Expression of the *c-myc* oncogene and the presence of HPV 18: possible surrogate markers for cervical cancer?

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ABSTRACT

The study aims to evaluate the cause of cervical cancer in a cohort of patients and to establish whether or not human papillomavirus (HPV) is the leading risk factor and to determine whether or not *c-myc* oncogene over-expression is a predicative marker for the disease. Cone biopsy samples are examined from 53 patients diagnosed with either adenocarcinoma or squamous cell carcinoma of the cervix. Results showed that 19% of the patients studied were positive for high-grade HPV 18 DNA by polymerase chain reaction (PCR). For the *c-myc* gene expression, only three (23%) of the 13 control slides were positive. Of 49 known cervical cancer patients examined, 41% were positive, 51% were negative and 8% were doubtful. Of those who were positive for HPV, only two were positive for a mutation in the *c-myc* gene and one slide gave a doubtful result. *P* value for hysterectomy patients was 0.23 and for cancer patients was 0.48. In the cervical cancer patients studied, the HPV 18 prevalence rate was very low compared to that found in other studies. Therefore, the presence of HPV and expression of the *c-myc* oncogene cannot be used as surrogate markers for cervical cancer.

KEY WORDS: Genes, myc.
Human papillomavirus.
Immunohistochemistry.
Uterine cervical neoplasms.

Introduction

Cervical cancer is one of the three major causes of malignancy in women worldwide[1–3] and about half a million new cases are diagnosed each year.[4] The incidence of cervical cancer varies worldwide, but approximately 25% of cases occur in India.[5] The progression of a benign infection to malignancy of the cervix is exacerbated by various factors such as life style and number of sexual partners.

Transmission of human papillomavirus (HPV) and the presence of genital warts have long been associated with sexual activity. In 1954, Barret, Silber and McGinley confirmed the sexual transmission of HPV. In 1976, two new morphological forms of cervical HPV were described and these flat, endophytic (inverted) condylomatous lesions have since been associated with cervical intraepithelial neoplasia (CIN) and invasive squamous cell carcinoma.

Initiation of the process leading to cervical carcinogenesis occurs when HPV DNA integrates into the host cellular genome. At this point, there is disruption in the early HPV genes. The *E2* gene responsible for replication is down-regulated, leading to high-level expression of the *E6* and *E7* genes,[6] resulting in genomic instability.

There is strong epidemiological evidence to support the theory that high-risk strains of HPV (i.e., types 16, 18, 31 and 33) act as carcinogens in cervical tissue.[7] There are also many possible gene mutations, hormonal imbalances and infections that may go undetected.

Although HPV infections lead to specific humoral and cell-mediated immune responses, the presence of virions will remain undetected unless found in the mucous membrane lining the cervix. To date, however, data on the influence of immunoregulatory mechanisms on the clinical course and outcome of infection are scanty.

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Although HPV infection is common in teenagers and older women, infection rate is highest in those between the ages of 18 and 30.[8]

According to a global study conducted in 2005 by Clifford and co-workers,[9] HPV type distribution varied in different regions, with European women significantly more likely to be infected with HPV16 than those in sub-Saharan Africa. Women from South America showed HPV-type distribution that fell between that seen in sub-Saharan Africa and Europe. Heterogeneity between areas of Asia was significant.

Although the role of HPV has been demonstrated experimentally and clinically to be pivotal in carcinogenesis, the process is multi-step and involves changes in cytogenetic equilibrium (e.g., chromosomal imbalance, allelic loss and structural aberrations).[10]

The *c-myc* oncogene found on chromosome 8 at locus 8q24 is responsible for cell-cycle regulation, and its down-regulation can lead to programmed cell death through apoptosis.[11] Up-regulation in the *c-myc* region has been

observed in several cancers and is thought to be a predicator for cervical cancer. Expression level is usually low in normal growing cells, but may be increased in a variety of cancers including cervical cancer. However, integration of high-risk HPV DNA into the cell genome is probably the most crucial event for tumourigenesis.[12]

This study seeks to explore the possibility that *c-myc* oncogene expression and HPV 18 are surrogate markers for cervical cancer.

