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Predictive Value of Radiation Induced Apoptosis in Response to Radiotherapy in Cancer Cervix Patients Attending Department of Radio Therapy, Govt. Medical College Thrissur, A Prospective Study

Authors
Dr Ajithkumar V R[1], Dr R Mahadevan[2], Dr Seena K B[3], Dr Jayakumar K L[4], Dr Jewel Joseph[5], Dr Prema K R[6], Dr Shehna A Khader[7]
[1]Assistant Professor, Radiation Oncology, [2]Professor, Radiation Oncology
[3]Associate Professor, Obstetrics and Gynaecology, [4]Professor, Radiation Oncology
[5]Senior Resident, Radiation Oncology, [6]Associate Professor, Radiation Oncology
[7]Associate Professor, Radiation Oncology
*Corresponding Author
Dr Seena K B
Department of Radiation Oncology, Government Medical College, Thrissur, India

Abstract
Aim: *To correlate the apoptotic index with respect to radiation therapy in patients with carcinoma cervix attending Govt. Medical college, Thrissur.*
Materials and Methods: *Patients both early stage and locally advanced cervical cancer has been included in the study. Between December 2013 and September 2014. 26 patients were included in the study. Pre and post Radiotherapy biopsy of patients were taken and they were examined for Nuclear Pleomorphism, Hyper chromasia, Apotosis, Mitosis, Keratinisation, and Necrosis.*
IHC was done using Bcl 2, Bax, and Ki - 67.
Results: *Apoptotic index and markers favouring apoptosis can positively predict disease outcome. Patients with good apoptosis faired well in the study. But this has to be analysed in large cohort studies.*
Bax is a predictive marker for apoptosis.

Introduction

Worldwide cervical cancer is the second most common cancer to affect women, in developing countries including India it is the second most common malignancy among females.

Incidence varies worldwide with the highest rates found in Latin America and the lowest among Jewish women in Israel.

The use of cervical screening has greatly reduced the incidence of invasive cervical cancer in the western countries, but it continues to be a major cause of cancer mortality in the rest of the world because majority of patients have locally advanced disease at presentation.

Patients with early stage disease (IB non bulky and stage IIA) can expect cure with either radical hysterectomy or radical radiotherapy.

The standard treatment for stage IIB to IV A cervical cancer has been radical radiotherapy alone, (external pelvic radiation combined with intracavitary brachy therapy). 5 year survival for patients with locally advanced (stage III/IV A)

disease is approximately 25% (10-45%) when treated with radiotherapy alone. Although treatment failures outside the radiation field may occur, the more common cause of treatment failure is the inability of primary radiotherapy alone to completely eradicate all pelvic disease. The radiotherapy failure rate for patients with stage IIB disease is approximately 20-50% and for patients with stage IIIB disease the failure rate ranges from 50% to as high as 75%.

Attempts at improving local control by increasing radiation dose is limited by the maximum tolerated dose of radiation to surrounding organs, beyond which morbidity becomes unacceptable. Complications are the upper limit of acceptability with doses currently used. As a result many strategies have been investigated to try to improve outcome in cervical cancer. These include hyper fractionated treatment schedules, modification of radiation treatment volumes, hyperbaric oxygen, hyperthermia, hypoxic cell sensitizers and neutron therapy, unfortunately, these modalities have not demonstrated improved therapeutic response or local control.

For at least 25 years, clinicians have been searching for ways of combining chemotherapy with radiation to improve local control in cervical cancer. Most of the early prospective randomized trials involved the use of neoadjuvant chemotherapy followed by radiotherapy. Despite encouraging tumor response to various combination chemotherapy regimens, there is no improvement in local control or survival.

Study Group

Patients both early stage and locally advanced cervical cancer has been included in the study. Between December 2013 and September 2014. 26 patients were included in the study. Written informed consent was obtained from of all patients stage IB/IIA lesions that measure 3 cm or less can be managed with definitive surgery or radiation.

Routine investigations and metastatic work up was done. This included complete biochemistry, abdominal CT scan if indicated and a biopsy. Initial biopsy was done in all patients. Patients were given radiotherapy 45 gy in 23 fractions, after 5 fractions on the first Saturday, a repeat biopsy is also performed. Biopsies were done in the first week itself, according to patient convenience. The biopsy material was examined for apoptosis and tumour cell proliferation rate. Residual disease at the time of ICR was also evaluated and studied.

