EXPRESSION OF CYCLIN A IN RECTAL MUCOSA
&
RECTAL CARCINOMA

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ADYAR, CHENNAI

CERTIFICATE

CERTIFIED THAT THIS IS THE BONAFIDE DISSERTATION DONE

BY

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AND SUBMITTED IN PARTIAL FULFILLMENT OF THE

REQUIREMENTS FOR THE

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THIS WORK IS DEDICATED TO ALL THE CANCER PATIENTS

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ABBREVIATIONS

APS - AMINOPROPYL TRIETHOXY SILANE

CDC - CELL DIVISION CYCLE

CDK - CYCLIN DEPENDANT KINASE

CR - COMPLETE RESPONSE

CRC - COLORECTAL CANCER

MDM2 - MURINE DOUBLE MINUTE 2

NA - NOT AVAILABLE

NED - NO EVIDENCE OF DISEASE

PBS - PHOSPHATE BUFFERED SALINE

PR - PARTIAL RESPONSE

1. INTRODUCTION AND REVIEW OF LITERATURE

1.1 Introduction of rectal carcinoma

Colorectal carcinoma is a common cancer all over the world. Colorectal cancer ranks second in terms of both incidence & mortality in more developed countries. Nearly 945,000 new colo-rectal cancers cases are diagnosed world wide each year and colo-rectal cancer is responsible for some 492,000 deaths. There is significant geographical variation in age standardized incidence as well as in cumulative 0-74 years incidence, high rates occurring in countries of Europe, North America, Australia and Japan (**Bernard W. et al 2003**). For carcinoma rectum, the crude incidence rate per 100,000 populations in Chennai, India and world for males are 3.0, 3.6, & 16.4 respectively and for females are 1.6, 2.6 and 14.8 respectively. The age standardized rates per 100,000 population in Chennai, India and world for males are 3.7, 4.7, and 19% respectively and for females are 1.9, 3.2 and 14.4 respectively (**GLOBOCAN 2000, IARC).** Geographic variation in colorectal cancer incidence implies and to a large extent proves the critical nature of environmental factors. The incidence rate for residents of Alaskan natives exceeds to 70 per 100,000 (**Brown**

MO et al, 1998), whereas that far residents of Gambia and Algeria is less than 2 per 100,000 (**Parkin, DM et al, 1999**).

The etiology of colorectal cancer is complex involving an interplay of environmental and genetic factors. Familial factors contribute to the risk of sporadic colorectal cancer. Involvement of at least one first degree relative with colorectal cancer doubles the risk of colorectal cancer (**Fuchs CS et al, NEJM 1994**). There is further enhancement of the risk if such a relative is affected before the age of 60yrs. Obesity and total caloric intakes are independent risk factors for rectal cancer (**Singh PN et al, 1998**). Ingestion of red meat is associated with an increased risk of colorectal cancer. Fried, barbecued and processed meats are associated with rectal cancer, with an odds ratio of 6 (**Chan J. et al, 1998**).

Vegetable, fruits and calcium has been implicated as having a protective effect on colorectal cancer development. A sedentary life style may account for an increased colorectal cancer risk. Association between alcohol consumption in men and risk of rectal cancer are strongest. Perhaps interference with foliate metabolism through acetaldehyde is responsible (**Seitz HK et al, 1990**). There is an inverse relationship between use of aspirin, NSAID and the incidence of both colorectal cancer and adenomas. In a cohort study, the relative risk of

colorectal cancer was 0.49 (95% confidence interval, 0.24 to 1.0) for regular NSAID users compared with non users (**Smalley W et al, 1999**). Familial syndromes associated with increased risk of colorectal cancer are FAP, HNPCC, and Peutz Jeghers syndrome, familial colorectal cancer syndrome.

The major malignant histologic type is adenocarcinoma. Other less common epithelial tumour types include mucinous adenocarcinoma, signet ring carcinoma, squamous cell carcinomas, adenosquamous carcinomas and undifferentiated carcinomas.

The college of American pathologists has published an expert panel consensus statement outlining its interpretation of the validity and usefulness of a large number of putatively prognostic and predictive factors in colorectal carcinoma (**Compton CC et al, 2000**). Variables were categorized as belonging to categories I through IV. The categories are defined as:

Category I : Those factors proven to be of prognostic importance based on evidence from multiple statistically robust, published trials and generally used in patient management.

Category 1 factors are the T, N, and M categories of the current AJCC-UICC staging system, blood or lymphatic vessel invasion,

residual tumor after surgery with curative intent (the R category), an elevation of the preoperative CEA level.

Category II A : Factors intensively studied biologically or clinically as both and repeatedly shown to have prognostic value for outcome or predictive value for therapy that is of sufficient importance warrant inclusion in the pathology report but that remain to be validated in statistically robust studies. Factors in category IIA included tumor grade, radial margin status (for resection of specimens with nonperitonealized surfaces), and residual tumor in the resection specimen after neo-adjuvant therapy.

Category II B : Factors shown to be promising in multiple studies but for which sufficient data are lacking for inclusion in category I or IIA. Factors in category IIB included histologic type, histologic features associated with MSI (i.e., host lymphoid response to tumor and medullary or mucinous histologic type), high degree of MSI (MSI-H), loss of heterozygosity (LOH) of 18q (DCC gene loss), and tumor border configuration (infiltrating vs. pushing border).

Category III : Factors felt to be not yet sufficiently studied to determine their prognostic value. Factors grouped in category III included the following: DNA content, all other molecular markers except LOH of 18q/DCC and MSI-H, perineural invasion,

microvessel density, tumor cell–associated proteins or carbohydrates, peritumoral fibrosis, peritumoral inflammatory response, focal neuroendocrine differentiation, nuclear organizing regions, and proliferation.

Category IV : Factors that have been adequately studied and have convincingly shown no prognostic significance. Those factors included in category IV (proven to be of no significance) include tumor size and gross tumor configuration.