Material and methods

Tissue sections were obtained from 53 cervical cancer patients and 13 hysterectomy patients after ethics approval was given by the government of Mauritius. Samples were from all the patients diagnosed with cervical cancer over a 12-month period. The tumours were classified according to the International Federation of Gynecology and Obstetrics FIGO classification. Patients who had undergone abdominal hysterectomy for irregular bleeding not associated with any form of malignancy provided the negative control material. Sections were cut at 5 µm and had an approximate surface area of 1 cm^2

DNA extraction from wax-embedded sections

Sections were dewaxed and brought to water. The tissue was gently detached from the slide and transferred to a 1.5 mL Eppendorf tube containing 0.75 mL lysis buffer (0.5% SDS, 0.15 mol/L NaCl, 2 mmol/L EDTA, 10 mmol/L Tris HCl [pH 7.8])[13] to which was added 10 µL proteinase K (20 mg/mL). DNA was extracted by an in-house method.[14] HeLa 229 cells were used as a positive control for HPV.

MY09/MY11 oligonucleotide primers[15] were used to detect high-grade HPV. Positive strand primer (MY 11) 5′ GCM CAG GGW CAT AAY AAT GG 3′, negative strand primer (MY09) 5′ CGT CCM ARR GGA WAC TGA TC 3′, where M=A+C, R=A+G, W=A+T and Y=C+T. Polymerase chain reaction (PCR) conditions were as follows: initial denaturing at 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 5 min. The resulting amplicons were run on a 1% agarose gel submerged in 1xTBE buffer for 1 h at 100 V. A 100 bp DNA ladder was used to size the amplicons (approximately 500 bp). The positive amplicons were sequenced and their identity found using BLAST search tools.

Immunohistochemical detection of **c-myc** *oncogenic expression*

Immunohistochemistry was performed to detect those samples over-expressing the product of the *c-myc* oncogene. The method used was modified from that described by Vijayalakshmi and colleagues.[16] Briefly, tissue sections were dewaxed and brought to water, washed thoroughly in distilled water and drained. The slides were transferred to a moist chamber, flooded with 3% hydrogen peroxide for 1 h to block endogenous peroxidase and washed thoroughly with distilled water. The sections were then blocked with 2% bovine serum albumin (BSA) for 1 h, washed and then flooded with anti-myc antibody mixed with a 1 in 5000 dilution of anti-mouse antibodies (Bio-Rad) and 2% BSA. The sections were incubated in a moist chamber at room temperature overnight. The sections were then washed and the colour was developed using an AEC immunohistochemical staining kit (Sigma) for 15 min. Following a brief wash, the slides were blotted and then viewed under oil immersion (x100) for evidence of over-expression of the *c-myc* gene.

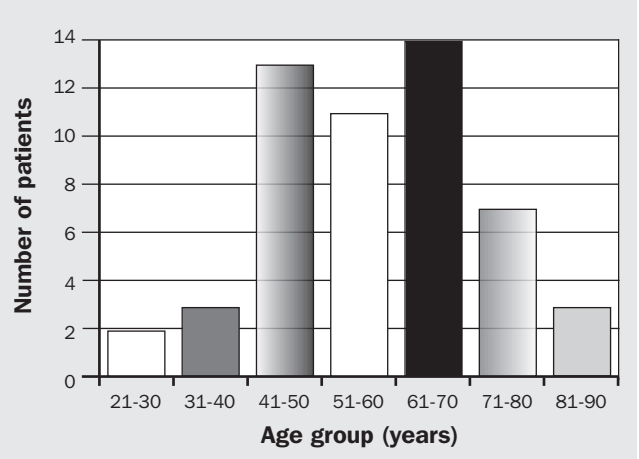

Fig. 1. Incidence of cervical cancer in different age groups.

Results

Figure 1 shows the incidence of cervical cancer in different age groups. The age of members of the cohort ranged from 28 years to 86 years (mean: 56.7 years) and they consisted of an ethnic mix with different cultural and religious backgrounds.

