - fixed tissues were embedded in paraffin blocks.
- Four to five micron thick sections were cut on a microtome and transferred to clean glass slides.
- Sections were deparaffinized in xylene and hydrated by down grading in alcohol grades (absolute, 70, 50 and 30%) for 10-15 minutes in each. The sections were then washed with distilled water and a final wash was given with Tris buffer (pH 7.6).
- The sections were treated with H₂O₂ (Reagent 1) for 5 minutes at room temperature to block the endogenous peroxidase.
- Normal swine serum (Reagent 2) was used as the blocking agent to reduce non - specific staining. The excess of serum was tapped off after 30 minutes of incubation at room temperature.
- The sections were then exposed to the primary antibody (Reagent 3), i.e. the rabbit antibody against papilloma virus antigen. Washing was done with Tris buffer (pH 7.6) after 30 minutes
of incubation at 37°C.

- The sections were incubated with anti-rabbit swine antibody (Reagent 4) for 20 minutes and subsequently washed with Tris buffer.

- An incubation with rabbit peroxidase antiperoxidase complex (Reagent 5) was given for 30 minutes followed by rinsing with Tris buffer.

- The sections were subsequently developed with the substrate (Reagent 6 + 7 + 8 in the ratio of 1:2:1) for 5-10 minutes. Reagent 6 contained the chromogen, amino-ethyl carbazol (AEC) while reagent 7 was the substrate buffer and reagent 8 was hydrogen peroxide.

- Counter staining of the sections was done with Mayer's Haematoxylin for 5 minutes. Excess stain was removed by washing the slides with tap water and mounted in 1% gelatin containing azide.

- A positive reaction was interpreted as a dark brown precipitate in the nuclear region of the cells mainly in superficial and intermediate layers of epithelium.

C. DNA HYBRIDIZATION ANALYSIS FOR THE DETECTION OF HUMAN PAPILLOMA VIRUS IN PATIENTS WITH CERVICAL CANCER.

C.1 Materials

C.1.1 Strain: E. coli HB101 [ F−hsdS20 (rB− mB−) recA13]
ara-14 proA2 lacY1 galK2 rpsL20 Str(R) xyl5 mtl1
supE44 leu thi] was procured from Microbial Type
Culture Collection (M.T.C.C.), Institute of
Microbial Technology, Chandigarh, India.

C.1.2 DNA: HPV-16:pBR322 plasmid DNA was a gift from
E.M.DeVilliers of Deutsches Krebsforschungszentrum,
Heidelberg, Germany.

C.1.3 Materials purchased from Sigma, USA: Silane,
Pronase, Salmon Sperm DNA (sodium salt), Ficoll,
Polyvinylpyrrolidone, Bovine Serum Albumin (Pentax
fraction V), Tris base, Agarose, Eco RI, HindIII,
Ampicillin (sodium salt), Chloramphenicol and RNase.

C.1.4 Materials purchased from Boehringer-Mannheim,
Germany: Biotin-16-dUTP, Streptavidin-β-galactosidase
conjugate, 5-Bromo-4-Chloro-3-Indolyl-β,D-galacto-
pyranoside (X-gal), Nitroblue tetrazolium (NBT),
pBR322 plasmid DNA and Nylon membranes.

C.1.5 Materials procured from Bangalore - Genei, India:
Gene clean kit and Bam HI.

C.1.6 Other materials: HindIII digested λ-phage DNA
(Biolabs, UK), Sephadex G-50 (Pharmacia Fine
Chemicals, Sweden), Nitrocellulose paper
(Schleicher and Schuell, Germany) and Nick
translation kit (Amersham, USA).
C.1.7 LB (Luria-Bertani) broth was prepared as follows: Tryptone-10g; Yeast extract-5g and NaCl-10g per liter of the deionized H₂O and the pH of the medium was adjusted to 7.2 with 1M NaOH. LB-agar was made by adding 1.5% of agar powder to the broth.

