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Expression profiling of G2/M phase regulatory proteins in normal, premalignant and malignant uterine cervix and their correlation with survival of patients

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ABSTRACT

Background: Cell regulatory G2/M phase proteins are the key regulators of mitosis and have been reported with abnormal expressions in various malignancies.

Aim: To determine the expressions of these proteins in neoplastic uterine cervix tissue.

Materials and Methods: This study evaluates the G2/M phase regulatory protein expression of Cyclin B1, Aurora-B, Pololike kinase 1 (PLK1) and LIM kinase1 (LIMK1) in tissues of 25 normal (control), 16 dysplastic (dysplasia) and 34 neoplastic (cancer) patients of uterine cervix. The expressions of different proteins were obtained by using Western Blot technique.

Statistical Analysis: One way analysis of variance (ANOVA), Pearson correlation, Kaplan-Meier and other tests are used for analysis.

Results and Conclusion: The level of expression of LIMK1 in cervical cancer patients was found to be significantly higher ($P<0.01$) than both the controls and dysplasia. The expression of Aurora B and PLK1 in cervical cancer patients was also found to be significantly higher ($P<0.05$) than controls but it did not differ with dysplasia. However, the expression of Cyclin B1 was similar among cervical cancer patients, dysplasia and controls ($P>0.05$). The expression of all the above proteins showed significant ($P<0.01$) and inverse relation with the survival of cancer patients. Among the selected candidate proteins, it was LIMK1 that showed the most positive correlation with the aggressiveness of the disease and negative correlation (r= -0.64; $P<0.01$) with the survival of patients.

KEY WORDS: Aurora B, cervix cancer, Cyclin B, LIMKinase1 (LIMK1), Polo Like Kinase1 (PLK1)

INTRODUCTION

Uterine cervical carcinoma is one of the most common gynecological malignancies affecting women worldwide. In India, about 130,000 new cases of cervix cancer occurs every year and constitute one-fifth of the total global burden, its age-standardized incidence rate is 30.7 per 100,000 and age-standardized mortality rate is 17.4 per 100,000 which are the highest in South Central Asia.[1] The number of deaths due to cervical cancer is estimated to rise to 79,000 by the year 2010.[2] The cancer mostly affects middle- aged women (between 40 and 55 years), especially those from the lower-economic status who fail to carry out regular health check-ups due to financial inadequacy. Cervical cancer is still diagnosed late and treatment outcome remains poor, hence early monitoring and management is needed. G2/M phase proteins like Cyclin B1, Aurora kinases, PLK1 and LIMK1 are essential for initiation of mitosis, chromatin segregations, cytokinesis and tumor invasion.[3-6] Most of the cell cycle studies for cervical cancer have focused mainly on G1/S phase and much remains to be known about the expression of G2/M regulators simultaneously in normal, pre-malignant and malignant tissues. Recently, efforts are being made to study cell cycle changes on G2/M phase and their regulatory proteins because efforts to increase G2/M arrest causes increased apoptosis.[7] G1/S phase indicates the process of replication of the DNA where as apoptosis, the final outcome of G2/M phase may be a key end stage regulatory process for the upregulation or the down regulation of the cell cycle, which further reflects that perhaps in cancer cell mitosis may be more important in cancer development than merely the replication of DNA. In view of this, the present study was planned to test G2/M proteins in carcinoma cervix, and its prognostic significance using the follow-up data and survival of the patients, an area not well understood so far.