Figure 2 shows the results of high-grade HPV PCR. The positive amplicons were then sequenced. The nucleotide sequence data obtained for each amplicon were checked for homology using BLAST (www.ncbi.nlm.nih.gov/BLAST).[17] The sequences have been lodged in GenBank (www.ncbi.nlm.nih.gov/Genbank/index.html) with the accession numbers DQ059009, DQ059013 and DQ058830.

Bioinformatics data from all positive cases gave very high expected (E) values for HPV 18. Ten (19%) samples were positive for HPV 18 DNA using the MY09/MY11 primers. The youngest patient was 42 and the eldest was 80 years old. Mean age for HPV-positive patients was 58.7 years.

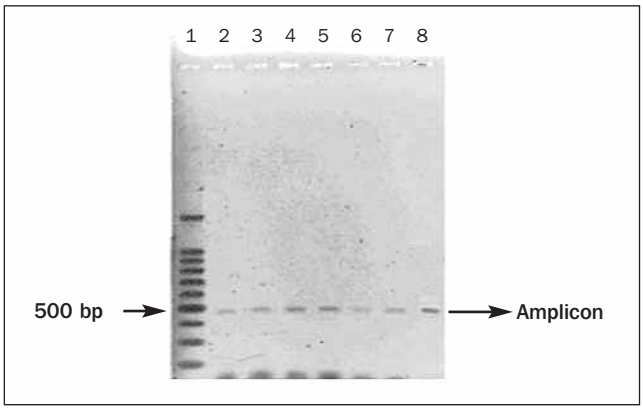

Fig. 2. High-grade HPV PCR. Lane 1 shows a 100-bp DNA marker with 500 bp highlighted. Positive amplicons (lanes 2–8) gave bands of approximately 500 bp.

c-myc *mutation detection*

A total of 62 sections were examined, 13 of which came from hysterectomy specimens. Only 49 samples from cervical cancer patients were used because four were insufficient for analysis. Negative samples were introduced blindly to prevent bias while examining the sections.

Of the 13 sections from hysterectomy patients, only three (23%) were positive and one (8%) was doubtful. Of the 49 known cervical cancer patients examined, 25 (51%) were negative, four (8%) were doubtful and 20 (41%) were positive.

On comparing those that were positive for HPV, only two were positive for mutation in the *c-myc* gene and one slide gave a doubtful result.

Testing the Null Hypothesis gave a Z value of 0.15, which, as it exceeds zero, implies that *c-myc* expression cannot be used as a predictive marker in cervical cancer patients.

Discussion

In the cohort studied here, only two patients were positive for both HPV 18 infection and the *c-myc* mutation. Similar findings were reported by Ngan *et al*.[18] and Kersemaekers *et al*.[19] who reported that over-expression of *c-myc* had no correlation with HPV detection and stage. Similar findings from another study[20] produced data to indicate that while *c-myc* amplification was a predicator of uterine cervical lesions, there was no relationship between *c-myc* status and HPV infection. Acting as a predictor of cervical lesions, it is perhaps not unusual to find a high level (23%) of *c-myc* expression in hysterectomy patients, most of whom undergo surgery to treat bleeding disorders.

Oncogenic activity for K-ras and C-myc proteins has been associated with many malignant and premalignant conditions.[21] When down-regulated, C-myc protein, which is responsible for cell-cycle regulation, can lead to programmed cell death through apoptosis.[11] Mutation in the *c-myc* oncogene can be used as predictor for cervical cancer. However, using *c-myc* as the sole marker for the diagnosis of cervical cancer can be controversial.

The high level of mutations seen in the hysterectomy patients may be attributed to other underlying conditions associated with cell death. One possible cause is hypoxia as a consequence of fibroid disease in patients undergoing hysterectomy. Similar findings were reported by Brenna *et al*.,[11] who reported an incidence of 40% in their cohort; however, the incidence of mutations can vary. Zhang *et al*.[22] reported an incidence of 25% in their study.

