Papers

Alterations in exon 1 of c-myc and expression of p62^{c-myc} in cervical squamous cell carcinoma

J J O'Leary, R J Landers, M Crowley, I Healy, W F Kealy, J Hogan, C T Doyle

Abstract

Aims—To examine human papillomavirus (HPV) positive and negative squamous cell carcinomas of the cervix for structural alterations in exon 1 c-myc; and to investigate the expression pattern of p62, the protein product of c-myc.

Material—Archival paraffin wax embedded tissues of cervical squamous cell carcinomas, stage I and II, retrieved from the files of the department of pathology, University College Cork, Ireland: 40 cases were examined for alterations in exon 1 of c-myc; 57 cases were used for immunocytochemical p62 analysis.

Methods—c-myc exon 1 PCR on HPV positive and negative stage I and II cervical squamous cell carcinomas was performed using primers designed to fragile sites in exon 1 of the c-myc oncogene, which are frequently involved in translocation phenomena and deletions in other neoplasms. This region is bordered by two promoter sequences P1 and P2. In addition, the expression of p62 was evaluated using the monoclonal antibody Myc1-9E10.

Results—Alterations in exon 1 of c-myc were shown in 7.5% of squamous cell carcinomas of the cervix. Changes in exon 1 and 2 of c-myc were also found in COLO 320 cells and Raji cells. These alterations were due to small deletions within exon 1 of c-myc, but point polymorphisms occurring within the priming sites (in one case) may also have occurred. The alterations uncovered appeared "clonal," as replicate samples showed the same amplicon band pattern. Expression of c-myc was variable, with cytoplasmic staining patterns predominating. All cases which showed exon 1 alterations were HPV positive and had strong nuclear positivity on p62 immunocytochemistry.

Conclusions—Alterations in exon 1 of c-myc occur in a minority of cervical cancers and there was increased expression of p62 in a cohort of HPV positive and negative cervical squamous cell carcinomas. Exon 1 alterations may provide an alter-

native route to c-myc activation in early squamous cell carcinoma.

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The c-myc oncogene is expressed in almost all differentiated cell types and the protein is thought to act as a transcriptional regulator.¹ The gene has been shown to have an important role in early embryogenesis,² the control of cell growth, cellular differentiation,³ tissue repair processes,⁴ and apoptosis.^{5,6} Expression of the gene is considered to be a major component in the regulatory processes associated with normal cell proliferation and differentiation. This view is reinforced by the demonstration of gene amplification or an increase in the level of the gene product p62 in a variety of pathological states in which there is perturbation of cell proliferation and differentiation. These include several malignant and premalignant conditions.^{7,8} The myc gene contains a transcriptional activation domain and a basic helix loop leucine zipper DNA binding and dimerisation domain. Acting as a heterodimer with a structurally related protein, Max, myc can bind DNA in a sequence specific manner, which acts as a transcriptional activator for many genes critical in regulation of cell growth.⁵ Max is not fully restricted to acting with myc and indeed forms homodimers and heterodimers with Mad and Mxi-1, which then act as antagonists to myc/Max heterodimers, by competing for similar DNA targets. In apoptosis, myc interacts with p53, Bax, and Bcl-x. Myc induced apoptosis appears to occur in cells deprived of certain growth factors or where growth has been arrested by cytotoxic drugs. The role of c-myc role in apoptosis requires binding to Max.⁹

Expression of c-myc protein has been shown to be closely associated with the entry of a cell from the G₀ or resting phase into the cell cycle, while almost complete shut off of c-myc expression accompanies the inhibition of proliferation associated with differentiation.¹⁰ However, this finding is not universal, with other reports suggesting that c-myc protein

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Figure 1 Schematic representation of the human *c-myc* gene. Exons are indicated as solid black boxes. The position of primers in exon 1 is indicated in (B).

synthesis is independent of the cell cycle.¹¹ Amplification of this oncogene has been observed in several human cancer cell lines (including cervix) and in solid tumour specimens.¹² Structural changes in the *c-myc* gene and abnormal expression of the *c-myc* product have been observed in numerous tumours, particularly B and T cell malignancies.¹³

