Chapter 2
ALTERATION/AMPLIFICATION OF C-MYC AND C-Ha-Ras ONCOGENE IN CARCINOMA OF THE UTERINE CERVIX

Introduction

Although experimental evidence strongly suggest that HPV infection per se is essential, it may not be sufficient to produce malignant phenotype (Crook et al., 1988, Zur Hauzen, 1991 a,b). It is likely that other factors such as alterations and/or interaction of certain cellular genes are required for progression to a fully malignant state. These observations are consistent with the idea that cancer is a multistep process (Land et al., 1983). If infection by human papillomaviruses seem to have a role in the initiation of cervical cancer, other factors may be necessary for malignant transformation and maintenance of the neoplastic phenotype.

Normal cellular genes which have the potential to induce neoplasia are called proto-oncogenes or cellular oncogenes. Regulatory or structural alterations of these genes have been implicated in various forms of cancer (Bishop, 1987). Genetic and biochemical evidence point to the existence of a small region in the viral genome whose expression is sufficient to trigger carcinogenesis. This small region is called viral oncogene (v-onc) and cellular counterpart to v-onc is invariably found for every retroviral oncogene discovered so far. Till date at least 20 retroviral oncogenes have been identified and characterized. Ras oncogenes were first identified as transforming principles of several strains of murine sarcoma retroviruses (Ellis et al., 1981). Other human oncogenes identified were also found to represent cellular homologues of a well characterized retroviral oncogenes. The oncogene c-abl implicated in the development of chronic myelogenous leukemia, has turned out to be the cellular homologue of v-abl, the oncogene of Abelson murine leukemia virus (Stivelman et al., 1985). These findings illustrate the existence of dominant oncogenes in human tumors. Certain proto-oncogenes, most commonly c-myc and c-Ha-ras, have been shown to have a role in the development and progression of many human cancers.
Proto-oncogenes can be transformed into the oncogenes as a result of (a) point mutations which result in protein products with strongly enhanced tumorigenic potential (Barbacid, 1987), (b) amplification with enhanced oncogene expression (Alitalo and Schwab, 1986), (c) aberrant expression and (d) specific chromosome translocations that alter the normal function of a proto-oncogene (Cory, 1985). Activated proto-oncogenes are believed to play an important role in tumor formation and/or progression (Klein et al., 1981). Most often, these oncogenes show genetic alterations and/or amplifications during the process of carcinogenesis. Detection of these oncogene alterations, amplification, mutations, deletions, or in some cases over expression in early cancerous lesions could be of immense importance in identifying "high risk" population.

Activation of c-myc expression by genetic rearrangement or amplification has been demonstrated in cell lines derived from Burkitt lymphomas (Taub et al., 1982; Dalla-Fevera et al., 1982), promylocytic leukemias (Collins et al., 1982) and colon (Alitalo et al., 1983) and lung (Little et al., 1983) carcinomas. These genetic alteration of c-myc have been implicated as a contributing factor in the development of the malignancies. Integration of viral DNA sequences into host cell genome has also been known to be one of the first steps towards progression to malignancy. Such integration may lead to activation of certain oncogenes that are associated with such cancers. In cervical cancer cell lines HPV-18 DNA HeLa sequences have been found to be integrated at 8q; 24 region near the c-myc which may be activated in this cell line (Durst et al., 1987, Conturier et al., 1991).

Recent developments in cancer research have included the identification of specific point mutations in ras oncogenes which have strongly been implicated in tumorigenic transformation. As in the case of c-mos, placement of normal ras genes under the regulatory control of retoviral LTRs turns them into oncogenes (Chang et al., 1982). However, the most common mechanism by which ras genes acquire their malignant properties is
by single nucleotide mutations (Tabin et al., 1982, Reddy et al., 1982). These structural alterations may not have any effect on the rate of expression of these genes (Tabin et al., 1982). The ubiquitous expression of ras oncogenes and their extreme degree of conservation during evolution indicates that ras genes are also indispensable for normal cellular functions. If so, it is not inconceivable that mutations that turn ras genes into oncogenes may trigger neoplastic behaviour in many cell types. Thus it is possible that ras gene activation may be mediated by the type of etiologic agent(s) responsible for either initiation or promotion of the tumor, by the timing of the carcinogenic insult or perhaps by genetic traits that may predispose certain individuals to the activation of these loci (Krontiris et al., 1985).

Previous studies (Riou et al. 1984, 1985 Ocadiz et al, 1987) on c-myc proto-oncogene in a limited number of cervical tumours had shown that this oncogene has been amplified in majority of cases suggesting that c-myc amplification may be associated with tumour progression. They also suggested that the c-myc and the c-Ha-ras proto-oncogenes may cooperate in the progression of cervical cancer (Riou et al., 1988). The present investigation has been carried out to see whether such amplification / activation of oncogenes also occurs during cervical carcinogenesis in Indian women.

Further, it would be interesting to establish biological markers whose presence in the early stages of cancer could predict the risk of recurrences. It would also be of great interest to examine if specific genetic alterations can be found in precancerous lesions which may serve as prepathogenic markers for early identification of high risk population who are likely to develop invasive cancer.
MATERIALS & METHODS:

Tumour Biopsies:

Tumor biopsies were obtained from 83 cases of invasive carcinoma of the uterine cervix from the Deptt. of Radiotherapy, Safdarjung Hospital, New Delhi. Biopsy materials were also obtained from 13 cases of mild to moderate and severe dysplasia from All India Institute of Medical Sciences, New Delhi. In all instances the tissue was frozen in liquid nitrogen as soon as possible after surgical excision and was later stored at -70° C until analysis.