Progression of CIN to cervical cancer is seen in 65% of gynaecological cancer patients diagnosed in Mauritius. Cervical cancer is the second most prevalent cancer with an incidence of 20.7 per 100,000, the most prevalent being breast cancer with an incidence of 23 per 100,000.[23]

Cancer progression has been reported to vary in different ethnic groups. Manraj *et al*.[24] reported an incidence of 18.8 per 100,000 for Hindus, 17.8 per 100,000 for Muslims, 8.6 per 100,000 for Chinese and 24 per 100,000 in the general populations (i.e. those not covered by the other three groups). In the same year, the Office for National Statistics in the UK published the incidence of cervical cancer as 9.3 cases per 100,000. The number of new cases documented in the UK that year was 2740, which represented a fall of 26% over the previous five years. This fall was attributed to the effect of a screening programme designed to identify and treat CIN before it developed into cervical cancer.

The sequence data generated from the PCR amplicons in the present study showed the presence of HPV 18 alone, a serotype that has been observed in invasive cervical cancer.[25] This suggests that serotype 18 is the prevalent HPV strain in Mauritius.

The primers (MY09/MY11) used in this study to target the *L1* gene in HPV have been cited extensively in the literature and have been used successfully to amplify DNA sequences from HPV 16 and HPV 33. However, in the present study, the only positive control used was HPV18 from HeLa cells (10–50 copies/cell).[26] As controls for HPV 16 and 33 could not be obtained, it is impossible to comment on the possibility that serotypes 16 and 33 would have amplified if present.

Furthermore, it has been suggested that it is difficult to amplify degraded DNA using MY09 and MY11.[27] However, given the widespread use of these primers and the positive results obtained for one serotype, it would seem unlikely that the other HPV types would have gone undetected. The solution would be to test these primers against DNA extracted from CaSki cervical cells that harbour HPV 16 (500 copies/cell).[28]

Karaloglu *et al*.[25] used the same set of primers and were able to amplify HPV 16 DNA. However, they extracted DNA from cervical cells collected from the transition zone of the cervix and the ectocervix using a cervical brush. Brewer *et al*.,[29] using the consensus primers MY09 and MY11, reported the presence of HPV 16 in 58% of their cohort. Their extraction method was similar to that used in the present study, whereby tissue was digested with proteinase K, and they used phenol for DNA extraction. However, the tissue used had been frozen at –70°C rather than processed to paraffin wax.

Sepp *et al*.[30] and Frank, Svoboda-Newman and Hsi[31] evaluated different extraction methods including proteinase K digestion for use on routinely fixed, paraffin wax-embedded tissue and concluded that this method produced suitable DNA templates from a wide range of paraffin wax-embedded tissues. Frank, Svoboda-Newman and Hsi[31] concluded that DNA isolated using proteinase K was useful for identifying viruses, immunoglobulin gene rearrangements and checking for heterozygosity in paraffin wax-embedded tissues without the use of radioisotopes. However, they faced some setbacks using the detergent SDS for cell lysis. They reported that SDS interfered with PCR although they had not included a phenol-chloroform extraction step. In the present study, which used SDS, the DNA was extracted twice with phenol-chloroform and once with chloroform before being precipitated in two volumes of ethanol. Hence, the effect of SDS would have been minimal.

Other possible reasons for detecting a low level of HPV in the cohort may be errors in processing the tissues. Karlsen *et al*.[27] reported difficulties in obtaining PCR products above 200 bp from cervical cancer specimens, even after re-amplification for an additional 35 cycles.

The MY09 and MY11 consensus primers amplify the *L1* region, producing amplicons of approximately 500 bp. This is considered to be the limit from formalin-fixed paraffin sections.[31] De Roda Husman *et al*.[32] used a different pair of consensus primers to target the *L1* gene in HPV and reported a PCR product size of approximately 145 bp.

Elsewhere, Tieben *et al*.[33] described a second set of primers (CP-1/CP-2) to target the E1 open reading frame (ORF) and produced an amplicon of 188 bp.

When Rolighed, Bichel and Lindeberg[34] compared two sets of consensus primers, using DNA from formalin-fixed tissue, they found that GP5+ and GP6+ (a set of consensus primers that detects the *E1* gene in HPV) gave better correlation with the MY09/MY11 primer set. However, the consensus primers designed to detect the *E1* ORF failed to amplify HPV 16 in 10% of samples. It is possible that use of the GP5+ and GP6+ primers would have resulted in better HPV DNA detection, but changing from MY09/MY11 would have made a difference if the quality of the DNA had been inferior.

