Original Article

DNA content can improve the detection and prognosis of carcinoma of the cervix

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Summary **In all malignant diseases, several clinical and histopathological tests are established as standard methods for diagnosis. Alternative diagnostic quantitative methods are still lacking or conflicting in cancer of the cervix. Although DNA analysis was one of the earliest applications in flow cytometry and the DNA content of fresh/frozen tissue have shown good prognostic results in many diseases including cervical cancer, prognostic ability cannot be easily clarified quantitatively by biochemical values. Thus, our objective was to determine whether the addition of a DNA content study using flow cytometry improves the detection of cervix cancer. The quantification of DNA content was done by high resolution DNA flow cytometry in fresh/frozen tissues of healthy cervix (control, $n = 38$) and from cancer of the cervix ($n = 62$). Results show that the mean fraction of Total S phase, Total Aneuploid and G2-M (Diploid) are significantly higher ($p < 0.01$); while G0-G1 (Diploid) and G0-G1 (Aneuploid) are significantly lower ($p < 0.01$) in cancer patients as compared to control. Among cell cycle parameters, G0-G1 (Diploid) shows a classifying ability of 97% and at a criterion/threshold value of ≤ 79.88, it discriminates cases with the highest sensitivity of 96.77 (88.8-99.5; 95% CI) and specificity 100.0 (90.7-100.0; 95% CI) and with Total S phase or Aneuploid, it discriminates cases with 100% sensitivity. G0-G1 (Diploid) also showed a direct and significant correlation ($r = 0.66$; $p < 0.01$) with patient survival indicating prognostic significance. Cell cycle parameters will be very helpful in false positive results or where the clinician is unable to diagnose the stage of cervix cancer clinically or histopathologically.**

Keywords: DNA content, G0-G1 (Diploid), G0-G1 (Aneuploid), Total S phase, flow cytometry

1. Introduction

Despite the introduction of a number of organized and ad-hoc screening test programs, cervical cancer continues to remain a major source of mortality and morbidity for women in developing countries and is still the second most common cancer among women worldwide, especially in middle and low income countries. In India, about 130,000 new cases of cervix cancer occur every year and constitute one-fifth of the total global burden. The age-standardized incidence rate is 30.7 per 100,000 and age-standardized mortality rate is 17.4 per 100,000 in India, which is the highest in South Central Asia (*1*). In all malignant diseases, several clinical and histopathological tests are established as standard methods for diagnosis. Alternative quantitative methods of diagnosis are available in many other cancers but they are still lacking or conflicting in cervix cancer. Cytomorphologically (Pap test) abnormal epithelial cells of cervical smears are generally used for screening. Unfortunately, a substantial number of premalignant and malignant lesions of the uterine cervix have a history of normal cervical smears. The false negative rate of diagnosis varies considerably

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between 15 and 50%, while the number of false positive is about 10% (*2*). Several ACCP (The Alliance for Cervical Cancer Prevention) studies have also found Pap test sensitivity in the range of 50% at best (*3*). Thus, limitations of these cytological tests as moderate to low sensitivity and false-positive results leading to unnecessary patient anxiety and costs, limits the usefulness of Pap examination as a sensitive tool for early diagnosis (*2*). Beside early diagnosis, staging is another important feature in the management of cancer, as it guides the mode of treatment and adjuvant therapy thereafter. Staging itself is a very challenging task and there is a marked inter-examiner variability for the same patient as staged by different clinicians. However, the present day treatment decisions are being made solely on the FIGO stage of the patient. Despite the sophisticated diagnostic techniques, it is not always possible to predict the individual proliferative potential and tendency of recurrence among tumors within the same histological subtypes. Even patients with tumors confined only to the cervix *i.e.*, stage IA or B develop vault recurrence in as many as 20% cases after the various treatment modalities (*4*). New biological parameters are, therefore, needed for more accurate diagnosis requiring more aggressive and individualized treatment schedules.