A total 26 patients with stage IIB or III (FIGO) were evaluated in the study and pre-treatment results correlated to radiotherapy outcome. A punch biopsy was taken from all patients, fixed in buffered formalin and processed for paraffin-was embedding. Sections 5|am thick were cut from the paraffin-embedded tissue and one section was stained by routine haematoxylin eosin staining for histopathological evaluation. Duplicate serial sections were used for immunocytochemistry. All patients in the study received radical radiotherapy (45Gy in 23 fractions for external-beam radiotherapy and 8 Gy to point A in 2 sitting or 7 Gy to point A in 3 sittings by ICR (HDR).

Subjecting to Repeat Biopsy

A repeat biopsy with the patient's consent was needed. The patient was explained in detail on the procedure and the benefits of understanding radiation response. The significance was explained and a consent obtained.

The study patients are to be followed for 5 years or even more to assess final disease outcome.

BAX, BCL 2, Ki67 Technique for Determination of Apoptosis

Estimation of apoptosis in tissue was determined using the BAX, BCL 2, Ki67 assay, employing an In Situ Cell Death Detection Kit. Briefly, sections were dewaxed in xylene hydrated with decreasing concentrations of ethanol and washed in distilled water for 10 minutes. The nuclei in tissue sections were stripped from proteins with 20 μg/ml of proteinase K diluted in sterile buffer (10mm Tris HCI/pH 7.4-8.0) for 15 min at 37°C and endogenous peroxidase was subsequently

quenched with 0.3% H2O2 in methanol for 30 minutes. The sections were covered with 50μl of a label mix containing the modified nucleotide and enzyme in a humidified chamber for 60 minutes at room temperature. After appropriate washing in PBS, the samples were first analysed under a fluorescence microscope. For further signal conversion analysis, 50JJ.I of converter peroxidase POD reagent was added for 30 minutes at room temperature. The substrate reaction was developed using diaminobenzidine (DAB) and then counterstained with haematoxylin. To confirm the reaction specificity of the BAX, BCL 2, Ki67 procedure, a negative control was also run, omitting Tdt from the reaction mixture. As a positive control, sections of involuting rat breast tissue were used.

Assessment of Apoptotic Cells

A cell was considered apoptotic only when unequivocal nuclear labeling was observed in areas of the tumor free from inflammation. Cells exhibiting necrotic nuclear karyorrhexis as well as those in necrotic foci were excluded. Moreover, all results were compared with the morphological analysis. Since the enzymatic reaction may also label diffuse areas of necrosis, only those labeled cells that showed additional characteristics of apoptosis, i.e. isolated localization within an intact cell complex without an inflammatory reaction was regarded as positive. Grading of the BAX, BCL 2, Ki67 reaction was done as explained earlier. Briefly, to evaluate differences in the various rates of BAX, BCL 2, Ki67 reactivity in each sample, 1,000 cells were counted at random under high power, and an apoptotic index (Al) was expressed as shown below.

$$A1 = \frac{Number\ of\ immuno\ reactive\ nuclei\ x\ 100}{Total\ number\ of\ cells\ counted}$$

A total of 1,000 cells were evaluated in all sections. Expression of PCNA was considered significant when characteristic nuclear immunoreactivity was seen in more than 10% of the cells. In addition, an expression index was used to evaluate PCNA expression as described earlier. This was done by classifying the protein expression into four categories based on the number of cells with positive expression. Thus, class 1 expression included those sample with less than 10% expression, Class 2 included samples showing 31% to 50% expression and class 4 included all cases with positive expression in more than 50% of cells.

Immunocytochemical Localization of K i67

Immunocytochemical analysis was carried out as described by us earlier. Briefly, sections were dewaxed in xylene and hydrated through graded alcohols to deionised water. Endogenous peroxidase was blocked by a 25-min incubation in 3% H2O2 in methanol. The sections were rinsed with distilled water and then incubated with 0.3% bovine serum albumin to reduce non-specific antibody binding. Sections were incubated overnight at 4°C with monoclonal antibodies. Sections were then incubated with biotinylated anti- (mouse Ig) at a dilution of 1:200 and peroxidase-conjugated streptavidin at 1:500 for 30 min each at room temperature. Washing was carried out in phosphate-buffered saline after each step and the peroxidase reaction was developed by application of daminobenzidine solution. The reaction was allowed to develop for 20 min after which it was stopped by washing in distilled water. The sections were then lightly counterstained with Mayer's haematoxylin, dehydrated in ascending grades of alcohol, fixed in xylene and mounted in Distrene Dibutyl phthalate Xylene. Grading of the immunoreactivity of growth factors was done as previously explained by us. Briefly, samples with less than 10% positive cells were considered negative. Samples with 11 %-30% were considered as showing mild expression. 30-50% moderate expression and those with over 51% positive cells as showing intense expression.