Histopathological examination:

Histopathology for all the samples was done at Department of Pathology, Safdarjung Hospital and confirmed histopathological diagnosis was obtained.

HPV and Oncogene Plasmids:
1. Recombinant plasmids containing HPV-16, 18, 6 and 11 DNA were kindly provided by Prof. Harald zur Hausen of German Cancer Research Centre, Heidelberg, Federal Republic of Germany.
2. Recombinant plasmids containing 9 kb EcoR I-Hind III fragment of c-myc subcloned into pBR 322 were obtained from ATCC, USA.
3. Recombinant plasmids containing 2.75 kb EcoR I fragment of c-mos oncogene were also obtained from ATCC, USA.
4. Ha-ras synthetic oligoprobes and primers are detailed in Table 5 and obtained/synthesised at NII, New Delhi.

Extraction of DNA from Biopsy material:

Total genomic DNA was isolated from tumor biopsy specimens which had been stored at -70° C. The tissue was grounded with mortar and pestle (following mincing with scissors) with 0.5 ml 1xTE and a pinch of Sea Sand. Lysis buffer (3% Sarcosyl in 2xTE) in the ratio of 1:2; protease K (Boehringer Mannheim, Germany) to a final concentration of 100ug/ml was added and incubated at 37°C for 1-2 hours. Equal volume of equilibrated
Table 5: Sequences of Amplimers & 32p-labeled 20-mer probes used in hybridization experiments

<table>
<thead>
<tr>
<th>Amplimers</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>1. Ha-ras/codons 12, 13</td>
<td></td>
</tr>
<tr>
<td>5' Amplimer</td>
<td>3' ATGACGGAATATAAGCTGGT3'</td>
</tr>
<tr>
<td>3' Amplimer</td>
<td>5'CGCCAGGCTCACCTCTATA 3'</td>
</tr>
<tr>
<td>2. Ha-ras/codons 61</td>
<td></td>
</tr>
<tr>
<td>5' Amplimer</td>
<td>5'AGGTGGTCATTGATGGGGAG 3'</td>
</tr>
<tr>
<td>3' Amplimer</td>
<td>5' AGGAAGCCCTCCCCGCTGCG 3'</td>
</tr>
</tbody>
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<table>
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<tr>
<th>Probe</th>
<th>Sequence</th>
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<tbody>
<tr>
<td>H12wt</td>
<td>CC-CAC-ACC-GCC-GGC-GCC-CAC</td>
</tr>
<tr>
<td>H61wt</td>
<td>ACC-GCC-GGC-CAG-GAG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/1</td>
<td>ACC-GCC-GGC-GAG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/1a</td>
<td>ACC-GCC-GGC-AAG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/1b</td>
<td>ACC-GCC-GGC-TAG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/2</td>
<td>ACC-GCC-GGC-CTG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/2a</td>
<td>ACC-GCC-GGC-CGG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/2b</td>
<td>ACC-GCC-GGC-CGG-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/3</td>
<td>ACC-GCC-GGC-CAT-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/3a</td>
<td>ACC-GCC-GGC-CAA-GAG-GAG-TA</td>
</tr>
<tr>
<td>H61/3b</td>
<td>ACC-GCC-GGC-CAC-GAG-GAG-TA</td>
</tr>
</tbody>
</table>

Triplets corresponding to the wild-type codons 12 and 61 are underlined. Probes H61/1, H61/2, and H61/3 consist of mixtures of oligomers containing different base substitutions at the first, second, and third positions respectively on codon 61 as indicated.
phenol was added and mixed thoroughly on overhead shaker for 30 minutes. Centrifugation was carried out at 6000 rpm for 45 minute at 4°C. A mixture consisting of equal parts of equilibtrated phenol and chloroform : isoamyl alcohol (24:1) is used to remove proteins from preparations of nucleic acids. The DNA sample was first extracted with phenol:chloroform and then with chloroform. Following phenol-chloroform, extraction DNA was precipitated with 3M Sodium acetate (NaoAc) plus 2.5 volume of chilled absolute ethanol and stored overnight at -20°C. After centrifuging the sample at 6000 rpm at 4°C for 45 minutes the DNA pellet was washed in 70% ethanol and the pellet was left for air drying. The DNA pellet was then resuspended in appropriate amount of 1xTE (10m Tris-Hcl+1mM EDTA). The quantity as well as quality of DNA were measured by running 1 or 2ul of the DNA sample in a minigel with a Hind III-digested Lambda DNA marker. The DNA amount was determined by matching the fluorescence intensity of bands.

**Extraction of RNA from biopsy material**

Tissues freshly harvested were weighed, cut into small pieces (1cm²) with a sterile scalpel, and placed directly into homogenization buffer. The homogenization buffer was prepared with 4.0 M guanidium thiocyanate, 0.1 M Tris Cl (pH 7.5) and 1% β-mercaptoethanol. Guanidium thiocyanate is used to disrupt the cells, and the resulting homogenate is then layered on a cushion of a dense solution of CsCl. The buoyant density of RNA in CsCl (>1.8g/ml) is much greater than that of other cellular components. During centrifugation, the RNA forms a pellet on the bottom of the tube, while the DNA and protein float in the supernatant solution. The pellet was washed with 70% ethanol, recentrifuged briefly and allowed the pellet of RNA to dry in the air. The RNA was dissolved in a small volume of water and stored at -70°C.