Although various DNA flow cytometric studies have been performed on cervix cancer, the role of DNA ploidy and the S-phase fraction (SPF) in predicting biological tumor behavior finally remains unresolved. Although DNA analysis was one of the earliest applications of flow cytometry and the DNA ploidy content have shown prognostic ability in many cancer diseases such as cervical cancer (*5*), head and neck (*6*), ovarian (*7*), colon (*8*), and endometrial (*9*), but the prognostic ability cannot be easily clarified quantitatively in cervical cancer. The threshold values are provided but only for S phase and not for other cell cycle parameters. One main drawback of these studies was that the researchers were trying to identify diagnostic and prognostic indicators within an identical subgroup of cancer patients. Keeping the above discrepancy and controversy in mind, the present study was designed to determine the diagnostic accuracy of the disease by correlating pathological and clinical parameters with cell cycle parameters and evaluating them by correlating these with patient survival time. Thus, our objective was to determine whether the addition of a DNA content study using flow cytometry improves the detection of cervical cancer.

2. Material and Methods

2.1. *Patient selection and sample collection*

The present study was done in clinically diagnosed and histopathologically confirmed 62 cases of cervical carcinoma and 38 control cases (sample size, $n = 100$). The cases were selected from the patients registered in the Department of Radiotherapy and Gynecology, Chhatrapati Shahuji Maharaj Medical University, Lucknow during the years 2004-2006. Criteria for choosing patients with carcinoma of the cervix for inclusion in the study were previously untreated patients without any concurrent malignancy or chronic illness and with normal renal and liver function tests. The control population consisted of individuals undergoing hysterectomy for benign conditions. The age of the subjects varied from 22 to 76 years (mean age 47.12 ± 11.12 years). All procedures were performed in accordance with our hospital's ethical guidelines, and approval for the study was granted by the university hospital's ethics committee. The voluntary written consent was taken from all the subjects for participation in the study. A detailed history and clinical examination was undertaken for all subjects in the study. The samples for the suspected cervix cancer (malignant) cases were collected when the biopsy was taken to confirm diagnosis. The biopsies were guided by colposcopy and taken at the squamous-columnar junction. The samples for the control cases were collected when the hysterectomy was performed and was being sent for pathological examination. All the cervical biopsy samples were collected in phosphate buffered saline and cases were finally classified in different groups according to the FIGO staging (*10*).

2.2. *Histopathological evaluation*

Histopathological examinations were done on hematoxylin and eosin stained sections. All selected cases were positive for squamous cell carcinoma of the cervix except controls. Histopathological examination of cervix for controls exhibited a benign normal appearance. The squamous cell carcinoma (SCC) cases were classified on the basis of a modified Broder's classification (*11*) and graded according to the degree of differentiation as well, moderately and poorly differentiated. According to the findings of clinical and histopathological examinations of patients they were scored with dummy numbers from 0 to 6 along with a control according to the disease progression and/or severity of carcinoma. Out of 8 clinical sub-stages of cancer, this study involved only patients of 6 sub-stages because during the study period no cases of initial (IA) and last sub-stage (IVB) patients were found. 8 patients were found in IB group, 2 in IIA, 19 in IIB, 1 patient in IIIA group, 30 patients in IIIB group, and 2 patients in IVA group. The groups were graded as 1, 2, 3, 4, 5 and 6, respectively.

2.3. *Methodology for preparation of single cells*

Samples for flow cytometric evaluation were prepared

with some modifications of the method originally described by Vindelov *et al*. (*12*) and MEDLINE search (*13*) which was finally standardized in our laboratory. To standardize the preparation of single cell suspension, different concentrations of trypsin were tried**.** The tissue was minced with curved scissors, and transferred in five different tubes. Each tube was then treated with 1 mL of trypsin (3× purified, SRL) in 1 N HCl solution at concentrations of 0.05%, 0.10%, 0.25%, 1.0% and 2.5%, respectively. The tubes were incubated at 37°C and mixed intermittently for 10-15 min. Cells were then gently dispersed by triturating (triturating: cell dispersion through mild pumping action). The suspension was then filtered through fine mesh. Cells were then allowed to settle and excess liquid containing enzymes was decanted after centrifugation at 500 × g. The pellet was then washed 2-3 times. Finally, the single cell suspension was obtained by re-suspending cells in ice-cold PBS buffer (pH 7.4). After the acquisition of all these suspensions, the suspension with 2.5% concentration of trypsin was found to be the best for control tissues and 0.05% for cancerous tissues. Suspensions of all tissue samples were prepared as above using 2.5% and 0.05% concentrations of trypsin. Flow cytometric acquisition and analysis followed.