Inclusion Criteria

1) Women with biopsy proven carcinoma of cervix or clinically carcinoma of cervix.

2) Patient should have a anatomy favouring cervical biopsy
3) WHO performance status 0-1
4) Age < 80 years.
5) Adequate haematologic function (WBC > 4000/mm3, platelet count >1,00,000/mm3 and haemoglobin >9gm%).
6) Normal renal hepatic function
7) Willingness for informed consent document

Exclusion Criteria

1. Poor performance status
2. Bleeding disorders
3. Poor pelvic anatomy
4. Previous history of pelvic irradiation or systemic chemotherapy.

Diagnostic Work Up

1. Patients underwent a diagnostic work up which included:
2. Complete blood count
3. Renal and liver function tests
4. Chest X-ray
5. Ultrasound scan or CT scan of abdomen and pelvis
6. Cystoscopy

Tumour size was defined as the maximum diamefer of the tumour measured by ultrasound or CT scan of the abdomen and pelvis or by clinical examination.

Treatment

Concurrent chemoradiation with Inj. Cisplatin40 mg/m2 weekly was given for all patients.

External radiotherapy was delivered by four-field box technique using Cobalt -60. The pelvic field extended from the upper border of L-5 to lower border of the obturator foramen or 3 cm below the lowest extent of vaginal involvement (whichever was lower), and laterally 1.5 to 2 cm beyond lateral margin of the pelvic brim. For the lateral fields, anterior limit was the anterior border of pubic symphysis and the posterior limit was the space between S-2 and S-3. Midline shield was is not used in either arms. Dose of external radiotherapy was 45Gy/23 Fr/4 weeks.

With the goal of keeping total duration of treatment less than 8 weeks, intracavitary brachytherapy was performed within 2 weeks (preferably less than one week) after the completion of pelvic radiation.

Evaluation of Response

During treatment initial assessment of response was done at the time of 1st intracavitary brachytherapy. Clinical assessment was done by pelvic examination and response scored as:

1. No residual disease
2. Maximum dimension of residual disease ≤ 1 cm
3. Maximum dimension residual disease ≥1 cm

Follow-Up

On completion of treatment patients were followed on November 1, 2014. Clinical assessment was done and investigations were carried out only even indicated.

The closing date of the study was November 1, 2014; All the patients without regular follow up till closing date, patients who were presented last to follow up and their current states was obtained either by reply paid post cards or by telephone enquiry.

Statistical Analysis

The Kaplan -Meier (K-M) method was used to calculate the overall survival (OS) and disease free survival (DFS). The association between variables was assessed using chi-square test. All statistical analysis was performed using SPSS software. As the patients sample size was 26 and the evaluable patients were 13, WS Signed Rank Test was used.

Observation and Results

During the period from December 2013 to September 2014 an altogether of 26 patients were accured into the study. Of which good biopsy specimens were obtained for only 13 patients. Usual H&E stains are done for all patients. 6 patients slides had good processing features and immunohistochemistry was done in those patients.

There were problems during preparation of wax blocks and slides.

The first patient had 11 months of follow up. The last patient had four months of follow up. One patient died due to progressive disease after radical treatment.

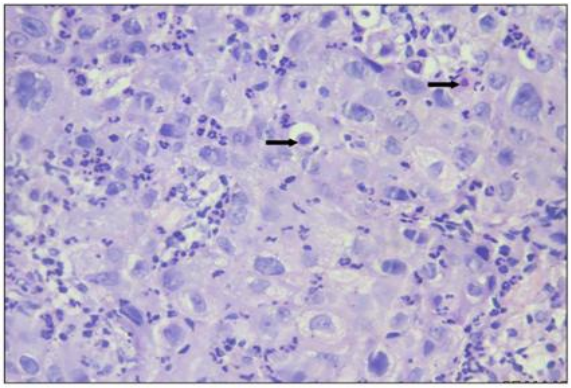


Fig.1.Carcinoma cervix pre radiotherapy showing apoptotic bodies

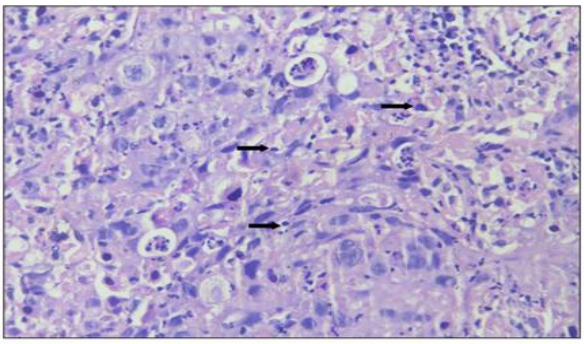


Fig.2. Carcinoma cervix post radiotherapy showing increased apoptosis, nuclear pleomorphism and hyperchromasia