**Preparation of Plasmid DNA:**

**Large scale preparation**

500 ml of *E.coli* cells carrying plasmid were grown at 37°C in the presence of ampicillin (50 ug/ml) to optical density of 0.4-0.5 (610nm). To
this chloramphenicol (250ug/ml) was added and cells were grown for another 16-18 hr. Cells were spun at 6000rpm for 15 min at 4°C in Sorvall RC 5C centrifuge. The cell pellet was suspended in 4.5 ml of TEG (Tris-EDTA-Glucose). To this 9.0 ml of freshly prepared NaOH-SDS was added, gently mixed and cells were allowed to lyse on ice for 30-60 min. 4.5ml of potassium acetate, was added to cell suspension, mixed and kept for another 10 min on ice. Supernatent collected after centrifugation at 12000 rpm for 15 min in Sorvall RC 5C was transfered to corex tubes and plasmid DNA was precipitated by adding two third volumes of isopropanol. The air dried pellet was dissolved in 2-4 ml of TE and supercoiled plasmid DNA was purified by cesium chloride density gradient centrifugation.

**Cesium Chloride density gradient:**

1.0gm of cesium chloride (IBI,USA) was added per ml of DNA and mixed thoroughly. To this 0.8ml per 10ml DNA solution of ethidium bromide (10mg/ml) was added. This was then transferred to gradient tubes and spun at 60000 rpm in Sorvall ultracentrifuge (OTD 65B, rotor no. TV 865) or Beckmann table top ultracentrifuge (TL100, rotor no.TLA 100.3) for 12 to 15 hr or 4 hrs respectively at 20°C. The covalently closed circular form of DNA was taken out with a needle and syringe by puncturing the gradient tubes.

**Vector-free insert preparation**

**Purification of DNA fragments by low gelling agrose:**

The following steps were followed :-

1) Run the DNA on 1% LMP agarose gel.
2) Cut the gel slice containing the fragment.
3) Melt at 65°C with 1/2 vol of TE for 30 min.
4) Add equal volume of phenol freshly saturated with TE.
5) Extract at 37°C for 15 min by intermittent vortexing.
6) Centrifuge in eppendorf centrifuge for 5 min.
7) Transfer the aqueous phase into fresh tube.
8) To phenol phase add 0.3 ml of TEamd repeat the extraction at 37°C as described in Step 5.
9) Repeat steps 4 to 8 once more.
10) Collect aqueous phase in one tube.
11) Extract aqueous phase three times with chloroform isoamyl alcohol mix (24 : 1 v/v).
12) Add to the aqueous phase 2.5 volumes of chilled ethanol and 1/10th vol of sodium acetate.
13) Precipitate the DNA overnight at -20°C.
14) Centrifuge at 4°C for 15 min.
15) Discard supernatant.
16) Wash pellet with 70% ethanol.
17) Dry the pellet and resuspend in requisite volume of TE.

**Purification of DNA by elutip column:**

Elutip columns of Schleicher and Schuell(Germany) were used and the purification procedure involved the following steps:

1. Prime the column with 3.0 ml of high salt buffer (1.0 M NaCl, 20 mM Tris-Cl (pH 7.5), 1mM EDTA).
2. Wash the column with 5.0ml of low salt buffer. (0.2M NaCl, 20mM Tris-Cl (pH 7.5), 1.0 mM EDTA).
3. Load the DNA.
4. Washing of the column is done with 3.0 ml of low salt buffer.
5. Elute the DNA adsorbed to the column with 0.4ml of high salt buffer.
6. Add 2.5 volumes of ethanol and 1/10 volumes of sodium acetate to the eluate and precipitate the DNA overnight at -20°C or for 2 hr at -70°C.

**Agarose gel electrophoresis:**

High molecular weight tumour DNA digested with Hind III and ECoR-I (New England Biolab, USA) restriction enzymes was electrophoresed on 0.8% or 1% agarose gels in 1X TAE or TBE buffer. Choice of the percentage of agarose and electrophoresis buffer system was made following the guidelines of Maniatis et al. (1982) with some modifications. Electrophoresis was carried out at 5 volts/cm gel, for 16-20
hours, depending on the size of the gel. DNA gel was stained in electrophoresis buffer containing 0.5 ug/ml ethidium bromide to visualise under a UV transilluminator (Fotodyne Inc., USA) and photographed using Polaroid type 667 film.

Southern transfer:

Restriction fragments of DNA separated on agarose gel were transferred onto nylon membrane (Gene Screen or Gene Screen plus\textsuperscript{Tm}) by the capillary blotting procedure of Southern (1975) and/or as described by Maniatis et al. (1982) with some modifications. The gel was exposed to short wave UV light for 5 minutes, prior to denaturation in a DNA denaturing buffer containing 0.5 M NaOH and 1.5 M NaCl for 1 hour (3 changes with new buffer) at room temperature. Gel was neutralized in a solution of 1 M Tris-HCl (pH 8.0) and 1.5 M NaCl for 1 hour at room temperature with constant shaking. Meanwhile, the nylon membrane was cut to the size of the gel and soaked in 1 X SSC. The gel was allowed to soak in 20 X SSC for 10 minutes at room temperature. The gel was placed on a plastic sheet on the bench, DNA slot side facing up. The nylon membrane was laid neatly on top of the gel, four Whatman 3MM sheets and a stack of ordinary filter papers cut to the size of the gel, were placed on top of the nylon membrane. A glass plate was placed on top of the stack and weighed down with about a 500 g weight. Allowed the transfer to proceed for 12-24 hours after which, the nylon membrane was removed from top of the gel inscribing the markers and dried at room temperature to fix DNA.

\textit{\textalpha^{-32}p} labelling of probe DNA

Probe DNA was labelled by random priming method (Feinberg and Vogelstein, 1984) using the protocol supplied with the kit (NEN, USA). Using \textit{\textalpha^{-32}p} dCTP (Amersham, England). The reaction mix was incubated at room temperature for 30 minutes to 3 hours.