1.2 Cell Cycle: Cell cycle is comprised of four phases G1 (Gap 1), S (DNA synthesis), G2 (Gap 2) and M (Mitotic).

Details of Cell Cycle:

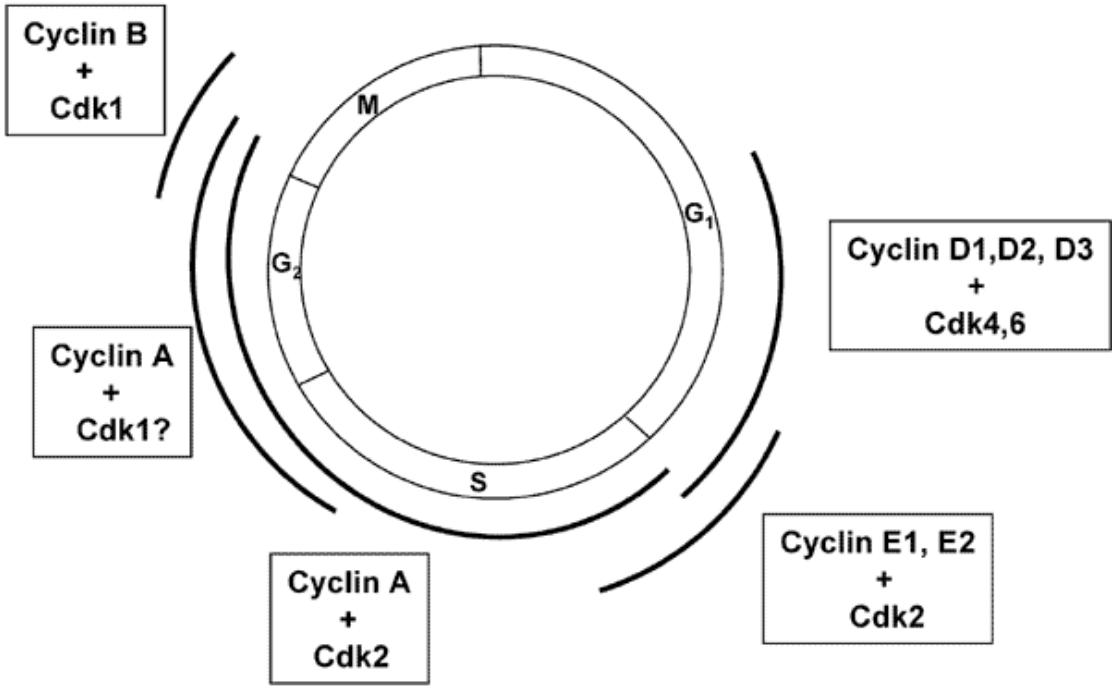

FIGURE 1

To summarize, three partially redundant D-type cyclins (D1, D2, and D3) activate two partially redundant cdks (cdk4 and cdk6). Although, unlike most other cyclins, D-type cyclins do not appear to be expressed with high periodicity in cycling cells, the interval at which their primary activating function is thought to occur is from mid to late G_1 to direct phosphorylation of the cell-cycle inhibitor pRb and related proteins p107 and p130. Phosphorylation of these proteins by cyclin D–cdk4/6 inactivates their negative regulatory functions, allowing progression into S phase. Unlike D-type cyclins, E-type cyclins (E1 and E2) are expressed with high cell-cycle periodicity, accumulating in late G_1 and declining during S phase. E-type cyclins activate cdk2, and the fact that premature expression of cyclin E1 leads to accelerated entry into S phase has suggested that the target(s) must be proteins responsible for initiation of DNA replication. However, the essentiality of cyclin E–cdk2 in this context has been put into question by the demonstration that cells from cyclin E1/E2 nullizygous mouse embryos can cycle with reasonably normal kinetics and can certainly initiate DNA replication. The most likely explanation for the dispensability of E-type cyclins for S-phase functions is redundancy with cyclin A, which also activates cdk2.

Cyclin A accumulates initially at the G_1/S-phase boundary and persists until prometaphase of mitosis. It has been best characterized as an activator of cdk2; however, it has also been reported to form complexes with cdk1. It is presumed that cdk2, activated by E-type cyclins and cyclin A, promotes cell-cycle progression from the G_1-S boundary through the G_2 interval.

At this time, B-type cyclins, in conjunction with cdk1, are responsible for getting cells into and through mitosis. Although mammalian cells express a number of B-type cyclins, only cyclin B1 appears to be essential. Cyclin B1 accumulates through S phase and G_2 and then is degraded at the metaphase-anaphase transition. It should be pointed out that the cdk family is extensive and that eukaryotes possess many additional cdks that ostensibly has nothing to do with cell-cycle regulation.

1.21 Cyclin Dependent Kinases:

The major transitions of the eukaryotic cell cycle are triggered by a family of serine threonine kinases called the Cyclin Dependent Kinases (CDKS). At least nine CDKs (CDK 1-9) are known so far (Morgan, etal 1997). CDK activation required the regulatory subunit called the cyclins and phosphorylation of a conserved threonine by the

CDK activating kinase (CAK) which itself is a complex of a regulatory cyclin H and catalytic CDK 7 subunit.

The typical CDK catalytic unit contains 300 amino acids. The catalytic subunit is completely inactive when it is monomeric and unphosphorylated. Cyclins are the primary regulators of the CDKs.

CDK1 constitute the homologous 34 KDa products encoded by the yeast cell division cycle (cdc) genes. p34 cdc2 (CDK1) is a highly conserved cyclin associated 34 KDa protein kinase that becomes activated on phosphorylation. Complexed with cyclin B it forms the maturation promoting factor (MPF). Without p34 cdc2 the cells are unable to divide (**Hamaguchi et al, 1992**), p34 cdc2 distribution is cytoplasmic during the interphase, shifts into the nucleus at the beginning of prophase and extends throughout the cell in the mitotic phase (**Bailly et al, 1989, Doussis et al, 1994**). As key regulators of the cell cycle, the activity of cyclin dependent kinases is controlled by four highly conserved biochemical mechanisms forming a web of regulatory pathways mismatched in this elegance and intricacy.