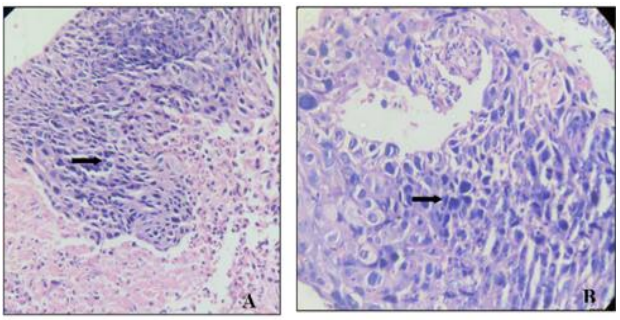


Fig.3. Keratinising SCC showing nuclear pleomorphism pre radiation (A) & post radiation (B)

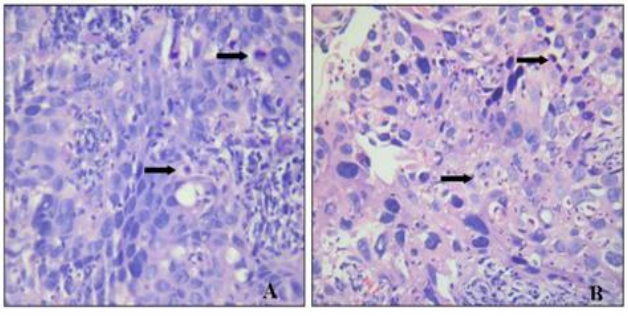


Fig. 5. Keratinizing SCC with mitotic figures & apoptosis pre radiation (A) and post radiation(B)

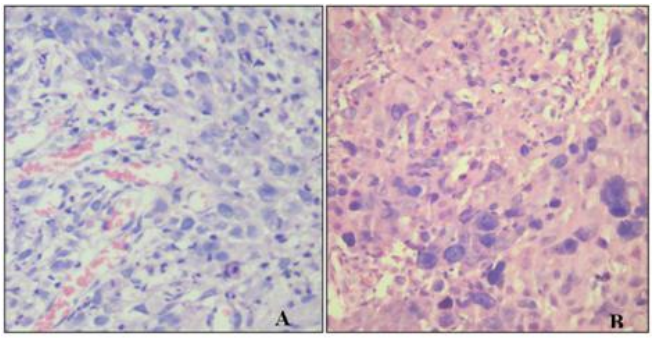


Fig.6. SCC cervix pre radiation (A) & post radiation (B)

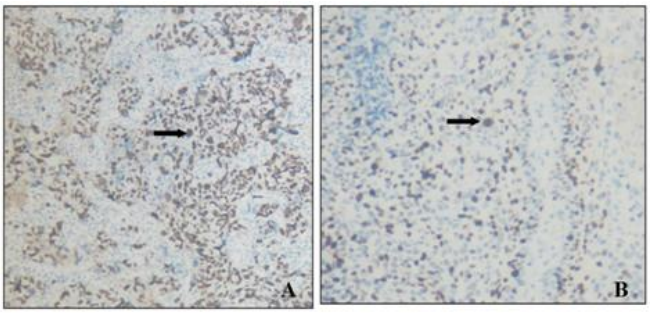


Fig.7. Ki 67 immunostaining pre radiation (A) & post radiation (B)

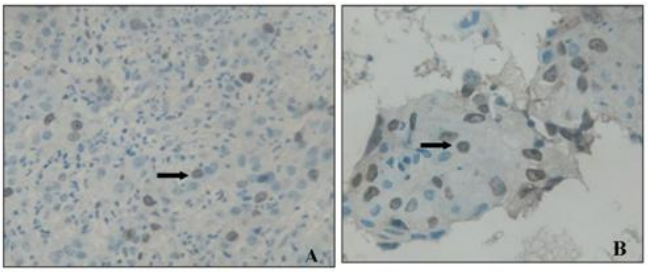


Fig.8. BAX immunostaining in carcinoma cervix pre radiation (A) a& post radiation (B)