MATERIALS AND METHODS

The cases were selected from the patients registered

in the Department of Radiotherapy and Gynecology. Patients selected were previously untreated without any concurrent malignancy or chronic illness with normal renal and liver function tests. Control population consisted of individuals undergoing hysterectomy for benign condition having normal cervix. 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 informed written consent was taken from all the subjects for their participation in the study. A detailed history and clinical examination was also undertaken for all the study subjects. All the subjects were classified in 3 groups - (I) 16 dysplastic cases (CIN; Cervical Intraepithelial Neoplasia patients), (II) 34 malignant patients of carcinoma of cervix (histologically confirmed as squamous cell carcinoma of cervix) and (III) 25 control subjects were recruited for the study. The samples for the suspected cervix cancer (malignant) and dysplasia (premalignant) cases were collected at the time when the biopsy was taken to confirm the diagnosis. The samples for the control cases were collected at the time when the hysterectomy was performed and tissue was being sent for the pathological examination. Each cervical biopsy sample was cut into two parts and collected in separate tubes, one part kept in the formalin was sent for histopathological examination (HPE) and the other part of the sample kept in the phosphate buffer saline (PBS) at -80°C for protein analysis. All the patients were staged according to the FIGO staging.[8] Only histologically proven cases of squamous cell carcinoma of uterine cervix were included in this study (n=34), which were then again classified according to the degree of differentiation into well, moderately and poorly differentiated carcinoma. The samples of the dysplasia cases were collected after the Pap smear examination and colposcopy report, for monitoring different degrees of dysplasia. After HPE report, they were also classified as mild, moderate and severe dysplasia cases. Similarly, the control samples were collected from the patients, who underwent hysterectomy for the unrelated reasons. HPE report of cervix for controls exhibited benign normal appearance. The carcinoma cases were further followed for 2 years for survival. *Antibodies:* Cyclin B1 monoclonal antibody labeled hamster anti-mouse, Aurora-B polyclonal antibodies labeled human anti rabbit, PLK1 polyclonal antibodies labeled human anti rabbit, LIMK1 polyclonal antibodies labeled human anti rabbit, β-actin and goat anti rabbit secondary antibodies **(***obtained from Cell Signaling Technology, Boston, Massachusett, USA).* To study the expression of the different proteins, the fresh frozen tissues of all 3 groups were homogenized in PBS at pH 7.4 and the desired 10% homogenate was used for further experiment. Protein estimation was done by Folin-Lowry method,[9] using the bovine serum albumin (BSA) as a standard protein. After preparing the homogenate, estimation of the protein was done using different concentrations. Towbin *et al*.[10] method for Western Blotting was standardized by using the sample protein of different concentrations. Finally, the best concentration (100 μg) and volume (40-45ml) were selected for loading homogenate along with the dye for 10% SDS PAGE vertical electrophoresis system *(Apparatus used was: Hoefer mini VE from Amersham Biosciences, Piscataway, NJ)*. The wells were loaded with the tissue homogenates of different groups in different lanes. In one of the lane, the marker was loaded. Electrophoresis was started and current was disconnected when the dye moved just before the end of gel. Before staining and destaining of the gel, the resolved gel proteins were blotted to the nitrocellulose membrane for 3 hours after preparing the sand-witch for semi-wet transfer method (Apparatus used was: *Mini Trans-Blot Electrophoretic Transfer Cell unit from BIO RAD Laboratories, LA, USA)*. Finally, the sand-witch was opened and the Ponceau stain test was done on membrane to ensure that the protein has been transferred from gel to membrane. The membrane was then kept in the blocking solution. The gel was also stained and destained to check any non-transferred protein. The membrane was now treated with the primary antibody and blocked overnight in the blocking solution [5% non-fat milk in TBST (Tris buffer saline -Tween 20; pH -7.6)] at 4°C. Next day, the primary antibody was washed using TBST buffer. Now the membrane was treated with the secondary antibody and left again overnight in the blocking solution. Next day, the secondary antibody was washed and the bands were developed using a buffer, (Tris HCl; pH=7.6), substrate (H_2O_2) and a dye (3, 3' diaminobenzidine; DAB) in dark. Electro-Chemi-Luminescence (ECL) kit supplied by *Amersham Pharmacia*, *USA was used t*o develop the bands of Cyclin-B protein. The experiment was repeated with each primary antibody Cyclin B1, Aurora-B, PLK1 and LIMK1 along with their respective secondary antibodies. The concentration of each antibody was used as suggested by the suppliers. For further densitometric analysis of these bands, they were scanned by scanner and saved in the computer as a tiff image file. The blots, on which the expression of proteins was seen, were further stripped and probed with β-actin (a house keeping gene) antibody which served as loading control. The densitometric analysis was finally done by using Bovis-Gel-Analysis software and the data was recorded and submitted for statistical analysis. Groups were compared by using one way analysis of variance (ANOVA) followed by Bonferroni post hoc test. Difference between two proportions was evaluated by proportion Z test using its correction for continuity. Associations between variables were done by Pearson correlation method. The Kaplan-Meier methods (Logrank χ^2 test and Cox proportional Hazard ratio) were used to compare the survival between two groups. A two-tailed test (α=2), $P<0.05$ was considered to be statistically significant. GraphPad Prism (version 5) and STATISTICA (version 7) were used for the analysis.