2.4. *Flow cytometric analysis*

A standard FACS Calibur (BD Biosciences, San Jose, CA, USA) equipped with a 488-nm argon laser and four-color filters was used to analyze the cellular DNA content using a standard protocol with the appropriate filter combinations for excitation and measurement of the fluorescence of propidium iodide with wavelength settings of 457 nm (FL1), 568 nm (FL2) and 645 nm (FL3) band-pass fluorescence filters and a 781 nm red diode laser with a 684 nm band-pass filter (FL4). A total of 10,000 events were analyzed by flow cytometry using an excitation wavelength set at 488 nm and its fluorescence was detected from 550 up to 670 nm. Cells present in single cell suspension of all the tissue samples were fixed with chilled ethanol for 30 min at 4°C. After adding propidium iodide (50 µg/mL), this mixture was stored in the dark for 1 h at room temperature before additional analysis. At least 10,000 events were counted in each sample. The cell cycle profiling of all samples was done in the same environment using the same instrument and by the same observer. Human lymphocytes were used as an external standard or for calibration. DNA histogram cell-cycle analysis was performed as described by Rabinovitch (*14*) using ModFit LT for Mac V2.0 software. For each sample, the percentage distribution of cells in the following 12 Cell Cycle Parameters *viz*.: Total S phase, Total Diploid, Total Aneuploid, DNA index (DI), G0-G1 (DIP; Diploid), G2-M (DIP), S (DIP), G2/G1 (DIP), G0-G1 (ANP; Aneuploid), G2-M (ANP), S (ANP) and G2/G1 (ANP) were estimated.

2.5. *Statistical analysis*

The overall (n = 100) average (± SE) coefficient of variation (COV) of diploid was 0.14 (± 0.01) while that of aneuploid was 0.23 (± 0.14). The respective value of these in controls (n = 38) was 0.10 ± 0.005 and 0.04 ± 0.004, respectively, while those in patients (n = 62) were 0.17 ± 0.01 and 0.35 ± 0.22, respectively. Seven parameters *i.e*., Diploid, DI, S (DIP), G2/G1 (DIP), G2-M (ANP), S (ANP) and G2/G1 (ANP) were not found statistically significant and either were not correlated ($r \leq 0.50$) well with clinical and histopathological gradings or found to be similar, and were excluded from further analysis. Thus, five total parameters *i.e*., Total S phase, Aneuploid, G0-G1 (DIP), G2-M (DIP) and G0-G1 (ANP) which were found clinically significant were submitted for statistical analysis. Cell cycle parameters of two groups were compared by independent Student's *t*-tests. Classification of cases were done using binary logistic regression while diagnostic, by receiver operating characteristic (ROC) curve analysis (Figure 2). Association between variables was done by Pearson correlation coefficient (r). Comparison of survival curve, was done by Log rank test. MS EXCEL (MS Office 97-2003), Graph Pad Prism (Version 5) and Med Calc (Version 3) were used for the analysis. A two-tailed ($\alpha = 2$) probability (p) value less than 0.05 ($p < 0.05$) was considered to be statistically significant.

3. Results

The baseline cell cycle parameters of control and patients are summarized in Table 1. Table 1 shows that the average fraction of Total S phase, Aneuploid

Table 1. Summary of DNA content of controls and patients

Parameters	Control (n = 38)				Patients (n = 62)				t-value (DF = 98)
	Min	Max	Mean ± SE	COV	Min	Max	Mean ± SE	COV	
Total S phase	0.00	11.44	5.47 ± 0.52	0.58	2.62	80.06	32.23 ± 2.47	0.60	8.39*
Aneuploid	0.02	27.82	4.91 ± 0.83	1.04	0.00	94.35	37.58 ± 3.23	0.68	7.80*
G0-G1 (DIP)	82.82	99.18	94.01 ± 0.62	0.04	0.00	89.72	30.77 ± 4.05	1.04	12.16*
G2-M (DIP)	0.00	17.18	0.65 ± 0.46	4.29	0.00	95.93	35.17 ± 4.64	1.04	5.81*
G0-G1 (ANP)	0.00	100.00	85.99 ± 5.00	0.36	0.00	100.00	39.48 ± 4.44	0.89	6.74*