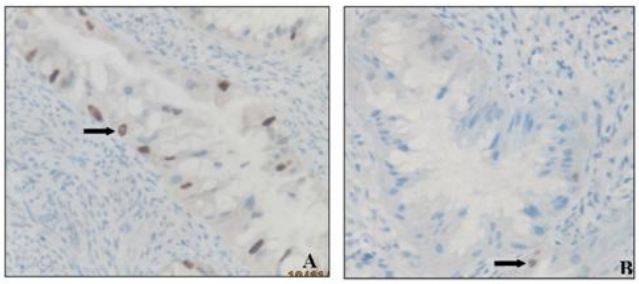


Fig.9.Ki 67 immunostaining in adenocarcinoma cervix pre radiation (A) & post radiation (B)

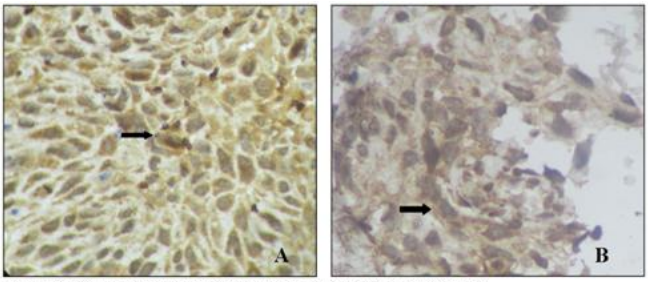
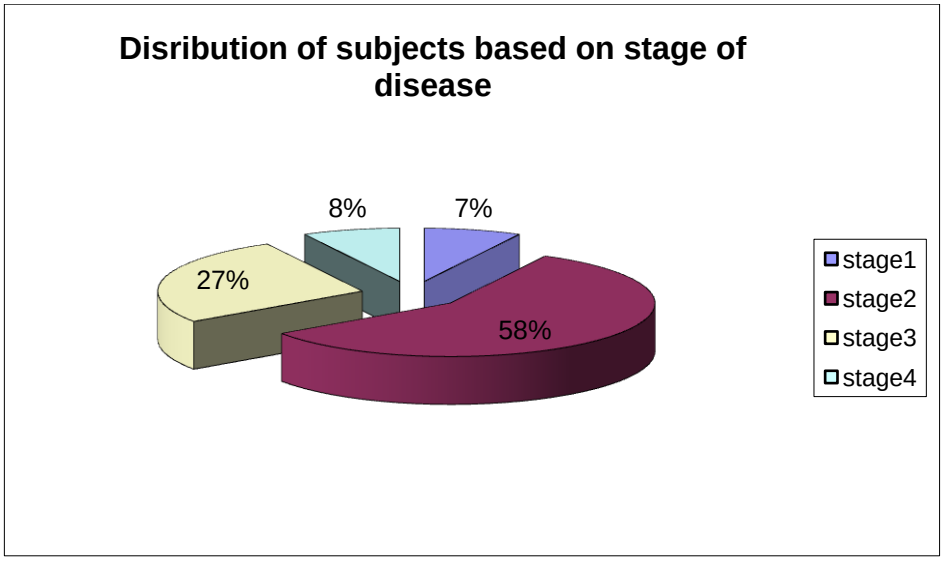


Fig. 10. Bcl 2 immunostaining in carcinoma cervix pre radiation (A)& post radiation (B)

Figure - 1

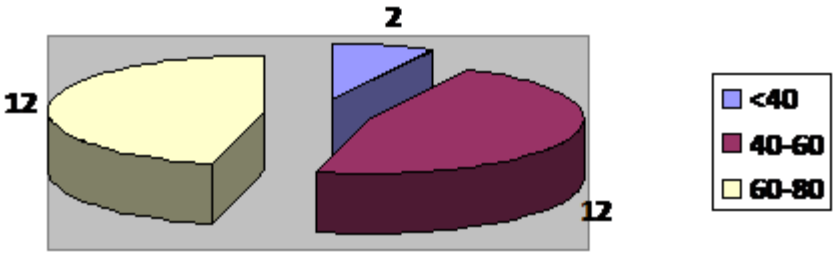


Stage1	2
Stage2	15
Stage3	7
Stage4	2

Chart showing age group of patients

< 40 years	2
40 – 60 years	12
60 – 80 years	12

Figure -2



Nuclear Pleomorphism

Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	1(a)	3.50	3.50
	Positive Ranks	6(b)	4.08	24.50
	Ties	4(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is significant 5.8%

Test Statistics(b)	
	POST - PRE
Z	-1.897(a)
Asymp. Sig. (2-tailed)	.058
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Hyper Chromasia

Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	1(a)	4.00	4.00
	Positive Ranks	7(b)	4.57	32.00
	Ties	3(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is significant 3.5%

Test Statistics(b)	
	POST - PRE
Z	-2.111(a)
Asymp. Sig. (2-tailed)	.035
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Apoptosis

Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	0(a)	.00	.00
	Positive Ranks	7(b)	4.00	28.00
	Ties	4(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is significant 1.1% which is of maximum significance in the groups.