The human cellular *myc* gene is made up of three exons separated by two large introns (fig 1). The first exon codes for a leader which contains termination codons on all three reading frames. The *c-myc* gene has two promoter regions (P1 and P2) and two transcription-initiation sites. Cellular *myc* sequences are transcribed in normal cells. Expression of *c-myc* is controlled during the cell cycle in non-transformed cells, but this control is lost after chemical transformation. Transcription of the *c-myc* gene increases markedly shortly after stimulating resting cells to enter the G₁ phase of the cell cycle. In various types of carcinoma it has been found that structural alteration of *c-myc* is accompanied by increased quantities of the *c-myc* oncogene product. Some of the alterations more often affecting *c-myc* gene expression in human cells are amplification, translocation, and somatic mutations.¹⁴⁻¹⁵ Gene amplification and high levels of *c-myc* expression have been observed in HL-60 promyelocytic leukaemia cells, small cell lung carcinomas, and in some breast tumours.¹⁶ Translocation activation of the *c-myc* oncogene has been found in Burkitt's lymphoma and in an undifferentiated T cell lymphoma as a result of specific chromosomal translocations. The *c-myc* oncogene is translocated in different types of tumours, the 8:14 translocation of Burkitt's lymphoma being one of the best studied.¹⁷

In Raji cells (which contain the 8:14 translocation), substantial alterations—deletions and point mutations—in the sequence of the first two exons are seen. These changes probably result from somatic mutations occurring during or after the translocation event. Changes observed in exon 2 of *c-myc* result in an altered

c-myc protein product, whereas those in the first exon can result in either *c-myc* overexpression or higher stability of *c-myc* mRNA.¹⁴ Exon-1 of the *c-myc* oncogene is important in transcriptional control of this gene and thus alterations at this site can lead to overexpression of the *c-myc* gene or higher stability of the *c-myc* mRNA.

Interestingly, in COLO 320 cells, normal and truncated (lacking exon 1) *c-myc* genes coexist and both are transcribed.¹⁸ Turnover studies of *c-myc* mRNA show that the normal mRNA has an *in vivo* half life of approximately 30 minutes, which appears to be approximately similar to the turnover time of mRNA in lymphoblastoid cells. However, the truncated mRNA (in COLO 320 cells) is known to be substantially more stable. This observation has also been made in Burkitt's lymphoma cell line which contains a translocated, truncated *c-myc* gene. Therefore truncation of the *c-myc* gene can cause the messenger RNA to be more stable than the full size product, suggesting that this may indeed be a crucial factor in the activation of the *c-myc* oncogene by exon 1 (such as in chromosomal translocation). Exon 1 may also play a role in *c-myc* degradation mechanisms.

Amplification of *c-myc* oncogene remains the predominant route for *c-myc* activation in cervical cancer¹⁹⁻²¹ and appears to be important in relation to prognosis in early carcinoma of the cervix.

In the light of this knowledge, we have examined a series of human papillomavirus (HPV) positive and negative squamous cell carcinomas of the cervix for structural alterations in exon 1 *c-myc* and have investigated the expression pattern of p62, the protein product of *c-myc*.

Methods

MATERIALS

Archival paraffin wax embedded tissues of cervical squamous cell carcinomas, stage I and II, were retrieved from the files of the department of pathology, University College Cork, Ireland. Forty cases were examined for alterations in

exon 1 of c-myc; 57 cases were used for immunocytochemical p62 analysis.

HPV IN SITU HYBRIDISATION

Three 5 µm sections (selected from two different areas of the tumour) were cut onto APES coated glass slides (PH106, C A Hendley, Essex, UK). Two areas from the included cervix were also examined.

Tissue dewaxing was carried out using xylene and rehydration through a graded alcohol series. Proteolytic digestion was done with 0.5 mg/ml proteinase K in proteinase K buffer (50 mM Tris HCl pH 7.6, 5 mM EDTA) at 37°C for 10 minutes. Slides were then immersed in phosphate buffered saline (PBS) containing 2 mg/ml glycine for five minutes and washed in PBS for a further five minutes. For alkaline phosphatase detection, slides were immersed in 20% (vol/vol) aqueous acetic acid at 4°C for 15 seconds. For peroxidase detection, slides were immersed in 3% sodium azide/hydrogen peroxide to abolish endogenous peroxidase activity. Slides were then washed in PBS for five minutes. A postfixation step was not carried out. Dehydration of the sections then took place through graded alcohols to water.²²

Cloned HPV probes (6, 11, 16, 18, 31, 33) (genital types; gifts of Harald Zur Hausen) were labelled with biotin or digoxigenin using a standard nick translation protocol.²² Probes were initially prepared at a concentration of 200 ng/ml.