C.1.8 Antibiotics: The stock solutions were filter-sterilized and stored at -20C in small aliquotes.

- Ampicillin was prepared by dissolving its sodium salt in water at the concentration of 50 mg/ml and was added to media at a final concentration of 50 μg/ml.
- Chloramphenicol was dissolved in absolute ethanol at the concentration of 34 mg/ml and was used at a final concentration of 170 μg/ml.

C.1.9 Buffers:

- TE: 10 mM Tris.Cl (pH 8.0) and 1 mM EDTA
- STE: 10 mM Tris.Cl (pH 8.0), 100 mM NaCl and 1 mM EDTA.
- TAE: 50 X stock solution per liter of distilled water.

Tris base - 242 g.
Glacial acetic acid - 57.1 ml.
0.25M EDTA - 200 ml.
- TBE: 5 X stock solution per liter of distilled water.
  Tris base - 54g.
  Boric acid - 27.5g.
  0.25 M EDTA - 40.0 ml.
- SSC: 20 X stock solution per liter of distilled water
  NaCl - 175.3 g.
  Sodium citrate - 88.2 gm.
  pH - 7.0

C.2 Samples
Punch biopsy was taken from the patients with cervical cancer in sterile distilled water in glass vials. These samples were taken from the patients visiting the Radiotherapy Department of PGIMER, Chandigarh and colposcopically directed biopsies from women with abnormal cytology were taken from the Department of Obstetrics and Gynaecology, PGIMER, Chandigarh.

C.3 Methods
C.3.1 Transformation of E. coli HB101
E. coli HB101 was transformed with the HPV-16 genome cloned in pBR322 plasmid DNA according to the method of Mandel and Higa (1970).
- Twenty ml of LB was inoculated with a single colony of E. coli HB101 and incubated at 37°C for overnight with shaking (200 rpm).
A fresh aliquot of 20 ml LB was inoculated with 0.2 ml of overnight inoculum and incubated at 37°C with vigorous shaking (200 rpm) to a cell density of about 5 x 10^7 cells/ml (OD_{600} = 0.2 - 0.3).

One ml of the culture was taken in an eppendorf tube and chilled in ice bath for 10 minutes.

The culture was centrifuged at 4000 rpm for 5 minutes at 4°C in a cold centrifuge (Remi).

The supernatant was discarded and the pellet was resuspended in 500 µl of ice cold 100 mM CaCl_2 solution made in 10 mM Tris.Cl buffer (pH 8.0). The culture was mixed properly and kept on ice for 15 minutes.

The culture was centrifuged at 4000 rpm for 5 minutes at 4°C and the pellet was resuspended in 100 µl of ice cold solution of 100 mM CaCl_2.

Approximately 40 ng of the HPV-16: pBR322 DNA was added to the treated cells and incubated in ice bath for 30 minutes. The cells were subsequently subjected to heat shock at 42°C for 2 minutes.

One ml of the prewarmed (37°C) LB was added to the transformed cells and incubated for an hour without shaking so as to allow expression of ampicillin resistance.
The transformed culture (200μl) was subsequently spread plated on to the ampicillin containing LB plates and incubated for overnight at 37°C.

The transformants obtained were picked up and restreaked on fresh LB - ampicillin plates to obtain the pure culture.

Glycerol culture was made for long term storage of the transformed strain by mixing 0.15 ml of sterile glycerol with 0.85 ml of transformed E. coli culture grown in LB - ampicillin broth.

C.3.2 Small scale isolation of plasmid DNA

Small scale preparation of the plasmid DNA from the transformant was made by alkali lysis method of Birnboim and Doly (1979) as modified by Ish-Horowicz and Burke (1981).

- A single colony of the transformant was inoculated in LB-ampicillin broth and incubated for overnight at 37°C with shaking (200 rpm.)
- The culture (1.0 ml) was taken in an eppendorf tube and centrifuged to pellet the cells.
- The pellet was resuspended in 100 μl of ice cold solution I and kept on ice for 5 minutes.