Test Statistics(b)	
	POST - PRE
Z	-2.530(a)
Asymp. Sig. (2-tailed)	.011
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Mitosis

Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	0(a)	.00	.00
	Positive Ranks	4(b)	2.50	10.00
	Ties	7(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is significant 5.9%

Test Statistics(b)	
	POST - PRE
Z	-1.890(a)
Asymp. Sig. (2-tailed)	.059
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Keratinisation

Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	3(a)	3.17	9.50
	Positive Ranks	3(b)	3.83	11.50
	Ties	5(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is not significant 83.2%

Test Statistics(b)	
	POST - PRE
Z	-.213(a)
Asymp. Sig. (2-tailed)	.832
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Necrosis

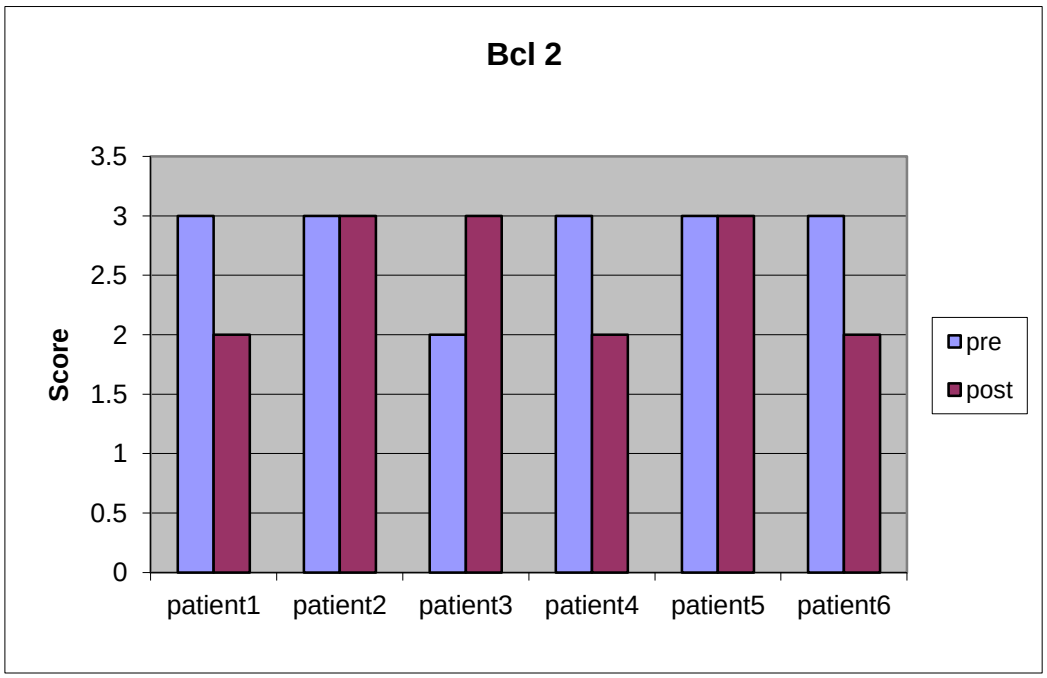
Ranks				
		N	**Mean Rank**	**Sum of Ranks**
POST - PRE	**Negative Ranks**	2(a)	2.50	5.00
	Positive Ranks	3(b)	3.33	10.00
	Ties	6(c)		
	Total	11		
a POST < PRE				
b POST > PRE				
c POST = PRE				

Test is not significant 48%

Test Statistics(b)	
	POST - PRE
Z	-.707(a)
Asymp. Sig. (2-tailed)	.480
a Based on negative ranks.	
b Wilcoxon Signed Ranks Test	