RESULTS

The number (in percentage) and mean age of subjects in different groups and subgroups are summarized in Table 1. The age of subjects in all three groups ranged from 22 to 76 years. The mean age of subjects in all three groups was identical (F=0.006; $P>0.05$). Among malignant uterine cervix

patients, the proportion of stage IIB was the highest. The cell cycle regulatory proteins expressions of three groups are summarized in Table 2 and also shown graphically along with blots in Figure 1. All cell cycle regulatory proteins showed increasing expression from control to dysplasia and highest expression were seen in cervical cancer patients. The expression (mean level) of Cyclin B1 was found to be statistically not significant ($P>0.05$) when compared among all the three groups. The expression of Aurora-B in dysplasia patients was higher than control but the difference was not statistically significant ($P>0.05$) while the expression of it in cancer patients was significantly ($P<0.05$) higher than the control but it did not differ ($P>0.05$) between dysplasia and cancer patients. The expression of PLK1 in all three groups showed similar trend as of Aurora-B but with more significant ($P<0.01$) over expression in cancer cases. In contrast, the expression of LIMK1 in cancer patients was found to be significantly ($P<0.01$) higher than both the control and dysplasia. It was found to be 4.6 and 3.0 fold higher than the control and dysplasia, respectively. The correlation of cell cycle regulatory proteins expression in cancer patients and their survival time (months) are summarized in Table 3. The cell cycle regulatory proteins expression in cancer patients showed significant ($P<0.01$) positive correlation with each other while their expression has significant ($P<0.01$) and negative correlation with the survival. Among cell cycle regulatory proteins, the expression of LIMK1 showed maximum inverse relation with the survival. Comparing 2 years overall survival [Figure 2] in cancer patients those having the expression of LIMK1 ≤ 1 and > 1, it was found that patients having expression > 1 have significantly lower survival as compared to those having expression ≤ 1 ($\chi^2=10.05$; $P<0.01$). Further, the hazard ratio (the slope of the

Table 1: Mean age and number (%) of subjects in all groups and sub groups

Groups	Number [n (%)]	Age (years): Mean ± SD
Control	25	44.68 ± 12.29
Dysplasia	16	44.75 ± 13.00
Cancer	34	44.41 ± 10.17
IA	0 (0.0)	
IB	6 (17.6)	
IIA	0 (0.0)	
IIB	15 (44.1)	
IIIA	1 (2.9)	
IIIB	11 (32.4)	
IVA	1 (2.9)	
IVB	0 (0.0)	

Table 2: G2/M phase cell cycle regulatory proteins expression profiling summary (mean ± SE) of control, dysplasia and malignant uterine cervix patients

Groups n	Cyclin B1	Aurora-B	PLK1	LIMK1
Control 25	0.46 ± 0.10	0.26 ± 0.05	0.18 ± 0.03	0.22 ± 0.05
Dysplasia 16	0.58 ± 0.11	0.36 ± 0.11	0.41 ± 0.13	0.35 ± 0.14
Cancer 34	0.66 ± 0.10	0.61 ± 0.10[c]	0.64 ± 0.11[c]	1.04 ± 0.13[cd]

*[c](control) is the significance in comparison with dysplasia and cancer, [d](dysplasia) is the significance in comparison with cancer (regular font: $P<0.05$, bold font: $P<0.01$)

Table 3: Correlation between cell cycle regulatory proteins expression and survival in malignant uterine cervix patients (n=34)

Variables	Cyclin B1	Aurora-B	PLK1	LIMK1	Survival
Cyclin B1	1.00				
Aurora-B	0.69**	1.00			
PLK1	0.57**	0.61**	1.00		
LIMK1	0.83**	0.77**	0.77**	1.00	
Survival	-0.44**	-0.59**	-0.63**	-0.64**	1.00