Cyclins are a class of structurally related proteins that bind and activate the catalytic subunit of the CDKS. To date there are eight type of cyclins, (cyclins A to H) and all of them share an 150 amino acid region of homology call the cyclin box which is responsible for the

CDK binding and activation **(Hunt et al 1991**). Cyclins can be roughly divided into two subfamilies, the G1 cyclins and the mitotic cyclins. The G1 cyclins (C, D, E) are short lived and have rapid turnover throughout cell cycle. Their levels are determined by levels of transcription of their mRNA. The mitotic cyclins (A, B) are very stable throughout the interphase but undergo rapid proteolysis by an ubiquitin dependent pathway during mitosis, compared to the mitotic cyclins the G1 cyclins have a longer C terminal sequence at the cyclin box and it is this part of the protein that seems to confer instability to the G1 cyclins (**Glotzer et al**, **1991**). The function of cyclins is primarily controlled by changes in the cyclin levels which increase at specific stages and are often categorized by the stage at which they are expressed. The transcription of the mitotic cyclins, cyclin A and B are cell cycle dependent and their levels are determined both by transcription and proteolysis (**Hunter et al, 1992, Minshull et al, 1990).** Protein degradation is an effective method for promoting in unidirectional cell cycle transitions because of its rapidity and irreversibility. Three major cell cycle transitions, entry into S phase, separation of sister chromatids and exit from mitosis, require the degradation of specific proteins via the ubiqutination by 26 S proteosome pathway.

The degradation of mitotic cyclins involves the ubiquitin dependent proteolytic machinery and a small sequence motif called the destruction box located at the N terminus. This region has a small cluster of conserved residues followed by lysine rich stretch. The destruction box region differs between cyclin A and B and probably accounts for the finding that in mitosis cyclin A is degraded before cyclin B.

G1 cyclins lack the mitotic destruction box but contain the PEST sequences as the peptide motifs that are important for proteolysis. PEST sequences (rich in proline, glutamic acid, serine and threonine) are frequency present in unstable proteins such as G, cyclins and contain specific sites of phosphorylation. Phosphorylations of PEST regions facilitate the destructions of PEST sequence proteins. It is not the PEST sequences per se but the specific motifs within them that actually control the PEST sequence recognition by the ubiquitination machinery (**Rechsteiner and Rogers et al, 1996).**

Cyclins are thought to target the CDK to specific substrate and sub cellular locations. Cyclin expression varies during cell cycle and the periodic expression of different cyclins defines the start of each phase of the cell cycle and also marks the transitions between the

various phases. Cyclins and their cognate CDK catalytic subunits noncovalently form 1:1 complexes to produce the CDK holoenzyme. Specific CDKs operate in distinct phases of cell cycles.

TABLE 1: CYCLINS AND CELL CYCLE PHASES

CYCLIN	CELL CYCLE PHASE
Cyclin D1	Early G1 phase
Cyclin E	G1 / S tansition
Cyclin A	S phase
Cyclin B	G2 / M phase

1.22 Cyclin D:

The first groups of cyclins that is expressed after the cells are stimulated to enter the cell cycle are the cyclin D. these act as growth factor sensors. Cyclin D has a relatively short half life of 20 minutes approximately and rapidly disappears with the removal of mitogenic stimuli as the addition of antiproliferative agents (Peter SG, 1994). Cyclin D helps in moving G0 cells into G1 and early G1 cells into the G1 / S transitions in response to the extracellular stimuli.

The D type cyclins belong to a distinct subset within cyclin family based on structural and functional criteria. There are three

genes identified under the family, Cyclin D1, D2 and D3 (**Inaba et al, 1992, Xiong et al, 1992a**)

TABLE 2: TYPES OF CYLIN D

1.23 Cyclin E:

After cyclin D induction, but well before the onset of S phase, cyclin E is transcriptionally induced and forms an active complex with CDK2. Cyclin E is essential for the cells to enter S phase. Cyclin E mRNA of and protein levels and the activity of cyclin E -CDK2 complex, all peak at the G1 / S transition sharply declining as cells progresses through mid and late S phase (**Guadagno et al, 1993).** When cyclin E is over expressed, the cells progress through G1 into S phase at a faster rate (**Ohtsubo et al, 1993**). Cyclin E shares some of the characteristics with cyclin A which regulate cells once they have entered the S phase. Both cyclin E and A in conjunction with CDK2 associate with pRB related proteins p107 and transcription factor E2F.

Over expression of cyclin E shortens the G1 phase, decreases cell size and diminishes the serum requirement for the G1 to S phase transition. Cyclin E activity gets down regulated as the cells reach senescence due to the induction of a family of inhibitory proteins that inactivate cyclin-CDK complexes. Cyclin E is deregulated in human

cancers with amplification of cyclin E gene, common in gastric and colorectal cancers (**Keyomarsi et al, 1997**).

1.24 Cyclin A:

Cyclin A is induced shortly after cyclin E. Cyclin A binds and activates CDK2 in S phase and CDK1 in G2 and M phase. A complex of CDK2 and cyclin A is required for the cells to progress through S phase and complex of CDK1 and cyclin B is required for mitosis (**Sherr, et al 1996).**

Cyclin A has been implicated in regulation of both DNA replication (**Wang et al, 1992)** and mitosis. There are two types of cyclin A, A1 and A2 and it appears that cyclin A1 is restricted to meiosis (**Minshull J. et al, 1990**). Cyclin A2 binds to both CDK1 and CDK2 in a cell cycle dependent manner. Cyclin A2 synthesis begins as cells enter S phase and at this point it is exclusively associated with CDK2 (**Tsai et al, 1991**). Cyclin A CDK2 complex is required for DNA replication though not for G1 to S transition. Cyclin A CDK2 is required for progression through S phase.