The hybridisation buffer contained 2× SSC (sodium chloride/sodium citrate), 5% dextran sulphate, 0.2% dried milk powder containing no vegetable extracts, and 50% formamide. Approximately 10–20 ng of the appropriate probe was used during standard in situ hybridisation protocols.

Medium stringency post-hybridisation washings were initially applied using 2× SSC at 60°C for 20 minutes, followed by 0.2× SSC for 42°C at 20 minutes then 0.1× SSC at room temperature for five minutes, and 2× SSC at room temperature for five minutes. Higher stringency washes included 0.2× SSC at 55°C and 60°C for 10 minutes, and 0.1× SSC at room temperature, 42°C and 55°C.

The hybridisation signal was detected using one step, two step, or three step techniques, as described previously.¹⁸ Colorimetric detection wash achieved using an NBT/BCIP substrate for alkaline phosphatase or aminoethylcarbazole (Zymed kit for peroxidase, California, USA).²² Sections were counterstained with 2% methyl green for alkaline phosphatase detection or haematoxylin for the peroxidase detection system.

Tissue controls for non-isotopic in situ hybridisation (NISH) included HPV positive cervical wart and myocardium (negative for HPV). Reaction controls included hybridisation buffer on its own, biotin/digoxigenin labelled plasmid sequences (pBR322), and irrelevant probe (herpes zoster virus, HZV). Labelled human placental DNA was used to check hybridisation efficiency.

Detection controls included omitting primary or secondary antibody steps and addition of the colorimetric substrate only.

SOLUTION PHASE POLYMERASE CHAIN REACTION

HPV E6 DNA sequences were derived from the EMBL genetic sequence database.²³ Oligonucleotide primers were synthesised on a Perkin Elmer Applied Biosystems DNA synthesiser (Perkin Elmer, Cheshire, UK), deprotected, and stored in liquid ammonia at –20°C. During oligosynthesis, a biotin reporter molecule with a 15 carbon atom linker arm was added to the 5' end of the oligonucleotide probe which was subsequently used as an internal probe to confirm product specificity. The nucleotide sequence of the primers are as previously detailed.¹⁹

Controls included tissues HPV positive by NISH and HPV plasmids. Reaction controls included omitting Taq DNA polymerase and running β actin PCR assays on each sample.

General HPV primers (which identify sequenced and unsequenced virus) were also used for a pan HPV screen, unrestricted by the type specific viruses chosen above.²⁴

Ten 5 µm sections were cut from the tissue blocks and placed in sterile Eppendorf tubes. Nucleic acid extraction was performed using proteinase K (0.1–0.5 mg/ml) in proteinase K buffer (100 mM NaCl, 10 mM Tris HCl, 25 mM EDTA, 0.5% sodium dodecyl sulphate, pH 8.4). Proteinase K incubation was carried out for three to five days at 37°C with adequate mixing of samples. Proteinase K inactivation was then carried out at 94°C for 10 minutes. DNA was purified using a standard phenol chloroform isoamylalcohol technique. Nucleic acid was precipitated using 3 M sodium acetate and ice cold ethanol. Strict anticontamination protocols were adopted throughout.

For type specific HPV polymerase chain reaction (PCR), the PCR solution consisted of PCR buffer (50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatine, 200 µM of each dNTP, 1.0 µM of each primer, 2.5 units of AmpliTaq DNA polymerase, and 100 ng of DNA template). Samples were then subjected to 40 cycles of PCR in a Perkin Elmer 480 DNA thermocycler. Cycling parameters were as follows: 94°C for one minute, followed by 94°C for one minute, 55°C for two minutes, 72°C for three minutes × 40 cycles, with a final extension set for 72°C for five minutes.