\textbf{Purification of the labelled probe:}

The radiolabelled probe was purified by a spun column procedure to
remove the unincorporated nucleotides. A sterile 1 ml tuberculin syringe was plugged at the lower end with siliconised glass wool. The syringe was then filled with Sephadex G-50 (Sigma, USA) which was equilibrated earlier with TE, upto the 1 ml mark in the syringe. The syringe was placed in a centrifuge tube and spun at 1500 rpm for 2 minutes to pack the column. The labelling reaction mixture, in a volume of 130 ul (30 ul reaction mixture + 100ul TE) was loaded on to the column. A sterile 1.5 ml eppendorf tube was put at the bottom of the column to collect the eluate. The column was respun at 1500 rpm for 2 minutes and the purified probe collected in the eppendorf tube. The unincorporated nucleotides remained in the column. The specific activity of the probes ranged from 1 X 10^7 to 5 X 10^8 cpm/ug DNA.

Southern blot hybridization:

Southern blot hybridization was carried out using the above labelled probes and under stringent conditions of hybridization(Tm-20°C). The details are as described in Chapter 1.

Dot blot hybridization

Nitrocellulose membrane filters (0.45 uM) or nylon membranes were first soaked in 2xSSC for 10 min and the air dried. Denatured DNA (5-7ug in 10ul) was applied to the membrane by directly spotting with Gilson pipetman, P20 or by using dot blot apparatus, Manifold I (Schleicher and Schuell Germany). DNA was fixed to the membrane by baking the filters at 80°C. Prehybridization was done at 65°C in 20 ml of prehybridization mix containing 6XSSC, 5X Denhardts solution and 0.5% sodium dodecyl sulphate. Hybridization was carried out at 65°C for 24 hours in the same solution with 2x10^6 cpm of α-32p labelled DNA probe. Filters were washed thrice, 30 minutes each in washing solution containing 2xSSC, 0.1% SDS at 68°C, air dried and exposed to X-ray film (Indu or Kodak) at -70 °C with intensifying screens.

Dot-blot hybridization in presence of TMACI:

Nylon filters (GeneScreen Plus, New England Nuclear) were
pretreated by incubation in, successively, distilled water and 10x SSC, and were finally dried at 60°C. 1 ul of the DNA amplified in vitro was spotted onto the filters. The filters were dried again at 60°C, rewetted in distilled water and illuminated with a 254-mm UV lamp (1.6 kJ/m2 to bind the DNA to the filter. The filters were prehybridized for 16 h at 56°C in 3.0 M tetramethylammonium chloride (TMACl), 50 mM Tris (pH 8.0), 2 mM EDTA, 100 ug/ml of sonicated denatured salmon sperm DNA, 0.1% SDS and 5X Denhardt's solution and subsequently hybridized for 1 h at 56°C in the same mixture containing approx. 100 pg·32p-labeled oligomer probe (specific activity 10⁹ dpm/ug). The filters were washed in 2xSSPE, 0.1% SDS for 5 min at room temperature, followed by a 5-min wash at various temperatures (67°C for Ha-ras 61, 73°C for H-ras 12) in 5x SSPE, 0.1% SDS. Subsequently, the filters were washed twice in hybridization buffer without Denhardt's solution and salmon sperm DNA and then incubated for 1 h in the same solution at 50-60°C. Finally the filters were exposed to Kodak XAR films at -70°C using intensifying screens.

Optimal conditions for Direct sequencing of double-stranded PCR products with Sequenase

To sequence linear double-stranded PCR products, the template is heat-denatured in the presence of the primer and the reaction is initiated after the shortest possible annealing phase. The recommended times and temperatures of incubation as well as DNA concentrations in the reaction mixture are given below. 1) The annealing temperature (T), to which the sample is transferred after denaturation by boiling was kept -70°C. 2) The time course (L) of the labeling reaction, which is carried out at room temperature, was taken between 15 and 45 seconds, 3) the primer:template ratio (R) in the reaction mixture was around 20:1. This set of optimal conditions leads to an efficient protocol for directly sequencing double-stranded PCR products.

Sequencing gel formulation:

A standard 6% polyacrylamide/urea sequencing gel mix was prepared
as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity 1</th>
<th>Quantity 2</th>
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</thead>
<tbody>
<tr>
<td>40% (w/v) acrylamide stock</td>
<td>75 ml</td>
<td></td>
</tr>
<tr>
<td>(acrylamide: bisacrylamide; 38:2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urea</td>
<td>230 g</td>
<td></td>
</tr>
<tr>
<td>10 x TBE</td>
<td>50 ml</td>
<td></td>
</tr>
<tr>
<td>H₂O, add up to</td>
<td>500 ml</td>
<td></td>
</tr>
</tbody>
</table>

Each time, 75 ml of this instant gel mix, 150 ul of 25% APS and 100 ul of TEMED were added and the gel poured immediately.

**Preparation of Sequencing gel:**

Glass plates were cleaned thoroughly, rinsed with deionized water, wiped dry, surface of one plate was siliconized, and finally plates were rinsed with alcohol. Gels were casted using the 0.4 mm side spacers. Plates were taped around the edges. To pour the gel solution into the plates, the plates were held at an angle of 45°C, gel was poured from a 250 ml beaker. An even flow was maintained to avoid trapping of air bubbles. Then laid the plates on the bench top, inserted sharkstooth comb, allowed it to polymerize for 2 hours, following which, the comb was pulled out and inverted so as to make wells.

**Fixing and drying:**

Gels were fixed in a solution containing 10% methanol and 10% acetic acid for 30 minutes. Then the gel was carefully transferred to Whatman 3 mm paper, covered with Saran Wrap and dried at 80°C under vacuum for 1 hour.