Cyclin A / CDK2 has the important function of histone phosphorylation. One of the events required for cyclin A transcription is a signal from surface adhesion molecule in either late G1 or very early S phase. Once cells progress into late G2

phase, cyclin A2 becomes associated with CDK1 (**Pagano et al, 1992**). The exact roles of cyclin A - CDK1 complex versus cyclin A - CDK2 has not been defined. One potential role for cyclin A - CDK1 / CDK2 in late G2 phase is in the re-organisation of the cytoskeleton in preparation for mitosis (**Verdeet et al, 1992**).

1.25 Cyclin B:

B type cyclins are necessary for the activation of the cdc2 kinase at the onset of mitosis. Cyclin B degradation is required for the inactivation of the H1 kinase in late mitosis. Cyclin B first forms an inactive complex with CDK1 (cdc2) on such a complex called pre-MPF (Maturation Promoting Factor) is activated by a post translational modification at the onset of mitosis. While the rate of cyclin B synthesis during cell cycle is linear, activation of MPF at G2/M transition is exponential suggesting that cyclin B accumulation alone does not trigger the initiation of mitosis. The levels of tyrosine phophorylation vary during the cell cycle increasing from G1 and G2 and disappearing during mitosis coincident with activation of the H1 kinase **(Draetta et al, 1988)**. During interphase, CDK1 (cdc2) and cyclin B associate and are readily phosphorylated on Thr-14, tyr-15 and Thr-161. This

inactive pre-MPF accumulates during S and G2 phases of the cell cycle. Dephosphorylation of CDK1 (cdc2) on Thr-14 and Tyr-15 is the rate limiting step controlling entry into mitosis.

After dephosphorylation of these residues, the cdc2-cyclin B complex becomes active and mitosis can start. Ubiquitination and subsequent degradation of cyclin B brings about the inactivation of MPF which is necessary for exiting from mitosis. After dephosphorylation of Thr-161 which probably occurs after cyclin degradation, monomeric unphosphorylated CDK1 (cdc2) is formed and the cell cycle can restart.

In contrast to cyclin A, cyclin B only associated with CDK1 and these complexes are the mitotic specific kinases. The sub cellular location of the cyclin B complexes is also cell cycle regulated. During S phase and G2 phases the complexes accumulate in the cytoplasm, becoming associated with the centrosomes late in the interphase **(Pines et al, 1991**). Once the cells reach mitosis complexes move into the nucleus. The cyclin B- CDK1 complexes enter the nucleus at the beginning of the prophase, before the nuclear envelope breaks down and serves as the protein kinase responsible for the phosphorylation and consequent dissolution of the nuclear lamina (**Peter et al, 1990**). Inside the nucleus, the cyclin B 1-CDK1 becomes associated with the

spindle especially the spindle caps and main spindle fibers. Cyclin B1 has profound effects on the microtubules (**Verde et al, 1992**) but their exact role in organizing the spindle is yet to be defined **(Karsenti et al, 1991**). The activity of cyclin B1-CDK1 complexes is negatively regulated by phosphorylation on the threonine 14 and tyrosine 15 residues in the ATP binding region of the CDK1 promoted by the weel and mik 1 protein kinase. In order for the cell to enter mitosis, the cyclin B1- CDK1 complexes are activated by CDC 25 phosphatase.

In this study the expression of cyclin A is assessed in normal rectal mucosa, rectal carcinoma and to assess the prognostic /predictive value of cyclin A over expression in rectal carcinoma.

1.3 Review Of Literature

Bondi J, et al ; Deregulation of cell cycle control is a hallmark of cancer. The primary cyclins (A, B1, D1, D3, and E) are crucial for cell cycle progression. Secondary cyclins (C and H) have putative indirect effects on cell cycle progression and have not previously been evaluated in colon cancer. This study examined cyclin protein expression and gene amplification in colon adenocarcinoma and the correlation with patient outcome. Immunohistochemistry and real time quantitative polymerase chain reaction were used to determine

cyclin expression and gene amplification in 219 tumours. RESULTS: Cyclin H was overexpressed in all tumours, cyclin C in 88%, cyclin B1 in 58**%, cyclin A in 83%,** cyclin D3 in 36%, cyclin E in 25%, and cyclin D1 in 11% of the tumours**. Extra gene copies of cyclin A were seen in 6.2% of the tumours,** cyclin B1 in 9%, cyclin C in 26.9%, cyclin D1 in 55%, cyclin D3 in 20.5%, cyclin E in 19.1%, and cyclin H in 5.1%. A significant correlation between protein overexpression and gene amplification was seen for cyclin C only. ***High expression of cyclin A was independently associated with improved survival***. Amplification of cyclin C was independently associated with an unfavourable prognosis.

Nozoe T, et,al. Immunohistochemical staining for cyclin A was performed for 167 colorectal carcinomas and the correlation between cyclin A expression and the clinicopathological characteristics was analyzed. One hundred and two carcinomas **(61.1%) had cyclin A expression** and the other 65 (38.9%) did not. The mean size of the tumors with cyclin A expression was significantly larger than that of tumors without cyclin A expression ($p = 0.012$). Survival in patients with cyclin A-expressing carcinomas was significantly worse than that in patients with carcinomas without cyclin A expression ($p = 0.004$). Cyclin A expression ($p = 0.030$), as well as lymph node metastasis ($p = 0.007$) and Dukes' stage of the tumors ($p < 0.0001$) were found to be

factors independently associated with unfavorable prognosis in patients with colorectal carcinoma. ***Our results demonstrated that immunohistochemical expression of cyclin A is an independent prognostic indicator in patients with colorectal carcinoma.***