For general primer PCR, amplification conditions were similar except 3.5 mM MgCl₂ was used and an annealing temperature of 40°C was applied.

Type specific HPV PCR products were confirmed by dot blot hybridisation as previously described.²³

PCR ANALYSIS OF EXON-1 C-MYC

PCR for c-myc was performed using primers designed for fragile sites in exon 1 of the c-myc oncogene which are frequently involved in translocation phenomena and deletions in

Table 1 Primer sequences for c-myc exon 1 polymerase chain reaction analysis

3' Universal primer	C-0 CCT GGT TTT CCA ATA CCC GAA
5' Primers	C-1 ATG CGA GGG TCT GGA CGG CTG
	C-2 TCC TGC CTC GAG AAG GGC AGG
	C-3 AGC CGG TTT TCG GGG CTT TAT
	C-4 AAG AGC CGG GCG AGC AGA GCT

Table 2 Amplified fragment size of exon 1 c-myc product

5' Amplimers	Amplified region	Size (use with universal 3' amplimer C-0) (bp)
C-1	2303–2882	580
C-2	2388–2882	495
C-3	2466–2882	417
C-4	2550–2882	333

other neoplasms. This region is bordered by the two promoter sequences P1 and P2 (fig 1).

To eliminate fixation effects of formaldehyde, human placental DNA fixed in neutral buffered formaldehyde was included in all assays. The samples had previously been examined with pyruvate dehydrogenase and β globin gene with no evidence of genome wide instability.

To test the validity of the exon-1 assay, we used COLO 320 cells (ATCC), which contain normal and truncated exon-1 genes,¹⁸ and Raji cells (ATCC) which contain three deletions in the intervening sequence between exons 1 and 2 and 25 base pair changes in exon 2 as compared to published human c-myc sequences.¹⁵ However, the deletions and point mutations described in Raji cells are not within the region amplified by outer primers C-0 and C-1.

The PCR solution consisted of PCR buffer (50 mM KCl, 10 mM Tris HCl pH 8.3, 1.5 mM MgCl₂, 0.01% gelatine, 200 μ M of each dNTP, 1.0 μ M of each primer (table 1), 2.5 units of AmpliTaq DNA polymerase, and 100 ng of DNA template). Samples were then subjected to 40 cycles of PCR in a Perkin Elmer 480 DNA thermocycler. Cycling parameters were as follows: 94°C for two minutes, followed by 94°C for one minute, 62°C for one minute, 72°C for one minute $\times$ 40 cycles, with a final extension set for 72°C for five minutes. The reaction was performed on two geographical areas selected and in triplicate on each sample to exclude PCR generated artefact.

Twenty microlitres of the reaction product were electrophoresed on a 2% agarose gel containing 0.5 μ g/ml ethidium bromide and visualised under ultraviolet transillumination, yielding product sizes as in table 2.

SEQUENCE ANALYSIS OF EXON-1 USING CYCLE SEQUENCING

Cycle sequencing using Taq FS has been found to provide the best results when symmetrical PCR templates are used. When using symmetrical PCR products, a purification step is needed. In this study the Qiagen QIAquick protocol system (Qiagen, West Sussex, UK) was used to purify PCR product according to the following protocol.

The amplified DNA fragment was excised from the gel with a clean sharp scalpel. The gel

slice was weighed in a 1.5 ml Eppendorf tube. Approximately 100 mg of gel (100 μ l equivalent gel volume) was used for each purification. Three volumes of buffer QX1 were added to the gel slice. The mix was incubated at 50°C for 10 minutes. To aid dissolution of the slice, the tube was inverted and gently flicked, every two to three minutes during incubation. One gel volume (100 μ l) of isopropanol was added to the mix and the pH of the solution checked using a pH strip. If pH was > 7.5, then 10 μ l of 3 M sodium acetate, pH 5.0, were added to the mix and the pH rechecked. (The technique only works when the pH of the solution is < 7.5.) A QIAquick column was placed in a 2 ml collection tube and loaded with the sample and centrifuged at 13 000 rpm for one minute. The flow through was discarded and the QIAquick column was replaced in the same collection tube; 0.5 ml of buffer QIAquick was added to the column and centrifuged at 13,000 rpm for one minute. The column was then washed with 0.75 ml of buffer PE (Qiagen kit) and centrifuged at 13 000 rpm for one minute. The flow-through was discarded and the column was again spun for an additional one minute at 13 000 rpm. The QIAquick column was then placed in a clean 1.5 ml microfuge tube and then 30 μ l of 10 mM Tris-HCl pH 8.8, or HPLC water was added to the centre of the column and centrifuged at 13 000 rpm for one minute. The eluted DNA as then stored at -20°C. For sequencing reactions, 50–100 ng of purified product were taken.