**Autoradiography:**

Autoradiography was performed in X-ray cassettes which hold the filter and X-ray film in close contact with intensifying screens.
Results

Amplification of c-myc oncogene:

Samples from 83 invasive carcinomas of uterine cervix and 13 dysplasias were examined for genetic alteration of the c-myc proto-oncogene. The c-myc oncogene may be genetically altered either by amplification or by alteration of its native arrangement in chromosome. Alteration of c-myc gene by point mutation has not been observed so far in different types of cancer. The samples were digested with Hind III and were checked by gel electrophorosis (Fig 7 A). Fig 7 B shows that after amplification of the c-myc gene multiple copies of the gene appear to be linked to each other at a single locus. Hence after digestion with Hind III, only a single band of 11.5 Kb corresponding to the full length c-myc appears. The level of amplification was estimated by comparing to the band intensity of single copy c-myc gene present in normal human blood lymphocytes. Using this approach, a total of 26 samples out of 83 (31%) showed clear amplification of the c-myc gene. Fig 7 B shows a representative panel of samples found to contain amplification of the c-myc gene. These samples were initially screened for HPV16 and were found to harbor HPV type 16 DNA sequences (Fig 11). These samples showed amplification of the c-myc oncogene when compared with the control human β-globin gene (Fig 7C). In general, a maximum of about 3 to 5 fold amplification of the c-myc oncogene was observed (densitometric study) in some samples (Table 6). Male lymphocyte DNA was used as a control (Fig 7A & B). Table 6 shows that all the tumor DNAs bearing an amplification of the c-myc proto-oncogene were invasive carcinomas except one of 13 dysplasia cases studied here (Fig 7B) and were having HPV 16 infection (Fig 11) which also showed c-myc amplification (Fig.7B).

Structural gene rearrangement of the c-myc gene:

The type of alteration detected with probe of c-myc, was the presence of a novel c-myc related fragments in two invasive carcinomas. Blots of tumor number 46 and 58 DNA that had been digested with EcoRI and Hind III were hybridized with probes corresponding to Exon I (probe a),
Exon II and III (probe b), exon III and 3' flanking cellular sequences (probe c) (Fig 10). All the probes detected a 8.2 kb germline band for the normal DNA and a rearranged 6.8 kb band in the carcinoma samples (Fig 9C). In addition, probe c detected a 5.2 kb fragment (Fig 9B). These results suggest that the genetic alteration occurred between the 5' Hind III and 3' EcoRI sites of the c-myc locus. The observation that probe b does not detect the non-germline 5.2Kb fragment that hybridizes with probe c indicates that the break point is in the vicinity of the 3' end of Exon 3. Insertion or deletion of foreign DNA at the 3' end of Exon II & III is ruled out as probe b detects only one band whereas probe c detects both the bands. Interpretation of the restriction enzyme analysis is not easily compatible with either the deletion or the insertion of genetic material at the point near to 3' end of c-myc locus. Instead the data suggests that a reciprocal translocation had occurred. The 5.2Kb EcoRI-Hind III fragment that reacts with probe c probably represents a portion of chromosome 8 that has been exchanged with another chromosome. Examination of recombinant clones of this rearranged c-myc gene should give insight into the identity of the translocation partner.

Expression of the c-myc gene:

Amplification or rearrangement of proto-oncogenes may not always be associated with their enhanced expression. We used total RNA samples from these carcinomas and found higher levels of c-myc RNA in all invasive carcinomas bearing an amplified c-myc than that of normal sample (Fig 12). Tumor tissues for all the cases were not sufficient to do RNA analysis.

Analysis of mutation in c-Ha-ras oncogene

To determine the position and type of point mutation responsible for activating the c-Ha-ras gene, we used oligonucleotide sequences spanning codon 12, 13 and codon 61.

PCR Amplification of codon 12, 13 and 61 of c-Ha-ras oncogene

Initially DNA amplification in vitro was performed as described by Saiki et al (1985, 1988). Polymerase chain reaction was done using specific
Fig. 7: Amplification of c-myc proto-oncogene in carcinoma of uterine cervix:

(A) DNA (10μg) from tumors and lymphocytes was digested with the restriction endonuclease Hind III and separated by (0.8%) agarose gel electrophoresis.

(B) Southern blot of gel shown in Fig 7 A following hybridization with the exon 3 of c-myc probe.
Lane 1: shows Hind III digested Lambda DNA size marker.
Lane 2: Male Lymphocyte DNA
Lane 3: DNA from tumor sample no. 26
Lane 4: DNA from Tumor sample no. 31
Lane 5: DNA from Tumor sample no. 42
Lane 6: DNA from dysplasia sample no. 12
Lane 7: DNA from dysplasia sample no. 8
All samples show amplification and apparently no amplification in lane 7 when compared with normal DNA shown in lane 1.

(C) Southern hybridization of the blot of gel shown in Fig 7 A with labelled human β-globin gene probe.
Fig. 8: Analysis of C-mos gene amplification in invasive carcinoma samples Nos 2-7 corresponding to patients nos 26,31,42,12,8 & 74 respectively were dot blotted and probed with a C-mos clone. No amplification of C-mos locus was observed in these samples when compared to normal lymphocyte DNA sample No 1. Sample No 8 is C-mos positive control.
Fig. 9: Southern blot hybridization of cervical cancer samples for analysis of alterations in the C-myc oncogene.

(A) Ethidium bromide stained (.8%) agarose gel picture of Eco RI and Hind III digested normal and tumor DNA samples.