Furthermore, deletion of the *L1* region in HPV is more than a remote possibility.[35] As the consensus primers MY09 and MY11 are directed against this region, the outcome would be highly suggestive of a negative PCR result. No observable signals were obtained and the possible explanation given was fragmentation of DNA or inhibition of the polymerase due to modification of nucleic acids. However, other authors have amplified DNA from 40-year-old archival samples,[36] although some DNA degradation was reported due to DNase activity. Iwamoto *et al*.[37] were able to obtain PCR products from their fixed samples. According to these authors the problem was not the time the tissue spent in paraffin wax, but the type of tissue, the method of tissue procurement or the time spent in fixative.

Based on results from the present study, only HPV 18 has been detected in Mauritius and only 19% of patients with gynaecological cancers are infected. Clearly, further studies must be undertaken using alternative testing methods to verify these findings. Munoz *et al*.[38] pooled data from 11 case control studies involving 1918 women with histologically confirmed squamous cell cervical carcinoma and a control cohort of 1928 women. Contrary to the present findings, they detected HPV DNA in 90.7% of their cohort with cervical cancer and in 13.4% of the controls. They used MY09/11 and GP5+/6+ primers and found that MY09/11 were less sensitive than GP5+/6+ in detecting HPV (63.4% in patients and 19.1 % in controls, and 96.9% in patients and 15.6% in controls, respectively). Using both sets of primers they were able to detect HPV 16 as the main culprit in cervical cancer (overall prevalence: 58.9%) followed by HPV 18 (overall prevalence: 15%). However, these studies were carried out in different countries.

The MY09/11 set of consensus primers was used in studies in Spain and Columbia, while the GP5+/6+ set was used in Mali and Brazil. Interestingly, in Mali, 96.9% of the cervical cancer patients and 33% of the controls were positive for HPV. As many Afro-Mauritians who constitute the general population come from the same geographical area as Mali, this high prevalence of HPV in Mali to some extent supports the finding of a high incidence of cervical cancer in the general population in Mauritius.

Another interesting finding by Munoz *et al*.[38] was that the incidence of HPV 16 declined with age. Interestingly, most of the cervical cancer samples in the cohort in the present study were from people whose average age was 56 years. Although it is unlikely that HPV 16 was absent, this finding could explain the failure to amplify HPV 16 DNA. As the present findings are not dissimilar to those of Munoz and co-workers,[38] and the primers used in this study have previously amplified HPV 16 DNA, it is not possible to confirm that HPV 16 was absent due to lack of positive controls for HPV 16 and 33 DNA.

Harington's data[39] suggest a high frequency (40–50%) of cervical cancer in several populations in southern Africa. As the general population of Mauritius (i.e. not Hindu, Muslim or Chinese) originate from Africa, a higher incidence in this community would be expected.

Over-expression of the *c-myc* oncogene was not detected only in tissues obtained from cervical cancer patients. The up-regulation of this gene, found on chromosome 8 at locus 8q24, is multifactorial. The gene has three exons encoding a 62 kDa nuclear protein that has a transcriptional factor with a half-life of a few minutes. Mutations in this locus have been brought about by the integration of HPV 16 DNA which acts as a potential mediator in the process of cervical carcinogenesis. If mutations in the 8q24 region caused by HPV 16 results in the up-regulation and expression of the C-myc protein, it is unclear why the cohort studied here had such a high level of positive results for *c-myc* expression, given the fact that only 19% of the patients had HPV, with HPV 18 the only serotype seen.

It is also unclear why a proportion of the samples from normal cervical tissue gave a positive immunohistochemistry results when treated with 9E10 monoclonal antibody. Mutations caused by HPV 18 integrating the 8q24 locus are not the sole cause of carcinogenesis and it would be difficult to ignore the role of p53 complexing with the HPV E6 oncoprotein. □

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