Li JQ, et,al. To clarify the role of cyclin A in multistage colorectal neoplasms, cyclin A, CDK2, and Ki67 were immunohistochemically stained in 22 normal mucosa, 9 hyperplastic polyps, 61 adenomas, 197 primary carcinomas, 21 lymph node metastases, and 10 hepatic metastases. To clarify the alteration of p27(kip1) during lymphatic invasion, p27(kip1) was also stained in 21 primary cancers and paired lymph node foci. Situated in nuclei, cyclin A expression gradually increased from mild through moderate to severe dysplasia in adenomas and from normal tissue through hyperplasia to adenoma to early carcinoma. Expression was significantly decreased in the hepatic metastases and in the primary cancers showing venous invasion, deep infiltration, lymph node metastasis, mucinous type, advanced stage, or short postoperative survival time. Lymph node metastases lost more p27(kip1) than primary foci and hepatic lesions. ***Thus, dysregulation of cyclin A and its control mechanisms may contribute to colorectal carcinogenesis; abatement of overexpression of cyclin A is associated with hepatic***

metastasis and cancerous invasion. Loss of p27(kip1) may promote lymph node metastasis.

Palozza P, et,al; The present study demonstrates that beta-carotene, a natural pigment widely present in fruit and vegetables, inhibits the growth of several human colon adenocarcinoma cell lines (COLO 320 HSR, LS-174, HT-29 and WiDr) by inducing cell cycle arrest in G(2)/M phase and apoptosis. At inhibitory concentrations beta-carotene reduced the expression of cyclin A, a key regulator of G(2)/M progression. Neither p21 nor p27, two cyclin kinase inhibitors, were significantly modified by carotenoid treatment. This study represents a novel aspect of the biological profile of beta-carotene and a new step in elucidating the underlying molecular mechanisms of its antitumor action. In addition, since cell growth inhibitory effects were reached at beta-carotene concentrations achievable in vivo following its supplementation, this study provides a rational approach for the use of beta-carotene in colon cancer.

Habermann J, et,al; Ulcerative colitis patients are at increased risk for developing colorectal carcinomas. Two patient groups were selected: group A comprised 8 patients with ulcerative colitis-associated colorectal carcinomas, group B comprised 16 ulcerative colitis patients with risk factors (duration of disease, extent of inflammation, epithelial dysplasias). A total of 683 paraffin-embedded

mucosal biopsies were retrospectively evaluated for inflammatory activity, grade of dysplasia, ploidy status, laminin-5 gamma2 chain and cyclin A expression. RESULTS: Mild or moderate inflammatory activity was present in 78% of all biopsies, low- or high-grade dysplasia in 5.5%. There was no difference in inflammatory activity and dysplasia between patient groups. In group A, 75% of the biopsies exhibited aneuploid DNA distribution patterns. Group B showed mainly proliferative-diploid cell populations (85% / P = 0.006). Laminin-5 gamma2 chain was expressed in 13% of all biopsies, with a higher frequency in group A (P = 0.002). ***Cyclin A expression was found in 98% of all biopsies, with a higher number of immunopositive cells in group A biopsies*** (P = 0.014). Combined nuclear DNA assessment, laminin-5 gamma2 chain and cyclin A expression may help to identify ulcerative colitis patients with an increased risk for cancer development.

Handa K, et,al; Our aim was to analyze the relationship between the proliferative activity of cancer cells, assessed using some cell cycle markers, and clinicopathological factors in colorectal carcinoma patients. Immunoreactivity was evaluated semiquantitatively using a scoring system to calculate a staining index (SI). The expression of cyclin D1, histone H3 mRNA and cyclin A correlated significantly with Ki-67 antigen expression. The SIs of Ki-

67, cyclin A and histone H3 mRNA were significantly higher in patients > or = 65 years of age than in those < 65. The SIs of Ki-67 and cyclin D1 in poorly differentiated adenocarcinomas were significantly higher than in the other tumor types. ***The overall survival was significantly lower in patients with cyclin A overexpression than in those without. Multivariate analysis indicated that cyclin A overexpression is an independent prognostic factor in patients with colorectal adenocarcinoma***. Our results indicate that cyclin D1 overexpression correlates with poor adenocarcinoma differentiation and tumor progression, and ***cyclin A overexpression is a superior indicator of poor prognosis compared with the other cell cycle markers tested.***

Wang A, et.al; Cyclin E was higher in the cancer tissue than in the non-neoplastic mucosa in 92% patients (35 out of 38 cases). However, the cyclin A expression of the mucosa was higher than that of the cancer tissue in 63% (25 out of 40 cases) cases, and only 4 (10%) cancers had higher cyclin A expression. Eleven cancers (27%) demonstrated expression equivalent to that in the mucosa. Equal expression of cyclin D1 in cancer and mucosal tissues was found in 51% cases (20/39), lower expression of cyclin D1 by cancer tissues was demonstrated in 41% cases (16/39) and only three cancers showed higher expression than the mucosa. Proliferating-cell nuclear

antigen immunohistochemistry revealed that the labeling index of the cancer tissue was 43.5 +/- 8.3% while that of the mucosa was only 14.8 +/- 5.1%. These results proved that ***colorectal cancers express high levels of cyclin E, consistent with a high rate of cell proliferation, whereas most of such cancer lose control of cyclin A and cyclin D1 expression.***

Bahnassy AA, et,al; Aberrations in the cell cycle checkpoints have been shown to be of prognostic significance in colorectal cancer. The expression of cyclin D1, cyclin A, histone H3 and Ki-67 was examined in 60 colorectal cancer cases . Immunoreactivity was evaluated semi quantitatively by determining the staining index of the studied proteins. The staining index for Ki-67, cyclin A and D1 was higher in large, poorly differentiated tumors. The staining index of cyclin D1 was significantly higher in cases with deeply invasive tumors and nodal metastasis. Overexpression of cyclin A and D1 and amplification of cyclin D1 were associated with reduced overall survival. Multivariate analysis shows that cyclin D1 and A are two independent prognostic factors in colorectal cancer patients. ***Cyclin A and D1 are superior independent indicators of poor prognosis in colorectal cancer patients.***

2. AIMS & OBJECTIVE

1. Expression of cyclin A in normal rectal mucosa & rectal carcinoma
2. Correlation of cyclin A over expression with stage & grade of rectal carcinoma.
3. Correlation of cyclin A over expression with response to neoadjuvant treatment and recurrence.