(B) Southern blot hybridization of gel shown in Fig 9 A with probe C (C-myc) with a Cla I fragment including exon III and adjacent 3' cellular sequences (shown in Fig 10). Lane 1 has Hind III digested lambda DNA size marker. Lane 2 shows DNA from a normal individual showing a band of approx 8 kb in the Eco R I and Hind III digest. Lane 4 & 6 are showing a rearranged 6.8 kb band and a non-germline 5.2 kb band in EcoR 1 and Hind III digests from patient no. 46 and 58. Lane 3 & 5 do not bear any DNA sample.

(C) Southern blot hybridization of gel shown in Fig 9 A with probe a & b (C-myc map shown in Fig 10). Lane 1 has Hind III digested lambda DNA size marker. Lane 2 shows DNA from a normal individual showing a band of approx 8 kb in the Eco R I and Hind III digest. Lane 4 & 6 are showing a rearranged 6.8 kb band. The non-germline 5.2 kb band seen in Fig 9 B is not seen here.
Fig. 10.
The different probes used are shown below the restriction map:
Probe a is an Xho–Pvu II fragment within exon I
Probe b is the Pst I P Ryc 7.4 fragment cloned from a cDNA representing part of exon II and exon III
Probe c is a Clal–Cla I fragment including exon III and adjacent 3' cellular sequences
Fig 11: Detection of HPV-16 sequences in invasive cervical carcinoma samples by dot blot hybridization. Samples 1-9 show presence of HPV DNA. Each sample contains 10 ug of tumor DNA. The dot blot was hybridized by HPV-16 DNA probe. No.10 contains HPV-16 plasmid DNA which was used as a positive control. All the nine samples shown here were checked for C-myc alteration/amplification. Samples 2, 6 & 7 here showed amplification of the C-myc locus in tumor sample no 26, 31 & 42 in Fig 7 A.
Fig. 12: Expression of C-myc oncogene in cervical carcinomas.
5-7 ug of cellular total RNA was blotted on to the Gene screen plus membrane and hybridized by C-myc DNA probe. Samples 1, 6, 7, 9 correspond to sample nos. 26, 31, 42 & 12 in Fig 7 A. Samples 2, 3, 4 and 8 are dysplasia samples. Sample no. 2 here corresponds to sample no. 8 in Fig 7A. Sample No. 5 is from a normal placental tissue used as a control to analyze C-myc m-RNA overexpression in invasive carcinoma samples compared to normal sample.
Fig. 13: Ethidium bromide stained (2%) agarose gel showing PCR amplification of codon No 12, 13 & 61 of c-Ha-ras oncogene in invasive carcinomas. Specific amplimers set for codon 61 and codon 12, 13 from C-Ha-ras oncogene was used. Lane 1 shows \( \phi \) X 174 Hae III digested DNA size marker. Lane 2-4 show 110bp specific amplification of codon no 12, 13. Lane 5-7 show 120 bp specific amplification of codon \( \not{1} \).
Fig. 14: DNAs from malignant cervical cancer cases were amplified in vitro using oligomers specific for codon 12, 13 of c-Ha-ras gene and 20 ul of PCR product was dot blotted and hybridized to the oligomers II-12 (wild type codon 12,13 oligoprobe shown in table 5) in presence of Tetra-methyl ammonium chloride (TMACl). None of the samples show any point mutation in codon 12,13 in these DNA samples. sample 1-11 are from invasive carcinoma patients and N is from Normal individual as positive control.
Fig. 15: DNAs from malignant cervical cancer cases were amplified in vitro using oligomers specific for codon 61 of c-Ha-ras gene and 25 ul of PCR product was dot blotted and hybridized to the oligomers H61. (wild type codon 61 oligoprobe shown in Table 5) in presence of Tetra-methyl ammonium chloride (TMACl). None of the samples show any point mutation in codon 61 in these DNA samples. Samples 1-10 are from invasive carcinoma patients and sample N is from normal individual used as positive control.
Fig 16: Direct sequencing of PCR amplified product containing codon No.61 of C-Ha-as oncogene from invasive cervical cancer patient No.46 (sample no 3 in Fig.15) showing no modification in codon no.61 CAG. Codon 61 is shown by thin arrows.
Table 6: Genetic alteration/amplification and mutation of C-myc and c-Ha-ras oncogene in advanced cervical carcinoma samples

<table>
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<tr>
<th>Number</th>
<th>Class</th>
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<td>Amp 3x</td>
<td>N.D.</td>
<td></td>
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</tbody>
</table>

* N.C. : No Change
* N.D. : Not Determined
x : degree of Amplification (Densitometric study)
amp : amplification
oligoprimers mentioned in Table 5 for codon 12,13 and 61 of c-Ha-ras oncogene. PCR technique was followed as detailed in Chapter 1. Fig 13 shows gel electrophoresis of 110 bp(codon 12&13) and 120 bp(codon 61) DNA fragments from carcinoma samples showing amplified codon 12, 13 in lane 2,3,4 & codon 61 in lane 5,6,7. Dot blots were made with amplified DNA products and were probed with the 20 mer probe homologous to the wild type sequence for both positions (Table 6). Out of 14 carcinomas and 8 dysplasia cases tested for the presence of mutation, none of 14 invasive carcinomas of uterine cervix exhibited the mutations; nor did 8 cervical intraepithelial neoplasia cases (Fig 14 & 15). Direct sequencing of the PCR products (Fig 16) was done to analyse point mutations occuring in codon 61. Sequencing of the PCR products showed no change in codon no. 61 in invasive cancer sample. (Fig 16).