A reaction premix was prepared as follows; 5 $\times$ sequencing buffer, dNTP mix, A dye terminator, C dye terminator, G dye terminator, T dye terminator, and AmpliTaq DNA polymerase FS (10 units). The amount of terminator added to the pre-mix was adjusted to give an optimal distribution of signal between 10 and 700 bases. If a stronger signal was required at the beginning of the sequencing run, then the amount of terminator was adjusted upwards to 0.6 μ l per reaction.

Positive and negative strand sequencing was performed on selected cases. Cycle sequencing was performed using 3.2 pmol of the positive or negative strand primer in the Gene Amp PCR system 9600 (Perkin Elmer) and the following thermocycling profile was adopted: rapid thermal ramp to 96°C, 96°C for 10 seconds, rapid thermal ramp to 50°C, 50°C for five seconds, rapid thermal ramp to 60°C, then 60°C for four minutes. The above thermocycling profile was repeated for 25 cycles. At end of thermocycling, the solution was rapidly cooled to 4°C and held at that temperature. Extension products were then purified as follows:

In a 1.5 ml Eppendorf tube, 2 μ l of 3 M sodium acetate pH 4.6, and 50 μ l of 95% ethanol were added. The entire cycle sequencing solution was then transferred to the 1.5 ml Eppendorf tube. The mix was vortexed and the tube placed on ice for 10 minutes. The tube was then spun at maximum speed in a bench microfuge for 15 to 30 minutes. The ethanol solution was carefully aspirated for the tube. The pellet at the bottom of the tube was rinsed

Table 3 Cellular localisation of c-myc protein product p62, using Mycl-9E10

	Predominant staining pattern		
	N>C	N=C	C>N
Squamous cell carcinoma of the cervix	4	9	44
Normal cervix (margin of tumour resected)	8	5	8

N>C, nuclear staining exclusively present in 90%+ of positively staining cells.
C>N, cytoplasmic staining present in 90%+ of positively staining cells.
C=N, areas of exclusively nuclear and cytoplasmic staining, both of which are present in different areas of the section, but neither pattern accounting for 90% or more of the staining pattern.

by the addition of 250 µl of 75% ethanol. The ethanol solution was aspirated off without disturbing the cell pellet. The pellet was then dried in a vacuum centrifuge and resuspended in 5 µl of water.

The ABI PRISM 377 (Perkin Elmer) was used for identification and resolution of sequenced product. A loading buffer containing deionised formamide and 25 mM EDTA pH 8.0, containing 50 mg/ml blue dextran in a ratio of 5:1 formamide to EDTA/blue dextran was prepared.

Samples were then spun in a microfuge and vortexed briefly, after which they were heated to 90°C for two minutes to ensure complete denaturation. The samples were then loaded

using 1.5 µl approximately of the reaction solution and loading buffer as above. In all cases, a 48 cm WTR 4.25% T 19:1 acrylamide:bis-acrylamide gel format was used, giving resolution up to 800 bases. Samples were run over a 12 hour period and then analysed using sequence navigator software on the ABI PRISM 377 DNA sequencer. Automated sequence comparison for polymorphism analysis was achieved using the ABI sequence navigator and auto-assembled on the ABI PRISM 377 DNA sequencer.