†**significant; $P<0.01$

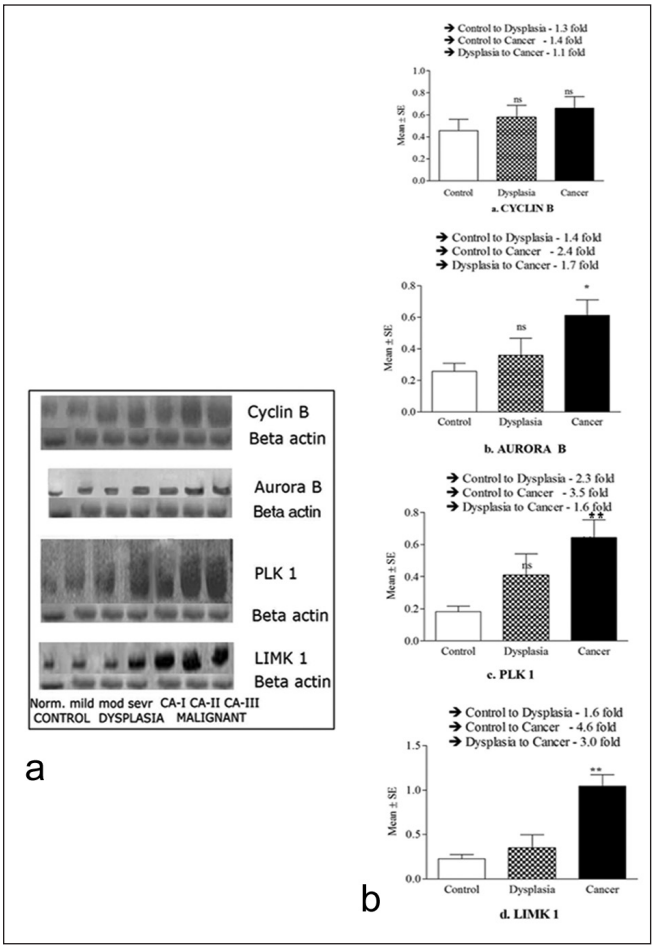

Figure 1: Representative Western blots (a) and Histograms (b) of Cyclin B1, Aurora B, PLK1 and LIMK1 protein expression in control, dysplasia and malignant uterine cervix patients. (a) Gradual increase in thickness of blots from normal to cancer can be noted in all proteins especially in LIMK1. Even mild change can be noted within the subgroups i.e. mild, mod. (moderate) and sevr. (severe) dysplasia and CA (cancer) stage I, II and III in Aurora B, PLK1 and LIMK1 protein bands. (b) Histograms demonstrating the gradual increase in the expression (mean values ±SE) of the proteins from control to cancer through dysplasia. Mean values were obtained by normalizing the intensity of protein bands with the intensity of β-actin protein bands. The fold increase within the groups has been shown along with the histograms for each protein. Significance (ns- $P>0.05$, *- $P<0.05$, **- $P<0.01$) has also been plotted in comparison with control. (ns= non significant, * significant, ** highly significant.)

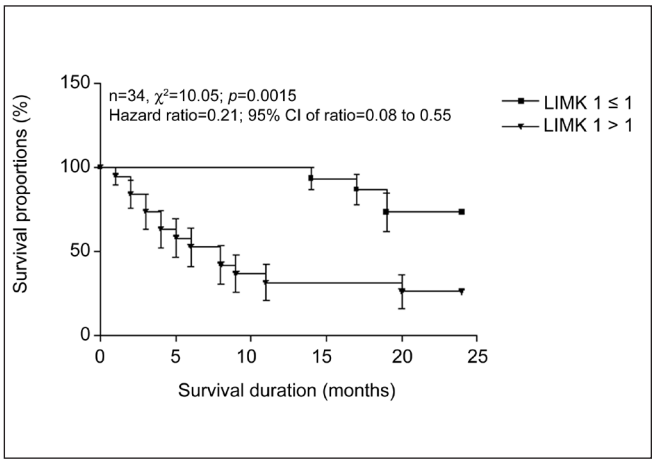

Figure 2: Survival curve showing 2 years overall survival in patients of uterine cervical carcinoma those having expression of LIMK1 ≤ 1 and > 1 with SE (vertical bar). The numbers of patients having LIMK1 ≤ 1 were 15 while > 1 were 19. The numbers of deaths in LIMK1 ≤ 1 were 4 (11.8%) while in LIMK1 > 1 were 14 (41.2%)

survival curve - a measure of how rapidly subjects are dying) was found to be 0.21, (95% CI of ratio=0.08 to 0.55) which indicates that the rate of death in patients having LIMK1 > 1 was 0.21 times more than the rate of death in patient having LIMK1 ≤ 1 *i.e.* the mortality rate is directly proportional to the concentration of the LIMK1. In addition, 3.5 fold more deaths were evident in patients having expression of LIMK1 > 1 as compared to those having expression ≤ 1 (11.8% vs. 41.2%, Z=2.38; *P*<0.05).