Bar charts on Immunohistochemistry

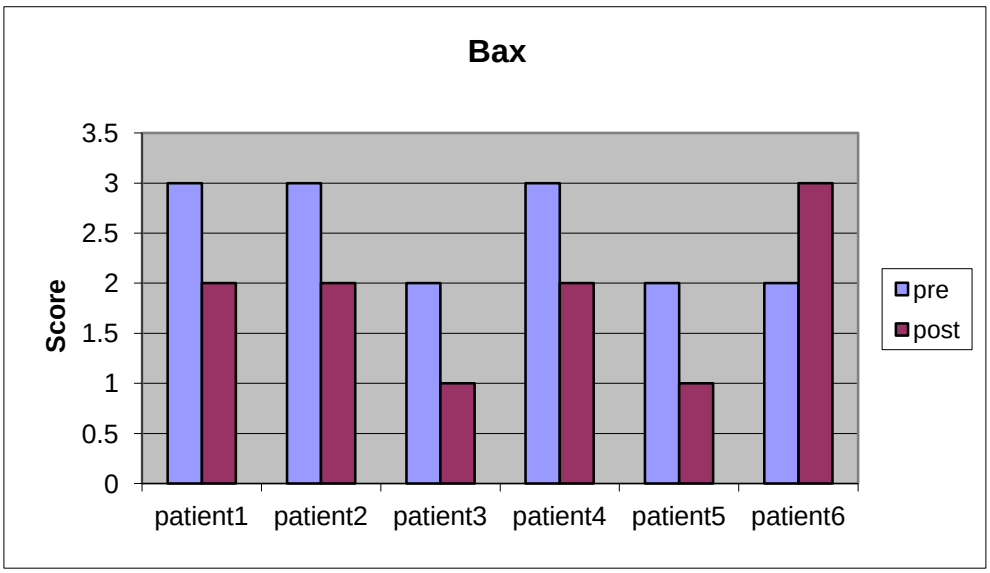
Figure -3



	Bcl - 2	
	Pre	Post
Patient1	3	2
Patient2	3	3
Patient3	2	3
Patient4	3	2
Patient5	3	3
Patient6	3	2

Test is not significant 31.7%

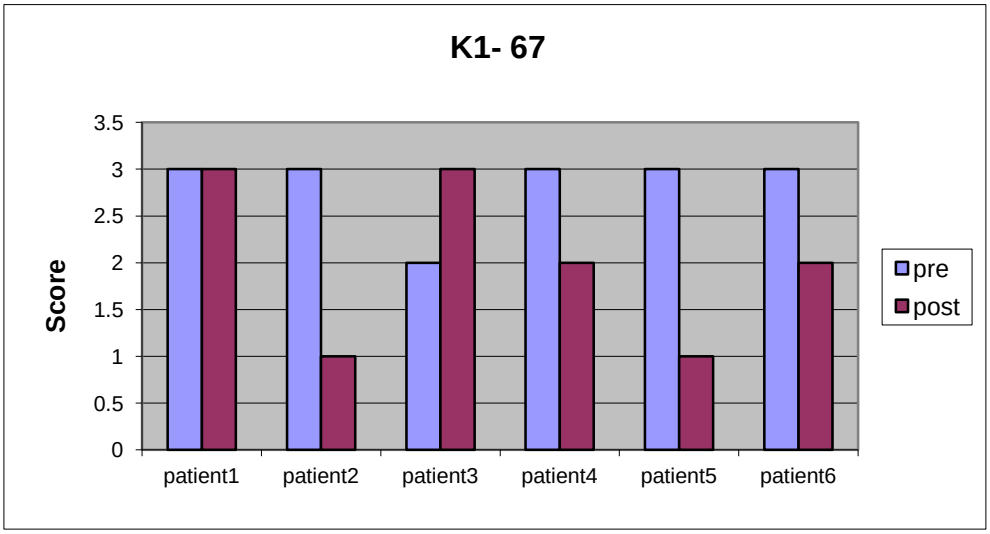
Figure -4



	Bax	
	Pre	Post
Patient1	3	2
Patient2	3	2
Patient3	2	1
Patient4	3	2
Patient5	2	1
Patient6	2	3

Test is somewhat significant 10.2%

Figure -5



	Ki - 67	
	Pre	Post
Patient1	3	3
Patient2	3	1
Patient3	2	3
Patient4	3	2
Patient5	3	1
Patient6	3	2