Discussion

Presently it is difficult to assess the total contribution of viral infections in the development of virus associated cancers in humans. Hepatocellular carcinoma (HCC) and nasopharengeal cancer are the most prevalent tumors linked to specific virus infections. These cancers including carcinoma of cervix account for approximately 80% of all virus-linked human cancers (zur Hausen, 1991b). The expression of specific viral oncoproteins may be required for the malignant phenotype of HPV-positive anogenital cancers. Functional modifications of host cell genes in the vicinity of the viral DNA integration site may influence the progression to malignancy (Saito et al., 1990). It is known that products of two transforming genes E6 and E7of HPV interact with tumor supressor protein p53 and RB p105 respectively, leading to loss of negative growth controls that release the break for tumorigenic progression.

a) Higher amplification, expression and rearrangement of cellular oncogenes, c-myc in cervical cancer

Endogenous host cell DNA modifications involve activation of protooncogenes which play an important role in tumor formation and/or progression (Klein et al, 1981). Activation of c-myc has been demonstrated
in tissue culture cell lines derived from Burkitt lymphomas (Taub et al., 1982; Dalla-Favera et al., 1982), promyelocytic leukemias (Collins et al, 1982) and colon (Alitalo et al., 1983) and lung carcinoma (Little et al., 1983). These genetic alterations of c-myc have been implicated as contributing factors in the development of these malignancies. Appropriate expression of the c-myc cellular gene has been shown to occur through at least four distinct mechanisms with characteristic tumorigenic effect. They are 1) by gene amplification 2) by chromosomal translocation 3) by retroviral promoter insertion and 4) by gene mutation.

There is evidence that amplification of proto-oncogenes in the myc gene family correlates with an aggressive or advanced primary tumour. Previous studies (Riou et al. 1984, 1985) on the c-myc protooncogene in a limited number of cervical tumors have shown that this oncogene was activated/amplified in the most advanced cancers, suggesting that c-myc activation may be associated with tumor progression. In the present study we have analysed the level of c-myc gene transcripts in tumor specimens obtained from untreated patients with primary invasive squamous cell carcinomas of the uterine cervix at different stages. Peripheral lymphocyte DNA preparations from these patients were used as standards to evaluate the extent of amplification. The human β-globin gene was used as internal control for the experiments (Fig 7C). Interestingly, no evidence for the amplification of c-mos was found (Fig 8). In contrast, an amplification of the c-myc proto-oncogene at varying degree was observed in about 31% of tumors. These results are in good agreement with Baker et al (1988) and Ocadiz et al (1987) who have found c-myc amplification in 32% and 48% respectively in carcinoma cervix samples. Our results indicate that amplification of the c-myc gene is not due to polyploidy as the c-mos gene, located in the same chromosome does not undergo amplification (Fig 8). This is confirmed by HindIII digestion pattern of the tumor samples which shows amplification of the c-myc gene (Fig 7B).

It has been observed that amplification and/or overexpression of the c-myc gene were more frequent in advanced carcinomas. In this study some patients with early stage cancers showed an overexpressed c-myc gene and had an early relapse. This prompted a study to examine the relative
prognostic importance of c-myc gene expression in relation to several other factors in predicting early relapse. We have found a significant association between the enhanced level of c-myc RNA in cervical cancers which provides a means of identifying patients at higher risk of early recurrence. This facilitates better and more individualized approach to treatment. These results are in agreement with other reports where similar c-myc amplification has been shown in carcinoma cervix and in breast cancer (Riou 1988, Escot et al, 1986). However, these observations are in contrast with Baker et al (1988) where they did not find apparent correlation with stage of disease and the prognostic significance of c-myc amplification could not be correlated in this study.

In our analysis of 83 invasive carcinoma of uterine cervix we found two DNA samples bearing non-germ-line c-myc-related restriction fragments. In both of them, we identified an apparent rearrangement with a break-point near the 3' end of exon 3. Interpretation of the restriction enzyme analysis is not easily compatible with either the deletion or the insertion of genetic material near c-myc. Instead the data suggest that a reciprocal translocation has occurred. The 5.2 kb EcoI-HindIII fragment that hybridizes with probe c but not with probe a & b suggests that the break-point has occurred near the 3' end of exon 3 of c-myc oncogene (Fig 9B). These results are in contrary to the observations made by Baker et al (1988) and Ocadiz et al (1987) where both the groups did not find genetic alterations in c-myc locus in the cervical carcinoma samples.

Several reports have indicated that in mouse plasmacytomas (MPCs), the variant 15;6 translocations occur on the 3' side of the gene, allowing the c-myc locus to remain intact on chromosome 15 (Banerjee et al., 1985, Cory et al., 1985). In these tumors immunoglobulin sequences are translocated to chromosome 15. Variant (8;2 and 8;22) translocations of the c-myc locus also occur in Burkitt's lymphomas. The affected c-myc allele in these translocations remains on chromosome 8 as an intact transcriptional unit and immunoglobulin sequences are introduced in a head-to-tail manner on the 3' side of the locus (Croce et al., 1983, Erikson et al., 1983, Hollis et al., 1984, Rappold et al., 1984, Taub et al., 1984 a,b). Detailed studies of rearranged c-myc gene should give insight into the identity of the
translocation partner and to determine whether the rearranged c-myc gene with a break-point near the 3' end of the gene as observed in this study represents variant translocation events as reported in mouse plasmacytomas (MPCs) and Burkitt's lymphomas.