* $p < 0.01$.

and G2-M (DIP) in patients were significantly high ($p < 0.01$) while G0-G1 (DIP) and G0-G1 (ANP) were significantly low ($p < 0.01$) as compared to control. Representive histograms of each group are shown in Figure 1. Corrected classifications (%) of cases of two groups are summarized in Table 2. All cell cycle parameters had high diagnostic value as they classified together (discriminated) control and cancer (patient) cases 100% correctly. Among these parameters, G0-G1 (DIP) alone classified cases 97% correctly, and with Total S phase or Aneuploid it discriminated cases 100% correctly. The percentage of correct classification using each parameter was higher in controls than in patients except Total S phase. The diagnostic value of cell cycle parameters or the accuracy of cell proliferation to discriminate controls and patients are summarized in Table 3. Among the cell cycle parameters, G0-G1 (DIP) showed the highest area under the curve (AUC = 0.997; SE = 0.007; 95% CI = 0.957 to 1.000; Z = 73.743; $p < 0.0001$), which indicates its highest diagnostic benefit, and at a criterion (threshold) value of ≤ 79.88 it discriminated the cases of two groups with

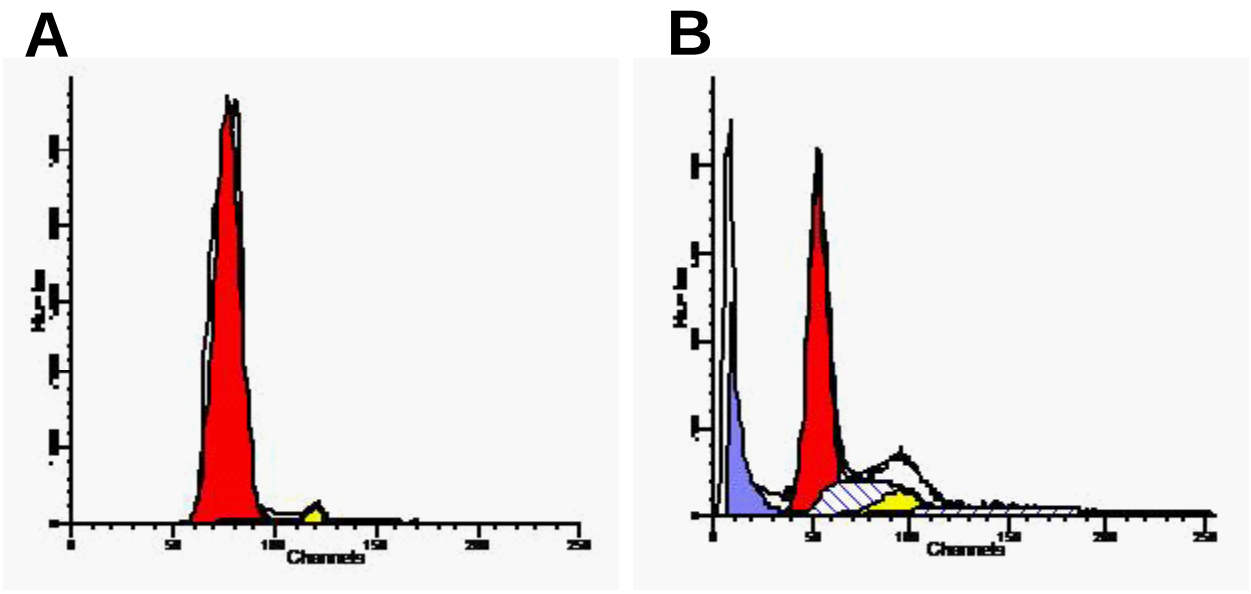

Figure 1. Typical patterns of flow cytometric histogramas of control and malignant cervical tissue in single cell suspension. **A**, Control; **B**, Cancer.