3. MATERIAL & METHODS

The expression of cyclin A was evaluated in normal rectal mucosa & rectal carcinoma by immunohistochemistry technique. Immunoreactivity was evaluated semiquantitively by determining the staining index of the studied protein.

3.1 Patient & tumor details:

Patients with a diagnosis of nonmetastatic rectal carcinoma were considered for the study. Patients came to cancer institute (WIA) during 2002 to 2003, were prospectively evaluated, treated with protocol treatment, re evaluated, operated, treated with adjuvant chemotherapy according to the stage & then followed up.

Punch biopsy was taken from the rectal growth & processed in pathology department, paraffin blocks were made & sections were cut for histological evaluation, 4 micron thin sections were cut for immunohistochemistry study.

After complete evaluation nonmetastatic rectal carcinoma patients who were considered fit for protocol treatment were considered for the study. Stage grouping of the rectal carcinoma was done according to the AJCC cancer staging, 6th edition.

3.2 TNM STAGING:

T Stage:

In **situ** adenocarcinoma (Tis) includes cancers confined to the glandular basement membrane or lamina propria.

T1 tumours invade into but not through the submucosa.

T2 tumours invade into but not through the muscularis propria.

T3 tumours invade through the muscularis propria into the subserosa or into nonperitonealized pericolic or perirectal tissue.

T4 tumours invade other named organs or structures (**T4a**) or perforate the visceral peritoneum (**T4b**). Tumours invading other colorectal segments by way of the serosa (i.e., carcinoma of the cecum invading the sigmoid) are classified as T4a. A tumor that is adherent to other structures or organs macroscopically is classified clinically as T4a; however, if the microscopic examination of the adhesions is negative, then the pathologic classification is pT3.

N Stage:

A **pN0** designation may be made even if fewer than the recommended numbers of nodes are present; however, the prognostic

significance of this pN0 designation is weaker. N0 denotes that all nodes examined are negative.

N1 includes tumours with metastasis in one to three regional lymph nodes.

N2 indicates metastasis in four or more regional lymph nodes. Metastatic nodules or foci found in the pericolic, perirectal, or adjacent mesentery without evidence of residual lymph node tissue are regarded as being equivalent to a regional node metastasis and are counted accordingly.

M Stage:

M0 disease if no evidence of distant metastases is present.

M1 disease if distant metastases are present. Involvement of the external iliac, common iliac, para-aortic, supra-clavicular, or other non-regional lymph nodes is classified as distant metastatic (M1) disease.

Stage grouping:

Stage I disease is defined as T1 to T2N0 disease in a patient without distant metastases (M0).

Stage II disease is defined as T3 to T4N0M0. T3N0 is classified as stage IIA and T4N0 is classified as stage IIB.

Stage III disease is defined node positivity in the absence of M1 disease. The current TNM staging system stratifies stage III disease into

IIIA --T1 to T2N1.

I**IIB** --T3 to T4N1.

IIIC--Any T, N2.

Stage IV disease is defined as metastatic disease**.**

3.3 Histological Grade:

The tumours were graded into

grade 1--well differentiated. grade 3--poorly differentiated.

grade 2--moderately differentiated. grade 4--undifferentiated.

3.4 Protocol treatment:

Protocol treatment comprises of neoadjuvant concurrent two cycles of chemotherapy (5 fluorouracil & mitomycin C) & 50 gray external beam radiotherapy to the pelvis. One month after completion of protocol treatment patients were reassessed & taken up for operative procedure (anterior resection /abdomino- perineal resection).

The specimens were examined & histopathological evaluation was done. Biopsies from normal rectal mucosa and rectal growth were taken for immunhistochemistry study.

3.5 Immunohistochemistry procedure:

3.51 Slide preparation:

The paraffin embedded sections of 4 micron size were placed on APES coated slides. The slides were treated in xylene consecutively twice for 8 minute each for dewaxing of the sections. This was followed by treatment in100% alcohol consecutively twice for dehydration for one minute each .The slides were washed in tap water for 8 minutes, taking care not to disturb the sections. Followed by tap water wash, the slides were placed in 0.3 % hydrogen peroxide for 30 minutes to quench the endogenous peroxidase activity in the tissues.

3.52 Antigen retrieval:

For the protein involved in the study needed antigen retrieval to expose the epitopes for immune reaction. Antigen retrieval was done by placing the slides dipped in 1mM EDTA solution at pH 8 in autoclave at 121 degree centigrade, 15 pound pressure for 0 minute holding time. After antigen retrieval the slides were cooled & than washed with fresh PBS solution for 5 minutes.

3.53 Blocking & antibody reaction:

The slides were arranged in a moist chamber to prevent drying of the sections & 100 Micro liter of 3 % BSA was added to each section, taking care to cover the whole section &than left undisturbed for 30 minutes. Following gentle tipping & drying of BSA off the slides the primary antibody (75 micro liter) in appropriate dilutions (1:50) was added to each section. Care was taken to omit the negative control. They were left for 45 minutes to incubate with primary antibody (cyclin A) at room temperature. The slides were washed with fresh PBS thrice for 5 minutes each to wash the excess of primary antibody. Secondary antibody (Anti cyclin A antibody, 75 micro liter) was added in appropriate dilutions (1:500 in 3% BSA) to each slide & incubated for 30 minutes. The slides were washed in PBS thrice for 5 minutes each followed by drying & addition of fresh ABC complex (75 micro liter) onto each slide and incubated for 30 minute at room temperature.