It is now widely believed that translocations involving c-myc lead to altered transcription of the gene and that this is how they contribute to malignant transformation. Because of translocation, the c-myc gene is moved to a new transcriptional locus, for example, in Burkitt's lymphomas and MPCs. Some investigators have suggested that disruption of regulatory control elements in the 5' flank of the locus can change the normal pattern of transcription in tumors which exhibit an 8;14 translocation involving exon I of the gene. (Taub et al.,1984b,Wiman et al., 1984). Translocation involving exon III may lead to altered gene product of c-myc which in turn leads to abnormal cell growth.In MPCs where 15;6 translocation occurs on the 3'side of the gene, despite the apparent minimal disruption of the c-myc locus, expression of normal-sized transcripts from the rearranged locus is enhanced to a substantial level (Cory et al., 1985). Given that the protein-coding sequences of the gene remain intact and that no qualitative changes in c-myc protein structure are believed to occur, the tumorigenic effects produced are most likely due to inappropriate levels of normal protein in the affected cells (Hann et al.,1984, Ramsay et al., 1984). Our results indicate that in some cancer cases amplification or rearrangement of c-myc protooncogene is associated with their enhanced expression. These results are in good agreement with Escot et al.,( 1986) and Couturier et al.,(1991), who observed genetic alteration in 3' end of the c-myc gene in breast cancer and cervical cancer. However, a correlation between genetic alteration of the c-myc domain and enhanced levels of c-myc RNA was not found in all the tumors studied.

b) Absence of alteration/mutation of c-Ha ras oncogene in cervical cancer:-

More than 50 different point mutations in five different ras codons have been identified. All such mutations activate 3T3 cell transforming function (Cichutek, et al., 1986). The 3T3 cell-transforming function of the
Harvey proto-ras gene from the bladder carcinoma was reduced to a single point mutation that changed the 12th ras codon of p21 from the normal glycine to valine (Reddy et al., 1982). Since such proto-ras genes behaved like dominant and autonomous cancer genes in this morphological assay, they were claimed to be cellular cancer genes (Tabin et al., 1982; Reddy et al 1982).

Specific point mutations in ras gene in human tumour cells have been identified (Barbacid, 1987). These mutations have been exclusively detected in codons 12, 13 and 61 of the three ras genes, c-Ha-ras, c-Ki-ras and N-ras. Detection of mutations have shown that ras gene mutations occur in human colorectal cancers. These results suggest that detection of ras gene mutations in precancerous lesions may provide the basis for early identification of patients at higher risk to developing cancers.

In the present study 22 human invasive cervical cancer, including 8 dysplasia cases, were listed to analyse for the presence of point mutation in the 12 amino acid and 61 amino acid of the c-Ha ras oncogene product. Hybridization of the PCR products with wild type oligosequences of Ha-ras 12th codon and 61 codon in presence of Tetramethyl ammonium chloride (TMACl), didn't reveal point mutations in codon 12,13 and 61 of c-Ha-ras in a limited number of twenty two carcinoma samples. We also tried direct sequencing of the PCR products in four carcinoma samples who showed no mutation in codon 61 by dot blot hybridization with wild type oligoprobe of codon 61 in presence of TMACl. This is to reanalyse and confirm our own probing results. None of the 22 samples exhibited any mutation. Thus the point mutation affecting these sensitive codons i.e. 12th and 61 codon of the c-Ha-ras gene may not play a role in the development of carcinoma of the uterine cervix. Similar findings have been reported by others for most human epithelial cell carcinoma of the bladder, colon or lung (Feinberg et al., 1983). These observations are, however in contrary to the observations made by Riou et al., (1988), who found 11% cervical carcinoma cases showing mutations at codon 12. It is difficult at present to have explanation for these contrasting results, except that the technical aproches were different. Riou et al (1988) have used restriction fragment length polymorphism (RFLP) technique on tumor DNA samples whereas we have
used PCR to amplify codons 12, 13 and 61. Our observations are further strengthened by the recent findings of Hurlin et al., (1991) who showed that at least codon 12 of ras oncogene is not altered when human epithelial cells are immortalized by HPV18 transfection. It will be interesting to examine if any of mutations at codon 12, 13 or 61 in c-Ha-ras exist in established HPV containing cell lines such as Hela, SiHa etc.

In a series of broadly analogous experiments two groups (Eliyahu et al., 1984, Parada et al., 1984) demonstrated independently that p53 expression constructs could cooperate with an activated ras gene in the malignant transformation of primary rodent cells.

Rb and p53 appear to play as yet poorly defined roles in the regulation of cellular DNA replication and the mitotic cycle. Interference with their function may lead to deregulation of the cell cycle and to chromosomal instability and aneuploidy that are regularly observed in individuals with high risk HPV infections (McCance et al., 1988, Pirisi et al., 1987, Durst et al., 1987). E6 or E7 proteins in low-risk HPV infections (HPV-6 or HPV-11) appear to bind either less actively or not at all to these host cell proteins (Dyson et al., 1989). They also fail to induce chromosomal changes. Thus the interaction of high risk HPV E6-E7 gene products with cellular Rb and p53 protein may represent an endogenous progression factor and may be important for the progression of premalignant lesions to malignant stages as the consequence of the induction of mutations and chromosomal instability. (p53 mutations have only been noted in HPV-negative cervical carcinomas but not in HPV16 or 18-positive tumours (Scheffner et al., 1991, Cuzick et al., 1992; B.C. Das, personal communication)

Activated ras gene alone are highly inefficient in transforming at least some types of primary rodent cells. However, if a second oncogene is present, then they readily induce transformed foci (Land et al., 1983). Oncogenes that can complement ras include myc, the E1A gene of adenovirus, myb, and SV40 and polyoma large T. Interestingly, all these genes share the ability to induce establishment or immortalization of primary
cells, and they all encode proteins that are localized primarily in the cell nucleus.

The present study suggests that though viral presence and c-myc amplification/alteration seem to be playing a role, co-operative effect of the c-myc and c-Ha ras seems to be absent during the development of cervical cancer. Further studies are needed to determine whether cellular oncogenes are activated by point mutation during the process of cervical carcinogenesis.