IMMUNOHISTOCHEMICAL DEMONSTRATION OF THE C-MYC ONCOGENE PRODUCT (P62) IN CERVICAL SQUAMOUS CELL CARCINOMAS

Fifty seven squamous cell carcinomas of the cervix were examined for the expression of c-myc protein product p62. In 21 cases, adjacent histologically normal ectocervical mucosa was available for examination, which serves as a control for the immunocytochemical staining (table 3). Two 5 µm sections were cut onto APES coated slides. Sections were dewaxed in xylene and washed in absolute alcohol. Endogenous peroxidase activity was quenched by incubation in 3% H₂O₂ in methanol for 15 minutes. The sections were then rehydrated through graded alcohols to Tris buffered saline (TBS, pH 7.6). Sections were washed for five minutes and were then incubated in 10% normal rabbit serum (NRS) followed by incubation in the mouse monoclonal antibody Mycl-9E10 at a dilution of 1:400. (This antibody is raised against a synthetic peptide of the C-terminus 408-432 of the human c-myc gene product and is a specific and sensitive probe for the protein on immunoblots.²⁵) Following incubation for one hour, sections were washed in TBS and treated again with NRS for 10 minutes. Sections were then incubated with biotin conjugated rabbit antimouse IgG (Dako, Bucks, UK) 1:400 in 10% NRS for 30 minutes. Sections were washed in TBS and then incubated for 30 minutes with Dako AB complex (which is freshly prepared). The sections were finally washed with TBS and the peroxidase reaction was developed by the application of DAB solution (1 mg/ml diaminobenzidine in 50 mM Tris-HCl pH 7.6, containing 10 mM imidazole, activated with 1 µl of 100 vol H₂O₂ immediately before use). The reaction was allowed to proceed for two minutes, after which it was stopped by washing in tap water. Sections were counterstained with Mayer's haematoxylin, dehydrated, cleared, and mounted.

Results

HPV PREVALENCE

Of the 40 cases examined for alterations in exon 1 c-myc, 35 were HPV positive (35 HPV 16; one HPV 18) and five negative for any HPV type by general primer HPV PCR. There was no discordance between HPV results with NISH and type specific PCR or general primer HPV PCR (data not shown).

Of the 57 cases examined by immunocytochemistry for p62 localisation, an additional 17 cases were added to the first study cohort

Figure 2 Two per cent agarose gel electrophoresis of c-myc exon 1 amplicons in cervical carcinoma, normal ectocervical margins, and fixed human placental DNA showing differential size patterns (see text for details).

Figure 3 (A) Area of control CIN 2 showing p62 staining using Mycl-9E10. antibody. (B) p62 cytoplasmic staining pattern in invasive cervical squamous cell carcinoma. (C) p62 nuclear staining pattern in invasive cervical squamous cell carcinoma.

above, including 16 HPV 16 positive and one HPV negative case.

ALTERATIONS IN EXON 1 OF C-MYC

We found alterations in exon 1 of the *c-myc* oncogene in 7.5% (three of 40 cases); all three cases were HPV 16 positive. The non-HPV cases showed no alterations.

No alterations were seen in formalin fixed human placental DNA, which therefore excludes a "fixative related" phenomenon, and no genomic instability was seen using β globin or PDH primers. The duplicate geographical samples from each case showed the same alteration, indicating the "clonality" of the observation. Control adjacent cervix in all three case had germline DNA configuration. The triplicate PCR results performed on all samples were concordant.

Figure 2 shows the alterations in some of the samples examined. Normal exon-1 *c-myc* yields amplifiable bands of 580, 495, 417, and 333 base pairs with the primers selected (as detailed in tables 1 and 2). The formation of smaller fragments than predicted suggests that deletions have occurred upstream of the targeted amplification site. Background faint bands (of the normal predicted size) represent the normal background cell population (for example, fibroblasts, inflammatory cells, and so on).

Sample A showed loss of the normal amplicon in lane 3. However, a background amplicon was also visible in lane 3 from background cells (fibroblasts, inflammatory cells, etc) Smaller amplicons than the predicted sizes were seen in lanes 1 and 2 of sample A. This suggests that deletions had occurred in the region amplified by C-0 and C-3 as detailed earlier. Sequence analysis showed a deletion of 62 base pairs, involving the C-3 priming site.

Sample B demonstrated loss of the normal amplicon in lane 2. Smaller amplicons in lane 1 were then found. This again suggests that a deletion had occurred within the region amplified by C-0 and C-2. Sequence analysis showed an 80 base pair deletion involving the C-2 priming site.