Table 2. Correct classification (%) of all cases, controls and patients by DNA content

Parameters	Control ($n = 38$)		Patients ($n = 62$)		Total ($n = 100$)	
	Number correct	Percent correct	Number correct	Percent correct	Number correct	Percent correct
Total S phase	33	86.8%	56	90.3%	89	89.0%
Aneuploid	34	89.5%	53	85.5%	87	87.0%
G0-G1 (DIP)	37	97.4%	60	96.8%	97	97.0%
G2-M (DIP)	36	94.7%	43	69.4%	79	79.0%
G0-G1 (ANP)	33	86.8%	49	79.0%	82	82.0%
All	38	100.0%	62	100.0%	100	100.0%
G0-G1 (DIP) and Total S phase	38	100.0%	62	100.0%	100	100.0%
G0-G1 (DIP) and Aneuploid	38	100.0%	62	100.0%	100	100.0%

Table 3. Diagnostic of all cases by DNA content ($n = 100$)

Parameters	AUC	Criterion	Sensitivity (95% CI)	Specificity (95% CI)	Z-Statistic
Total S phase	0.949	> 11.44	88.71 (78.1-95.3)	100.00 (90.7-100.0)	21.15*
Aneuploid	0.932	> 11.26	83.87 (72.3-92.0)	94.74 (82.2-99.2)	17.41*
G0-G1 (DIP)	0.997	≤ 79.88	96.77 (88.8-99.5)	100.00 (90.7-100.0)	73.74*
G2-M (DIP)	0.827	> 2.88	69.35 (56.3-80.4)	97.37 (86.1-99.6)	8.10*
G0-G1 (ANP)	0.844	≤ 80.59	82.26 (70.5-90.8)	86.84 (71.9-95.5)	7.91*

* $p < 0.01$.

the highest sensitivity (96.77%) and specificity (100%) as similarly shown from ROC curve analysis (Figure 2). The correlation between gradings of malignancy and P/V examination findings of clinical, biopsy reports and cell cycle parameters of all cases are summarized in Table 4. Table 4 showed that all cell cycle parameters are significant and correlated well with the gradings of clinical and histopathological examinations ($p < 0.01$) and the correlation with both clinical parameters were higher than the histopathological findings. Correlation of two-year survival of the patients (excluding 12 censored cases) with their pre-clinical, histopathological and cell cycle parameters was examined. As shown in Table 5, pre-clinical and histopathological parameters are not significant and correlated ($p > 0.05$) with survival, while the cell cycle parameters especially Aneuploid, G0-G1 (DIP) and G2-M (DIP) are significant and correlated with survival at $p < 0.05$ or $p < 0.01$. A hypothetical two-year overall (including censored cases) survival (%) was investigated in patients with G0-G1 (DIP) ≤ 25 and > 25. The median survival of patients with G0-G1 (DIP) > 25 was found to be 24 months while that with ≤ 25 was 14 months (Figure 3). The median survival of patients with G0-G1 (DIP) > 25 was 1.7 times more and significantly higher than that with ≤ 25 ($p < 0.01$).

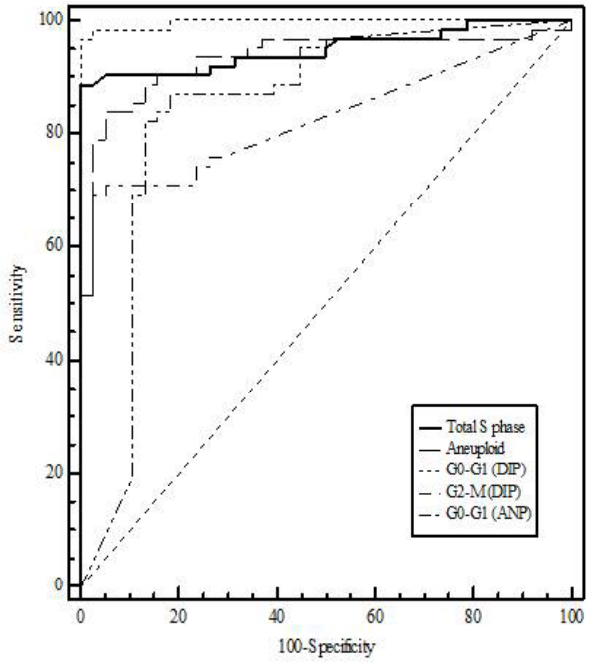

Figure 2. Comparative diagnostic of cell cycle parameters from controls and patients (n = 100) by ROC curve analysis. Different lines represent the sensitivity and specificity of cell cycle parameters. Here G0-G1 (DIP) and Total S phase shows the highest 100% specificity and sensitivity of 96.77% and 88.77%, respectively.