Solution I : 50 mM Glucose

25 mM Tris. Cl (pH 8.0)
10 mM EDTA. Na₂

- Freshly prepared 200 μl of the solution II was then added and the contents were properly mixed
by inverting the tube for two to three times and stored on ice for 5 minutes.

[Solution II : 0.2 N NaOH
1.0% SDS]

- An ice cold solution III (150 µl) was added and the contents were properly mixed followed by storage on ice for 5 minutes.

[Solution III : 5M potassium acetate - 60.0 ml
Glacial acetic acid - 11.5 ml
Distilled water - 28.5 ml]

- The solution was centrifuged at 10,000 rpm for 5 minutes at 4°C and the supernatant was transferred to a fresh tube. The DNA was precipitated with two volumes of ethanol at room temperature.

- The DNA was recovered by centrifugation at 12,000 rpm for 5 minutes at room temperature.

- The supernatant was discarded and the pellet was drained thoroughly by inverting the tube on a filter paper.

- The pellet was washed with 70% ethanol and air dried.

- The DNA was dissolved in 50 µl of TE (pH 8.0).

- The DNA sample was digested with appropriate restriction enzymes according to Sambrook et al. (1989).

- The digested DNA was electrophoresed in 0.8%
agarose gel to check the presence of plasmid DNA after staining with ethidium bromide.

**C.3.3 Agarose gel electrophoresis**

Electrophoresis was done to identify, separate, purify and determine the molecular size of DNA using agarose (0.8 - 1.0%) in TBE buffer. DNA samples were mixed with 0.1 volume of the loading buffer containing 0.25% bromophenol blue in 40% W/V sucrose as the tracking dye, added into the wells and electrophoresed at appropriate voltage for appropriate period of time. The gel was stained with 0.5 μg/ml solution of ethidium bromide for 30 minutes at room temperature and destained in distilled water for 30 minutes after the completion of the run. The DNA was visualized by illuminating the gel in the UV trans-illuminator (UVP, San Gabriel, CA, USA). *HindIII* digested λ – phage DNA was used as the molecular size marker exhibiting seven fragments of sizes (kb): 23.13, 9.42, 6.55, 4.37, 2.32, 2.03 and 0.56.

**C.3.4 Amplification and large scale preparation of plasmid DNA**

Plasmid DNA was amplified following the method of Sambrook *et al.*(1989).

- LB broth (10 ml) containing ampicillin was inoculated with a single bacterial colony and incubated at 37°C for overnight with shaking (200 rpm).
- A fresh aliquot of LB - ampicillin (25 ml) in a 100 ml flask was inoculated with 0.25 ml of overnight grown culture and incubated at 37°C with vigorous shaking (300 rpm) for 2.5 hr. The OD600 of the culture was around 0.6 (late log culture).

- LB-ampicillin medium (500 ml) was inoculated with 25 ml of late log culture in a 2 liter flask and incubated exactly for 2.5 hr at 37°C with vigorous shaking (300 rpm) (OD600 ~0.4).

- Chloramphenicol solution was then added and the culture was further incubated at 37°C with vigorous shaking for 16 hr.

- Bacterial cells were harvested by centrifugation at 4000g for 10 minutes at 4°C.

- The pellet was washed with 100 ml of ice cold STE buffer.

- The culture was then centrifuged at 4000g for 10 minutes at 4°C followed by resuspension of the pellet in 10 ml of solution I and 1.0 ml of freshly prepared lysozyme (10 mg/ml in 10 mM Tris.Cl, pH 8.0).

[ Solution I : 50 mM Glucose
  25 mM Tris.Cl (pH 8.0)
  10 mM EDTA. Na2 ]

- Freshly prepared 20 ml of solution II was added and the contents were mixed by inverting the
tube several times. It was allowed to stand at room temperature for 10 minutes.