DISCUSSION

In the present study, we evaluated the expressions of G2/M regulatory proteins with the degree of aggressiveness of the disease. As a key cell cycle modulator of the G2-M transition, Cyclin B1, Aurora-B, PLK1 and LIMK1 are considered to play an important role in various human tumors.[5,11-13] Increased expressions of Cyclin B1 gene were reported in seven out of twenty (35%) types of breast cancer cell lines.[14] In colon cancer cells, it has also been shown to increase gradually according to the differentiation of the cells.[15] In our study, there was a gradual increase in the expression of Cyclin B1 observed starting from control to dysplasia to cervical cancer. However, this difference was not statistically significant between the three groups. Zhao *et al*. showed that there was significantly (*P*=0.019) greater Cyclin B1 expression in invasive cervical cancer than in normal cervical tissue.[11] This difference could possibly be due to the observations at the transcript level as has been reported by Zhao *et al,* whereas we studied the Cyclin B1 protein at the expressed protein level. Over expression of a stable form of Aurora-B has been shown in different cancer cells lines viz. colorectal,[16] Hela cells,[17] and in tumors of oral carcinoma,[18] which alone induces multinuclearity, aneuploidy and are important during human cancer development. It has also been demonstrated in tumors of central nervous system.[19] The study on endometrial carcinomas demonstrated that the expression of Aurora-B significantly increased in grade 3 tumors, compared with grade 1 and 2 tumors,[20] which supports the findings of our study. Elevated expression of PLK1 has been shown in different carcinomas like colorectal cancer,[21] ovarian,[22] He La cells[23] and Prostate cancer.[5] The present study in the tissue homogenates showed a similar trend which also correlates with the findings of the above studies. In our study, we found expression of LIMK1, which is a serine-only protein kinase expressed during G2/M phase, to be significantly (*P*<0.01) higher in both dysplasia and cancer patients as compared to controls. Similar findings were also observed with BPH-1cells (benign prostatic hyperplasia), prostate tumors and in prostate cancer cell lines,[24] i.e. according to the aggressiveness of the disease.

LIMK1 expression is correlated negatively with overall survival of patients in our study which was similar for cell survival in other studies.[25] The survival studies on Cyclin B1and Aurora-B also show the negative correlation in breast cancer[26] and endometrial carcinoma[20] respectively. The over expression of PLK1 has also been associated with over-all poor survival, which all supports the finding of our study in cervix cancer patients. Its expression has been shown to have prognostic value for predicting outcomes in patients with many cancers such as lung cancer,[27] squamous cell carcinomas of the head and neck,[28] ovarian,[29] endometrial carcinomas,[30] breast cancer[31] and esophageal cancer.[32] In the present study, the positive correlation has been observed among the expression of all the four G2/M regulatory protein and negative correlation was seen with survival of carcinoma cervix patients which further results in LIMK1 to be the most positively correlated with the aggressiveness of the disease and negatively correlated with the survival of the patients. G2/M cell cycle regulatory proteins show robust increasing changes in their expression in cervical cancer patients vs. control and progressing through dysplasia. Among the selected candidate proteins, it was LIMK1 that showed the most positive correlation with the aggressiveness of the disease and there was an inverse relation with the survival. However, the molecular mechanisms of these proteins are not completely clear in cancer prognoses. Further, it is suggested to study the expression of all these proteins at genesis: gene level, as well as at transcript: mRNA level and expressed: protein level, simultaneously in all the grades and stages of cervical carcinoma. Studies may also be undertaken to evaluate the suitability of these G2/M phase cell cycle regulatory proteins as a therapeutic target for better outcome in this cancer, which is one of the most common cause of morbidity and mortality in women worldwide.

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