4. Discussion

Clinical staging has been the most important single parameter influencing the choice of treatment as well as outcome. However, clinical staging has some shortcomings and allows significant variations in tumor volume within stage. The challenge in the last few years has been to find a measurable prognosticator which can identify biologically more aggressive cervical cancer. In contrast to many conventional prognostic factors such as lymphnode metastasis, size of primary tumor, myometrial invasion, lymphovasucular involvement, tumor volume, cell type, grade of differentiation, vessel invasion, microvessel density (*15*,*16*), nuclear DNA content can be measured objectively and reproducibly. This is an important criterion in the prognostic factor evaluation outlined by McGuire in 1991 (*17*). In the present study, ROC curve analysis of all the above cell cycle parameters further gives the threshold value for G0/G1 (Diploid), Total S phase, Total Aneuploid involving the quantitative value which can more precisely differentiate and predict the disease. However, G0/G1 (Dipoid) values in the present study was the most sensitive parameter among all other cell cycle parameters such as, Total S phase, S (Diploid), S (Aneuploid), Total Diploid, Total Aneuploid, and DNA index as estimated in all other studies. However, as the restriction point that marks the first transition from G1 to S phase, the other being from metaphase to anaphase (the spindle checkpoint) and between anaphase and telophase when mitotic cyclins are degraded, the "G0-G1; diploid" and "G0-G1; aneuploid" may show no significant difference between control and patients. This can occur, because "cancer" can override restriction points (R-Point) on cell cycles and consequently can avoid apoptosis or

Table 4. Correlation between clinical and histopatological gradings and DNA content of all cases (n = 100)

Parameters	Sub-stage	Exam P/V	Biopsy report	Total S phase	Aneu ploid	G0-G1 (DIP)	G2-M (DIP)	G0-G1 (ANP)
Sub-stage	1.00							
Exam P/V	0.96**	1.00						
Biopsy report	0.50**	0.65**	1.00					
Total S phase	0.58**	0.63**	0.49**	1.00				
Aneuploid	0.62**	0.64**	0.42**	0.47**	1.00			
G0-G1 (DIP)	– 0.76**	– 0.79**	– 0.59**	– 0.64**	– 0.79**	1.00		
G2-M (DIP)	0.52**	0.53**	0.42**	0.22*	0.38**	– 0.66**	1.00	
G0-G1 (ANP)	– 0.43**	– 0.50**	– 0.50**	– 0.42**	– 0.05ns	0.30**	– 0.48**	1.00

ns, not significant; *, $p < 0.05$; **, $p < 0.01$.

Table 5. Corrletion of survival time (month) with clinical and histopatological gradings and DNA content of patients (n = 62)

Parameters	Correlation (DF = 60)
Sub-stage	-0.14^{ns}
Exam P/V	-0.10^{ns}
Biopsy report	0.08^{ns}
Total S phase	-0.01^{ns}
Aneuploid	-0.45^{**}
G0-G1 (DIP)	0.66^{**}
G2-M (DIP)	-0.29^{*}
G0-G1 (ANP)	-0.33^{**}

^{ns}, not significant; *, $p < 0.05$; **, $p < 0.01$.