[ Solution II : 0.2 N NaOH

1.0% SDS ]

- Ice cold solution (15 ml) of potassium acetate [made by mixing 60.0 ml of 5M potassium acetate with 11.5 ml of glacial acetic acid and 28.5 ml of H₂O ] was added to the tube and mixed gently. It was kept on ice for 10 minutes.

- The cell DNA and bacterial debris were pelleted by centrifugation at 20,000 rpm for 20 minutes at 4°C.

- The supernatant was transferred to the centrifugation tubes and 0.6 volumes of isopropanol was added to each tube. The contents were mixed properly and allowed to stand at room temperature for 10 minutes.

- The DNA was recovered by centrifugation at 12,000 rpm for 15 minutes at room temperature.

- The pellet was washed with 70% ethanol after discarding the supernatant.

- The ethanol was drained and the pellet was air dried to remove all traces of ethanol.

- The DNA was finally dissolved in 3.0 ml of TE (pH 8.0).
C.3.5 Purification of plasmid DNA

The plasmid DNA prepared was purified by equilibrium centrifugation in cesium chloride - ethidium bromide gradient according to the protocol given by Sambrook et al. (1989).

- The volume of the DNA to be purified was measured and 1.0 gm of cesium chloride per ml of solution was added and mixed thoroughly (refractive index was 1.4).
- Ethidium bromide (0.5 ml of 10 mg/ml in H₂O) was added per 10 ml of the cesium chloride solution.
- The solution was then transferred to corex tubes and centrifuged at 14,000 rpm for 20 minutes at room temperature. The bacterial proteins aggregated at the top and bulk of RNA formed a pellet.
- The supernatant was transferred to quick seal tubes to fill these to 3/4. The rest of the space was filled with paraffin oil.
- The solution was centrifuged at 45K for 24 hr in Beckman type 50 Ti rotor.
- The DNA resolved into two bands with pellet of RNA at the bottom.
- The lower band of covalently closed circular plasmid DNA was recovered by inserting a # 21 hypodermic needle into the tube just below the band.
- Ethidium bromide was removed by adding equal volume of water saturated isoamyl alcohol to the solution and mixing it thoroughly. To separate the aqueous phase it was centrifuged and the isoamyl alcohol (organic phase) containing ethidium bromide was aspirated. This extraction process was repeated till all the pink colour disappeared from the aqueous solution.
- The salts were removed from the DNA preparation by diluting it with three volumes of TE followed by addition of two volumes of ethanol at room temperature. The contents were mixed and centrifuged at 10,000 rpm for 10 minutes.
- Finally the DNA pellet was washed with 70% ethanol and dried before dissolving in TE (pH 8.0). Aliquots of 100 µl of DNA were made and stored at -20°C for further use.
- The purity of the DNA was checked by electrophoresis in 0.8% agarose gel.
- The OD of the DNA was taken at 260 and 280 nm and the concentration was estimated.

C.3.6 Recovery of HPV-16 DNA from the vector (pBR322)

The cesium chloride purified plasmid (HPV-16 : pBR322) DNA was linearized by subjecting it to Bam HI digestion. The digested DNA was resolved by electrophoresis in 0.8% agarose gel using TAE electrophoresis buffer.
followed by elution of HPV-16 DNA by gene clean kit according to the following protocol:

- The separated HPV- DNA band was excised from the gel with a sterile sharp blade while keeping the exposure to UV light to the minimum.
- The slice of gel was weighed and put in an eppendorf tube and 2.5 volumes of sodium iodide solution (provided in the kit) was added. The specific gravity of the gel was taken as 1.0.
- The gel was solubilized by warming it at 45-55°C for 5 minutes and the contents were thoroughly mixed.
- The DNA was adsorbed on the glass by adding 5 µl of glass solution (provided in the kit) per 5 µg or less of DNA and keeping at room temperature for 5-10 minutes with occasional mixing.
- The sample was microfuged at 12,000 rpm for 5 second and the supernatant was discarded.
- The pellet was washed with 200 µl of wash buffer (provided in the kit). Mixing was done on a vortex mixer and centrifugation at 12,000 rpm for two minutes. All traces of buffer were removed carefully.
- DNA bound to glass was eluted by adding 50-100 µl of TE and incubating at 45-55°C for 5 minutes.
- DNA was recovered by centrifugation at 12,000 rpm for 5 seconds. The supernatant containing
the DNA was transferred to a fresh tube. Purity and elution efficiency were checked by gel electrophoresis.