ABC solution is made by 9 microliter of Avidin & 9 micro liter of biotin mixed in 982 microliter of fresh PBS.

3.54 Treatment with chromogen:

The slides were washed in PBS thrice for 5 minutes each to wash the excess of ABC and the slides were treated in a solution of

DAB (150 ml of water + 150 ml of PBS + 100 micro liter of hydrogen peroxide + 150ml of DAB) for 5 minutes. The excess of DAB was washed off by keeping the slides in tap water for 10 minutes and counter stained in haematoxylene for 2 minutes. Followed by water wash for 10 minutes. The slides were fixed in lithium carbonate saturated solution and washed under tap water once again for 10 minutes. The slides were drained well and treated with 100 % ethanol twice for 30 seconds each. This was followed by treatment in xylene consecutively for 5 minutes each and sections were mounted using DPX.

3.55 Statistical procedure:

The results of the immunohistochemical study were examined for correlation of cyclin A over expression with clinico-pathological parameters using the Chi square test for contingency tables.

3.56 Photographs

PHOTOGRAPH 1

This photograph is of invasive carcinoma of cervix.

Positive Control Slide — The brown nuclei in the photograph denote cyclin A over expression.

PHOTOGRAPH 2

Negative Control Slide

PHOTOGRAPH 3

This photograph is of rectal carcinoma, the brown nuclei denotes cyclin A over expression.

PHOTOGRAPH 4

Signet ring cell carcinoma with very low cyclin A over expression.

4. RESULTS & DISCUSSION

4.1 Cyclin A expression in normal rectal mucosa & rectal carcinoma:

TABLE 3

Cyclin A Expression	Normal rectal mucosa (3 cases)	Rectal carcinoma (18 cases)
Nil	0	4 (19%)
<10%	3 (14.4%)	0
>10%	0	14 (66.6%)

Nuclear cyclin A expression was less then 10% in normal rectal mucosa. Nuclear cyclin A expression was not seen in 19% of rectal carcinoma. Nuclear cyclin A over expression (i.e.>10%) was seen in 66.6% of rectal carcinoma.

4.2 The age & sex distribution:

Table 4

83.3% of the rectal carcinoma patients are male & 16.7% are female. The age distribution in male are 44.4% patient was in age group of 41 to 60 year, 16.5% in age group of 61-80% & 22.2% in age group of 21-40 years. Amongst females 11% were of age group 41-60year 5.55% in age group of 21-40 years.

4.3 Distribution of rectal cases carcinoma according to histology type:

Table 5

Histologic type	No of cases	18=100%
Adenocarcinoma	12	66.6 %
Mucin secreting Adenocarcinoma	4	22.2 %
Signet ring cell Carcinoma	2	11.1 %

Adenocarcinoma of rectum constitutes 66.6% of cases, 22.2% case were mucin secreting adenocarcinoma & 11.1% were signet ring cell carcinoma.

4.4 Distribution of rectal carcinoma according to nuclear grade:

Table 6

Nuclear grade	No. of cases (18)	18=100%
1	1	5.5%
2	14	77.7%
3	3	16.6%

77.7% of rectal carcinoma were nuclear grade II, followed by 16.6% grade III & 5.5% grade I.

4.5 Distribution of rectal carcinoma according to primary tumor stage:

Table 7

T stage	No. of cases(Total=18)	% (18=100%)
T 1	0	0
T 2	1	5.5
T 3	13	72.2
T 4	4	22.2

72.2% of rectal carcinoma was in T3 primary tumor stage, 22.2% in T4 & 5.5% in T2 primary tumor stage.

4.6 Distribution of rectal carcinoma according to recurrence:

Table 8

Recurrence	**No. of cases(18)**	**18=100%**
present	7	38.9%
No rec.	11	61.1%

The follow up duration of patients ranged from 7 month to 44 month, 38.95 of patients developed recurrence.

4.7 Response to neoadjuvant protocal treatment:

Table 9

Response	**No. of cases**	**18=100%**
Complete	3	16.7
Partial	15	83.3

In 83.3% of carcinoma rectum patients partial response was achieved, & 16.7% of patients complete response was achieved.

4.8 Status of cyclin A over expression in rectal carcinoma:

Table 10

Cyclin A over expression	No. of cases (18)	18=100%
Absent	4	22.2
Present	14	77.7

77.7% of rectal carcinoma patients over expressed cyclin A & 22.2% cases did not express cyclin A.

4.9 Correlation of age & cyclin A expression:

Table 9

Age years	Cyclin A over expression	
	Present (%)	Absent
0-20	0	0
21-40	4 (80%)	1(20%)
41-60	7 (70%)	3(30%)
61-80	3 (100%)	0
81-100	0	0

4.10 Correlation of histologic type & cyclin A over expression:

Table 10

Histologic type	Cyclin A over expression(18 cases)	
	Present(%)	**Absent(%)**
Adenocarcinoma-12, cases	10 (83.3)	2(16.7)
Mucin secreting-4,cases	3 (75)	1(25)
Signet ring cell-2,cases	1 (50)	1(50)

The over expression of cyclin A in adenocarcinoma is 83.3%, in mucin secreting carcinoma is 75% & in signet ring cell carcinoma is 50%.

4.11 Correlation of nuclear grade & cyclin A over expression:

Table 11

Nuclear grade 18 cases	Cyclin A over expression	
	Present (%) 14 cases	**Absent(%) 4 cases**
1	0	1(100%)
2	12 (85.7%)	2(14.3%)
3	2 (66.6%)	1(33.4%)
4	0	0

pValue: 0.23

Nuclear grade I rectal carcinoma did not express cyclin A. Nuclear grade II rectal carcinoma, 85.7% over expressed cyclin A. Nuclear grade III rectal carcinoma, 66.6% over expressed cyclin A. Cyclin A over expression is more in high grade carcinomas.