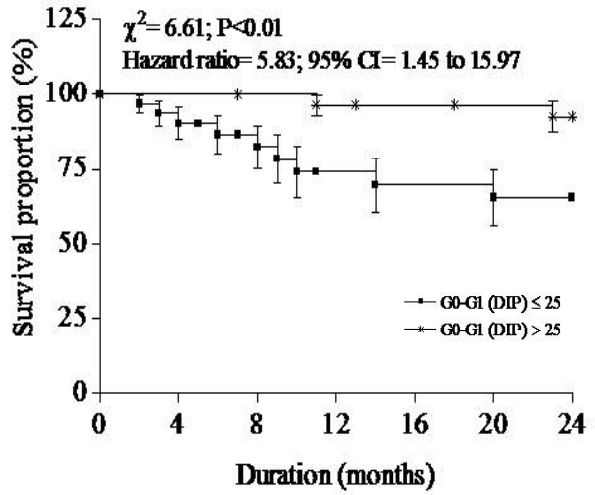

Figure 3. Comparison of 2-year overall survival proportions (%) in patients (n = 62) between G0-G1 (DIP) ≤ 25 and > 25 with 95% CI (vertical bar). The horizontal line with dots shows cumulative survival in patients having G0-G1 (DIP) ≤ 25 and horizontal line with stars shows survival of patients having G0-G1 (DIP) > 25. Star and dot represents deaths in respective groups.

senescence (*18*). This study proves that DNA content in fresh/frozen tissues may have a screening ability as diagnostic as well as prognostic significance. G0-G1 fraction (DIP) has significantly higher prognostic value, suggesting that patients with low G0-G1 (DIP) at all stages of disease were at higher risk. Seven cell cycle parameters *viz*., Diploid, DI, S (DIP), G2/G1 (DIP), G2-M (ANP), S (ANP) and G2/G1 (ANP) were not found statistically significant for diagnostic or prognostic value in patients with or without cervical neoplasia. Although DNA aneuploidy has been detected in fixed materials from uterine cervix in previous studies (*19*), it has been suggested that DNA aneuploidy is less preferred as an indicator of malignancy (*20*) and/or has no impact on prognostication of cervical carcinoma (*21*). In contrast, several researchers have found DNA aneuploidy in cervical lesions, which focused on the DNA content of fresh material from tumors (*12*) and a high S-phase fraction has been associated with poor prognosis (*5*). In this study, the prognostic value of Total S phase was also not found to be significant. Differential results in previous studies might be due to different preparation, staining and measuring techniques; different quality standards and lack of sophisticated computer software. Several previous studies reported an increased occurrence of aneuploid tumors in patients with an advanced stage of cancer (*4*). Cervical lesions with an abnormal DNA ploidy profile are more likely to persist or progress than normal diploid. In early cervical carcinomas, it has been shown that aneuploidy correlates with a high frequency of lymph node metastasis and tumor recurrences, irrespective of treatment with radiotherapy or surgery (*22*). Therefore, our results reinforce the hypothesis that DNA ploidy may be associated with progression of cervical carcinoma particularly with survival of patients. Wilailak *et al.* (*5*) concluded that patients with DNA diploid tumors have a better survival rate when compared to patients with aneuploid tumors. This study also concluded the same in terms of G0-G1 (DIP). Many investigators have reported that an abnormal DNA index is specific for malignancy, particularly in solid tumors (*23*) and statistically higher recurrence rates are seen in tumors with aneuploidy (DI > 1.5) in each of the stages IB-III of cervical cancer. Lai *et al*. (*24*) have shown that DI (DNA index) alone has promising prognostic value. However, the present study did not find DI a prognostic variable as reported in previous studies. The drawback of this study is that we could not get cervical tissue of patients during or at the end of treatment. Hence changes in cell cycle parameters during radiation treatment could not be evaluated. The prognostic evaluation of patients was done by correlating patient survival time with pre-clinical, histopatholigical examinations and cell cycle parameters. In the present study the digestion of the unfixed material was done with trypsin preferred over pepsin (*25*) and then fixed with ethanol and stained with propidium iodide. RNase was not used thus the broader CV might be due to contamination of RNA or it might be also because of particle variations *i.e*., if the samples were from the general population they might have different unknown disorders. The broadening of CV could also be because of preparation of single cell suspensions rather than single cell nuclei.

The present study concluded that G0-G1 (Diploid) may help in the diagnosis of carcinoma of the cervix which correlates well with histologically confirmed varied gradings of cervical cancer as well as patient survival. G0-G1 (Diploid) with Total S phase and Total Aneuploid fraction discriminates cases of both disease groups correctly with 100% sensitivity and specificity. Findings of this study may have clinical significance for false positive results. In future, it is suggested that if this study could be done on unknown smear samples, the threshold value of G0/G1 might result in early diagnosis of the disease as well.

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