C.3.7 Labelling HPV-16 DNA

HPV DNA was labelled with biotin-16-dUTP by nick translation using Amersham 5500 (UK) nick translation kit according to the basic method of Rigby et al. (1977).

Labelling reaction was set for the final volume of 50 µl. The composition of the reaction mixtures was as follows:

- Nick translation buffer - 1 X
- Unlabelled ATP, GTP and CTP - 1.0 nmol each
- Unlabelled TTP - 0.1 pmol
- Biotin-16-dUTP - 0.3 nmol
- E. coli DNA polymerase I - 2.5 units
- DNase - 50 pg
- HPV-16 DNA - 500 ng

The reaction mix was incubated at 15°C for 60 minutes and the reaction was stopped by the addition of 5 µl of 0.25 M EDTA.

C.3.8 Spun column chromatography

The unincorporated deoxyribonucleoside triphosphates were removed by centrifugation through Sephadex G-50 packed column.
- **Preparation of Sephadex G-50:** 3.0 gm of Sephadex G-50 (medium grade) was dispensed in 25 ml of TE (pH 8.0) and autoclaved for 15 minutes at 15 lb/in². The supernatant was decanted and replaced with equal volumes of TE (8.0).

- **Preparation of column:** Disposable syringe of 1.0 ml was plugged with sterile and siliconized glass wool. Swollen Sephadex G-50 was poured in the syringe to pack it to 0.9 ml capacity and the column was washed with several column volumes of TE (pH 8.0) before use.

- **Chromatography of nick translated DNA:** The volume of the nick translated DNA was made to 100 µl with STE and applied to the column and spun for 4 minutes at 1600 rpm (Remi centrifuge) to collect 100 µl of the effluent in a decapped eppendorf tube.

C.3.9 **Siliconization of glass wool**

The glass wool was dipped in 5% solution of silane, made freshly in chloroform, for 10 minutes at room temperature. It was then baked in a glass petri dish at 180°C in an oven for 2 hr and washed thoroughly in sterile water before use.

C.3.10 **Verification of biotinylation of HPV-16 DNA**

The nick translated HPV-16 DNA was checked for biotinylation prior to its use as probe for hybridizations.
The nick translated DNA (5-10 ng) was spotted on the nylon membrane and exposed to UV light on a UV transilluminator for 3 minutes to bind the DNA.

The membrane was washed briefly with buffer 1.

[Buffer 1: 100 mM Tris.Cl (pH 7.5) 150 mM NaCl]

The membrane was then incubated with 5X Denhardt’s solution (0.2 ml per cm²) in a water bath at 65°C for 45 minutes in order to block the non-specific DNA binding sites [Denhardt’s stock solution was diluted with buffer 1].

[Denhardt’s stock: Ficoll solution (50X)
Polyvinylpyrrolidone - 5 g
Bovine Serum Albumin - 5 g
Distilled water - 500 ml]

Streptavidin-β-galactosidase conjugate (0.2 ml per cm²) was added after draining the blocking agent. The membrane was incubated with the conjugate for 30 minutes at room temperature [The conjugate was reconstituted by suspending the stock of 500 U in 1.0ml of sterile distilled water. The working solution (1: 1000) was made fresh in buffer 1].

The membrane was washed three times for 5 minutes each with buffer 1 after draining the conjugate solution.
- Another washing was given with buffer 2 to equilibrate the membrane.