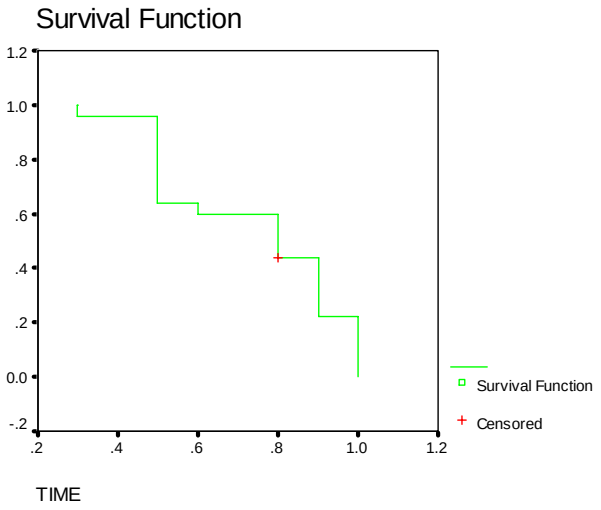
Test is not significant 12.9%

Summary of the Tests

No.	Parameter	Positive percentage	Z value	Statistical significance
1.	Nucelar pleomorphism	4.08	1.897	5.8%
2.	Hyper cromatia	4.87	2.111	3.5%
3.	Apoptosis	4	2.53	1.1%
4.	Mitosis	2.5	1.89	5.9
5.	Keratinisation	3.83	0.2	83.2
6.	Necrosis	3.33	7.07	48

No.	Parameter	Positive percentage	Z value	Statistical significance
1.	Bcl2	2.5	1	31.7%
2.	Bax	3.5	1.633	10.2%
3.	Ki67	2	1.518	12.9

Kaplan Meier Chart



The survival curve described above is not an ideal curve because the follow up period is only 11 months. Ideally for cancer cervix the survival curve will be a straight line.

Discussion

Bcl2 down regulates apoptosis whereas Bax promotes apoptosis. Ki67 is a proliferation marker.

In this study the Bax expression in PRT specimen and post radio therapy specimen has minimal statistical significance. This is because Bax favours apoptosis.

Bcl2 level in post radio therapy specimen has a strong correlation with apoptosis (post radiotherapy) 2.4%, pre radiotherapy necrosis 2.4%, post radiotherapy Keratinisation 2.6%, post radiotherapy necrosis (2.6%). Bcl2 always down regulates apoptosis. The results are correlating with Wootempoom et al study, in which necrosis in turn indicates low apoptosis and this will decrease tumour control. **Keratinisation** means well differentiation and this makes less radio sensitive.

Ki67 in the post radio therapy specimen has correlation with apoptosis because in the initial phases of radiotherapy there is tumour proliferation more and it leads of more of apoptosis. This is because of cellular re population. Second biopsy was done after 5 fractions. This is also reflected in the **Keratinisation** (8.4%) and necrosis 2.3%. All of them are due to repopulation of cervical carcinoma cells. When a correlation between age and stage was done there was no significant correlation. Out of the 26 patients 15 patients were of stage II. This is because increase of health education and literacy in the state. Patients are using papsmear and the newer diagnostic test for the diagnosis.

Carcinoma of the uterine cervix is an attractive model system for studying the clinical applicability of laboratory based predictive assays on tumour response to radiotherapy. It is a disease that in many centres including ours is managed primarily by radical radiation therapy. The clinical correlations emerging for tumour and normal tissue radio sensitivity and patient response to treatment is critical issue in radiation biology. If achievable such findings would bring about greater individualization of patient treatment by radio therapy.

We present a hypothesis that would explain the paradox of high apoptotic values that predict metastatic phenotype and poor survival. Part of the hypothesis is addressed previously in the statement that cancer is a multi step disease and progression through the various phases results from accumulation of genetic aberrations as cells repeatedly divide and duplicate the DNA (replication errors). Thus progression will be directly related to the number of multiplication cycles a tumour has had since its inception. Furthermore tumours with higher apoptotic values due to higher rate of cell loss require a greater number of tumour cell multiplications to attain a particular size or volume compared with low apoptotic values. Furthermore clinical detection requires the tumour to attain a critical mass and tumours with high apoptotic values wood attain a critical mass after a greater number of tumour cell duplications than tumours with low apoptosis values. Hence tumours with high apoptotic values are likely to have acquired greater number of genetic aberrations and this in turn would relate to

subclinical metastases and therapy related resistance.

This prospective study has limitation because sample size is only 26 and the follow up period is only 11 months.

Conclusion

Apoptotic index and markers favouring apoptosis can positively predict disease outcome. Patients with good apoptosis faired well in the study. But this has to be analysed in large cohort studies.

Bax is a predictive marker for apoptosis.

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