Sample C showed amplification of adjacent normal cervix in sample B, with the formation of normal predicted size amplicons.

In sample D (one of the replicates of sample B), the normal amplicon was missing in lane 2. A smaller amplicon than predicted was found in lane 1. A faint background normal amplicon was also seen in lane 1. This is due to amplification of background cells as described for sample A.

Sample E showed complete amplification of neutral buffered formaldehyde fixed human placental DNA. Similar results were obtained with COLO 320 and Raji cells.

The other case showing an abnormality of exon 1 by PCR did not give convincing evidence of deletion on direct sequencing, because of the high proportion of normal contaminating cells in the biopsy. Tumours with a normal exon-1 PCR pattern revealed the germline *c-myc* sequence on positive and negative strand sequencing ($n = 4$).

PATTERN OF EXPRESSION OF P62 IN CERVICAL SQUAMOUS CARCINOMAS

Table 3 shows the pattern of staining obtained with the monoclonal antibody Mycl-9E10. The predominant staining pattern achieved was cytoplasmic in the tumour samples examined, with nuclear staining seen in some cases (fig 3). In the normal cervical margin, nuclear staining predominated, which is in keeping with the pattern of staining in normal differentiated squamous epithelium from cervix and other sites. The localisation of *c-myc* product p62 in the cytoplasm of malignant squamous epithelial cells is statistically significant ($p < 0.001$, χ^2 analysis with Yates correction).

Discussion

The *c-myc* gene is apparently set up to be rapidly modulated, since both the corresponding m-RNA and protein have a short half life. Therefore any decreases in *c-myc* m-RNA concentration would naturally be followed rapidly by a loss of *c-myc* protein. Overproduction of *c-myc* messenger RNA can result from gene

amplification. However, it is unlikely that c-myc gene activation is achieved only through this mechanism.

Recently, it has been suggested that c-myc exon 1 sequences play an important role in either transcriptional control or in post-transcriptional mRNA degradation.¹⁸⁻²⁶ It is known that c-myc rearrangements can lead to accumulation of c-myc transcripts. While in normal cells it might be necessary to provide external growth stimulation to induce c-myc mRNA appearance after each round of cell division, the accumulation of c-myc messenger RNA and subsequently the c-myc protein by deletions, mutations, or single base changes, may effectively stimulate tumour cells to continuous growth. This fact is borne out in the findings that c-myc has been found rearranged and amplified in most tumours, but not in normal tissues, and it therefore seems clear that alternations of this gene may be important in the genesis or progression of carcinomas in the uterine cervix, as pointed out by Ocadiz and colleagues.²⁶ Gariglio *et al*¹⁴ have proposed that human c-myc gene expression is regulated by a post-transcriptional mechanism, and comment on substantial alterations in the sequence of the first two exons (both single base pairs and small deletions) in Raji cells. Changes in exon 2 can theoretically result in altered c-myc protein product, whereas changes in the first exon (single base pair changes or small deletions) can result in either c-myc overexpression or higher stability of its messenger RNA.

In this paper, we demonstrate alterations in exon-1 of c-myc in a minority of squamous cell carcinomas of the cervix. Other changes at different sites within exon 1 and 2 of c-myc have been described in COLO 320 cells and Raji cells. The alterations described in this paper are due to small deletions within exon-1, but point polymorphisms occurring within the priming sites could not be excluded in the third case. The findings are concordant with those of Ocadiz *et al*²⁶ and Huang *et al*²⁷ who have previously described alterations in c-myc in cervical carcinoma. To our knowledge, this is the first report in relation to exon 1, however. Importantly, the alterations uncovered appeared "clonal" as replicate samples showed the same amplicon band pattern. PCR replicative error has been excluded in this experiment (see Methods). In addition, the changes appear tumour specific, as no alterations were found in the adjacent normal cervix in these cases. All cases which showed alterations were HPV positive, although drawing a link between HPV and such alterations is highly speculative. Previously, the integration sites of HPV in relation to c-myc and N-myc have been examined.²⁸ All cases which show exon-1 changes had strong nuclear positivity on p62 immunocytochemistry, suggesting that such alterations may provide an alternative route to c-myc activation in early squamous cell carcinoma.