  [ Buffer 2 : Tris.Cl (pH 7.5) - 100 mM
    NaCl - 100 mM
    MgCl₂ - 50 mM ]

- Freshly prepared colour reagent was put on the membrane (0.1 ml per cm²) and incubated in dark at 37°C for 4 hr. A positive reaction was indicated by a dark blue precipitate at the site of reaction.

  [Colour reagent : NBT stock solution :- 45 µl
    (1.5 mg/ml in 70% dimethylformamide)
    X-Gal stock solution :- 35 µl
    (1.0 mg/ml in dimethylformamide)
    Buffer 2 :- 10 ml]

C.3.11 Extraction of DNA from leucocytes

DNA from blood sample was extracted according to the method of John et al. (1991).

- Blood sample (2.5 ml) was collected in a sterile vial containing EDTA.

- The volume was made to 5.0 ml by adding 2.5 ml of solution 1.

  [Solution 1: Tris.Cl (pH 7.6) - 10 mM
   KCl - 10 mM
   MgCl₂ - 10 mM ]
- The cells were lysed by adding 60 µl of Nonidet P40 (BDH).
- The lysate was centrifuged at 2000 rpm for 10 minutes.
- The nuclei were lysed by resuspending the pellet in 800 µl of solution 2.

[ Solution 2 : Tris.Cl (pH 7.6) - 10 mM
  KCl - 10 mM
  MgCl₂ - 10 mM
  NaCl - 0.5 M
  SDS - 0.5%
  EDTA - 2 mM ]

- The contents were transferred to an eppendorf tube containing 400 µl of distilled phenol (saturated with 1M Tris.Cl, pH 8.0).
- Aqueous phase was separated by centrifugation at 12,000 rpm for 10 minutes and transferred to a fresh eppendorf tube.
- Second extraction was done with 400 µl of phenol-chloroform solution containing equal amounts of distilled, saturated phenol and chloroform. (Chloroform used was chloroform : isoamyl alcohol in the ratio of 24:1). The contents were mixed thoroughly by inverting several times.
- Phase separation was achieved by centrifugation at 12,000 rpm for 1.0 minute and the aqueous
phase was transferred to a fresh eppendorf tube.
- Final extraction was carried out with 700 μl of chloroform and aqueous phase was transferred to a fresh tube.
- DNA was precipitated by adding 2 volumes of ice cold ethanol and centrifuging at 12,000 rpm for 10 minutes at 4°C.
- The pellet was washed with 70% ethanol and dried to remove all traces of ethanol.
- The pellet was resuspended in 50 μl of TE (pH 8.0).

C.3.12 Extraction of genomic DNA from biopsies:

The protocol given by Hallam et al. (1989) was followed for the extraction of DNA from the tissue.
- The cervical tissue was cut into small pieces and washed several times with distilled water to remove the blood cells.
- The cells were lysed by adding 500 μl of TE (pH 8.0) containing 1% SDS and 1mg/ml pronase and incubated at 37°C for overnight.
- The digested tissue was extracted with phenol-chloroform and chloroform (3 times each).
- The extract was treated with RNase (50 μg/ml) at 37°C for 30 minutes and reextracted with phenol-chloroform and chloroform, once each.
- The aqueous phase was transferred to a fresh tube and mixed with one tenth volume of 5M NaCl and two volumes of ice cold ethanol.
- The DNA was spooled onto the sterile glass rod which was then washed with 70% ethanol and dried in air.
- The DNA was dissolved in 500 μl of TE (pH 8.0).
- The approximate quantitation of the DNA was done by gel electrophoresis in 0.8% agarose and ethidium bromide staining and by comparing the intensity of tissue DNA to those of known concentrations of pBR322 (Boehringer Mannheim) which gave a single band of 4.36 kb.
- The final concentration of the DNA samples was adjusted according to the requirement either by dilution in TE or concentration by ethanol precipitation.

C.3.13 Dot blot DNA hybridization

The detection of HPV-16 DNA in the cervical tissues was carried out by dot blot hybridization following the method used by Hallam et al. (1989).