4.12 Correlation of primary tumor stage & cyclin A over expression:

Table 14

Primary tumor stage 18 cases	**Cyclin A over expression**	
	Present (%) 14 cases	**Absent(%) 4 cases**
T1 0, case	0	0
T2 1	1 (100%)	0
T3 13	9 (69.2%)	4(30.8%)
T4 4	4 (100%)	0

pValue: 0.37

The primary tumor stage T2, 100% of patients over expressed cyclin A, as the number of cases are less correlation b/w T2 & cyclin A over expression may not right.

Primary tumor stage T3 the cyclin A over expression was seen in 69.2% & stage T4 the cyclin A over expression was seen in 100%.

4.13 Correlation of response to neo-adjuvant treatment out come & cyclin A over expression:

Table 15

Response to treatment ***18 cases***	***Cyclin A over expression***	
	Present (%) ***14 cases***	***Absent(%)*** ***4 cases***
Complete response 3,cases	2(66.6)	1(33.4%)
Partial response 15,cases	12(80%)	3(20%)

pValue: 0.79

80% of partial responders to neoadjuvant chemo-radiotherapy & 66% of complete responders to the neoadjuvant chemo-radiotherapy were over expressing cyclin A.

4.14 Correlation between recurrence & cyclin A over expression:

Table 16

Recurrence 18 cases	**Cyclin A over expression**	
	Present (%) 14 cases	**Absent(%) 4 cases**
Present 7,cases	4(28.6)	3(75)
Absent 11,cases	10(71.4)	1(25)

pValue: 0.14

Patients who recurred after complete treatment 28.6% over expressed cyclin A, whereas patients who did not recur the cyclin a over expression was seen in 71.4%

5. DISCUSSION

In this study Cyclin A over expression is studied in normal rectal mucosa and rectal carcinoma. The normal rectal mucosal expression of cyclin A is very less (14.4 %), when compared with over expression of cyclin A in rectal carcinoma (66.6 %). In the study done by Li. J.Q et al., Cyclin A expression gradually increased from mild through moderate to severe dysplasia in adenomas and from normal tissue through hyperplasia to adenoma to early carcinoma indicating that over expression is a relatively early event in colon carcinogenesis which is possibly responsible for the pathological changes in the mucosa preceding neoplastic transformation. More recently Bahanassy et al., Handa et al., Habermann et al., Nozoe et al., and Bondi et al., reported up regulation of cyclin A in 80%, 70%, 68%, 61% and 83% of their studied cases.

Cyclin A over expression is also reported in carcinoma of the breast by Buckholm IR etal, and cervix by Shiohara S etal & Vijayalakshmi etal.

Cyclin A is over expressed in carcinoma because large fraction of carcinomatous cells are in cell cycle phases.

Cyclin A over expression varies with the histological type. In our study, the cyclin A over expression is more in adenocarcinoma than mucin secreting adenocarcinoma and least in signet ring cell carcinoma. The study done by Li.JQ etal, they inferred that cyclin A over expression significantly decreased in mucinous type of colorectal adenocarcinoma. The decreased expression of cyclin A in mucinous and signet ring cell carcinoma may be due to presence of mucin which interferes with the immunoreaction. It needs a detailed study in a large number of cases and a correlative study to know the cause & its implications as prognostic and predictive markers.

In our study, the Cyclin A over expression in nuclear grade 2, 3 are 85.7%, 66.6% respectively. The nuclear grade 1 tumor did not express Cyclin A. Higher the nuclear grade the probability of cyclin A over expression is also more. Higher the nuclear grade the more the tumor is poorly differentiated hence more fraction of cells are in cell cycle phase & more expression of cell cycle markers. Bahanassy etal also concluded in his study that as the grade of primary tumor increases the cyclin A over expression increases.

The cyclin A over expression in correlation with primary tumor stage does not show any increasing & decreasing trend. It may be because as the primary tumor stage increases the size of the tumor also increases and more fraction of carcinoma cells are in the resting phase of the cell cycle these cells does not over expression cyclin A. It also depends from which site the sections are taken from the tumour. In the study done by Li.JQ et al they concluded that cyclin A over expression was significantly decreased in the advanced stage tumor, whereas Bhanassy etal concluded that staining index for cyclin A over expression is higher in large tumours.

Cyclin A over expression in partial responders to neoadjuvant treatment is 80% and in complete responders is 66.6%. The Cyclin A over expression presence or absence does not correlate with the response to neoadjuvant treatment. It needs further detailed study in large number of patients to come on a definitive conclusion.

In our study patients with over expression of cyclin A in rectal carcinoma 71.1% did not recur and patients with absence of Cyclin A over expression developed recurrence in 75% of cases. Bondi etal also concluded in his study that over

expression of cyclin A was independently associated with a better out come in colonic adenocarcinoma.

In the patients who developed recurrence after complete treatment the correlation of the presence & absence of cyclin A over expression does not show a trend, this may be because the recurrence of carcinoma also depends upon the completeness of resection of the carcinoma with a safe margin.

The number of patients & duration of followup in the present study is less. The duration of followup ranged between 7 to 44 months & only seven patients came for followup for more than 24months. Nine patients were lost to followup.

6. **CONCLUSION**

Cyclin A expression is seen in normal rectal mucosa & it is over expressed in rectal carcinoma.

Cyclin A over expression is less in mucin secreting adenocarcinoma & signet ring cell carcinoma of rectum in comparison to adeno carcinoma of rectum.

The over expression of cyclin A is more in higher grade rectal carcinoma.

As the primary tumor stage of rectal carcinoma increases the over expression of cyclin A also increases.

Presence or absence of cyclin A over expression does not correlate response to neoadjuvant treatment.

The correlation of recurrence of the rectal carcinoma after neoadjuvant chemo-radiotherapy followed by surgery & cyclin A over expression needs further evaluation in a large number of patients with a longer duration of follow up to come to a definitive conclusion.

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