Secondly, we have examined the immunohistochemical localisation of c-myc protein product p62 using the monoclonal antibody Mycl-9E10. Increased expression of the cellular

oncogene c-myc has been described in a variety of human tumours. The gene encodes a polypeptide with predicated molecular weight of 49 kDa, but which shows aberrant electrophoretic mobility on polyacrylamide gel electrophoresis to give an apparent molecular weight of around 62 kDa (p62 c-myc). Biochemical and immunofluorescence studies of cell lines in vitro have indicated that the protein is normally located in the cell nucleus. Its biological half life is approximately 15 to 20 minutes. Although p62 c-myc in vitro binds non-specifically to DNA, in vivo the greater proportion of the protein appears to remain unbound, having a rather weak association with the nuclear matrix.²⁵ During mitosis, p62 becomes dispersed throughout the cytoplasm, having no association with the condensed chromosomes. Apart from this situation, very little p62 is normally found in cytoplasm.

The monoclonal antibody Mycl-9E10 used in this study was raised against a synthetic peptide of residues 408-432 of p62 c-myc and has been shown by its originators to recognise an epitope of the human c-myc protein p62.²⁵ It has been proved to be sensitive in immunoblotting experiments. However, immunohistochemical studies with monoclonal Mycl-9E10 and its closely related Mycl-6E10 have produced conflicting results when used with formalin fixed tissues. Mycl-6E10 appears to recognise a protein present in both the nucleus and the cytoplasm. Suggestions for this unexpected distribution of p62 have included poor tissue fixation condition or extraction of p62 by salt or low temperatures. Alternatively, it is possible that this antibody may cross react with an unrelated antigen in the cell.²⁹

In this study, the cellular localisation of c-myc protein was examined in 57 cases of squamous cell carcinoma of the cervix (including the cases examined for exon-1 alterations). The predominant staining pattern was cytoplasmic in 44 cases (table 3, fig 3). Equal nuclear and cellular staining was seen in nine cases, which consisted of areas of exclusively nuclear and cytoplasmic staining, both of which were present in different areas of the section, neither pattern accounting for 90% or more of the staining pattern. Greater nuclear than cellular staining was seen in four cases. To serve as an internal control, the normal cervical margin in some of the excised squamous carcinomas (where available) was used to assess the predominant staining pattern in these regions. The pattern of results in the normal cervical margin reflects those in normal tissues, where there is a definite correlation between p62 nuclear localisation and normally proliferating cells. Interestingly, Hughes *et al*²⁹ showed higher c-myc positivity in normal epithelium (63%) than in specimens with cervical intraepithelial neoplasia grades 2 or 3 (20%) or invasive carcinoma (0%), as assessed by flow cytometry. The finding of p62 localisation in normal relative to dysplastic epithelium may be a reflection of increased turnover of protein in dysplastic cells. Pulacz *et al*³⁰ have previously shown pancellular c-myc expression in endocervical mucosa in

90% of glandular intraepithelial neoplasia and in 100% of adenocarcinomas.

The results of our study again emphasise that fixation procedures in immunolocalisation of the c-myc product must be taken into account when analysing results. There appears to be a shift from the normal nuclear localisation of p62 to the cytoplasmic compartment in the malignant squamous ectocervical cell. The precise nuclear association of p62 in carcinomas may be more labile because of pH changes within the malignant cell, ATP concentration, or release of proteolytic enzymes or other undefined cellular/nuclear factors. One possible explanation for this cytoplasmic shift phenomenon may be a biologically significant difference representing accumulation of newly synthesised p62 in the cytoplasm of these malignant cells, or a relative or absolute deficiency of an undefined nuclear transport mechanism for p62. In addition, the newly formed protein may be unable to bind in the nucleus or its degradation or inactivation may be accentuated.

CONCLUSIONS

In summary, we have shown alterations in exon 1 of c-myc in a minority of cervical cancers, and increased expression of p62 in a cohort of HPV positive and negative cervical squamous cell carcinomas. Alterations in exon 1 may provide the malignant cell with an additional route to activation/stabilisation of c-myc, independent of amplification. Larger studies are now under way to determine the precise prevalence of such alterations and whether similar phenomena occur in exon 2.

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