- Nitrocellulose membrane strip was prepared for hybridization by soaking in distilled water and then in 6X SSC. The membrane was dried before spotting the samples.
- The DNA samples were denatured with 0.5M NaOH at
room temperature for 15 minutes and spotted on the membrane.

- The spots were air dried and neutralized with 1.5M NaCl and 0.5M Tris.Cl (pH 7.5).
- The membrane was baked at 80°C for 2 hr in vacuum.
- Prehybridization was carried out in prehybridization solution (0.2 ml per cm²) at 42°C (low stringency) or 68°C (high stringency) for 2 hr in a sealed plastic bag. Air bubbles were removed before sealing the bag.

[Prehybridization : 6 X SSC solution]

5 X Denhardt’s reagent
100 μg/ml denatured salmon sperm DNA
0.5% SDS

- Prehybridization solution was replaced with hybridization solution (50 μl per cm²) and hybridized under similar (42°C for low or 68°C for high stringency) conditions for 16-18 hr. Air bubbles were removed from the bag before sealing.

[Hybridization : 6 X SSC solution]

100 μg/ml denatured salmon sperm DNA
0.5% SDS
50 ng/ml HPV-16 probe].
- The membrane was washed with 2 X SSC, 0.1% (W/V) SDS solution, twice for 5 minutes each at room temperature.
- For high stringency conditions, the membrane was washed additionally with 0.1 X SSC, 0.1% SDS solution twice for 5 minutes each at 68°C.
- The membrane was then processed for spot development for the detection of hybridized DNA as was done in section C.3.10 (steps 2 - 7).
- The results of the blot were documented by photography.

C.3.14 Southern blot DNA hybridization

It was carried out according to the protocol used by Matsukura et al. (1989).
- Total cellular DNA (5-10 µg) of the cervical tissue was digested completely with Bam HI or HindIII as per the instructions of the manufacturers.
- Digested DNA was electrophoresed in 0.8% agarose gel at 50 V using TBE as the running buffer. The DNA cut with HindIII was used as the molecular size marker.
- The gel was stained with ethidium bromide (0.5 µg/ml) for 20-30 minutes, destained and photographed.
- The gel was trimmed from the well side and
soaked in several volumes of 0.25 N HCl for 10-15 minutes.

- The denaturation of DNA was achieved by soaking the gel in several volumes of 1.5M NaCl and 0.5M NaOH solution for 45 minutes at room temperature.

- Neutralization was carried out in several volumes of 1.5M NaCl and 0.5M Tris.Cl (pH 7.5) solution for 45 minutes at room temperature.

- A platform was made by wrapping the Whatman 3 MM paper for resting the gel in a baking dish filled with 10 X SSC to the top of the support. Whatman paper hanging at the two ends was used as the wick.

- The gel was placed upside down on the damp Whatman paper.

- The nitrocellulose paper was cut to the size of the gel, immersed in 2 X SSC for 5 minutes and placed on the gel with no air bubbles trapped in between.

- Whatman 3 MM paper was cut to the dimensions of nitrocellulose paper and placed on it after wetting in 2X SSC. All the air bubbles trapped in between were removed.

- A stack (5-8 cm high) of ordinary filter papers with the dimensions of nitrocellulose paper was placed on the Whatman paper strip and was
compressed with some weight (around 500g).

- The transfer of DNA from the gel to the nitrocellulose membrane was allowed to proceed for 12-24 hr.

- The stack of filter paper was removed and orientation of the samples was marked on the nitrocellulose paper.

- The nitrocellulose strip was then soaked in 6X SSC for a few minutes, air dried and baked at 80°C for 2 hr in vacuum.

- The peeled off gel was restained with ethidium bromide for 1 hr and checked for the transfer of DNA.

- The membrane was subjected to prehybridization and hybridization with HPV-16 probe at 68°C (Section C.3.13, steps 5-8) and developed for the location of DNA bands (Section C.3.10, Steps 2-7) as described previously.

- The results were documented by photography.