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IMMUNOHISTOCHEMICAL ANALYSIS OF P21 EXPRESSION IN GASTRIC ADENOCARCINOMA. A RETROSPECTIVE ANALYSIS IN A TERTIARY CARE CENTRE.

Pathology

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ABSTRACT

BACK GROUNND: Gastric cancer used to be the second most commonest cancer, but diagnosed late when the disease is already in advanced stage. Hence we need a molecular marker that helps in early diagnosis, treatment protocols and to know the disease progression. **METHODS:** Histopathological details such as tumour type, grade, depth of invasion, lymph node and peritoneal deposits and liver metastasis of 50 gastric adenocarcinoma patients were tabulated. Paraffin blocks with tumour of those patients were retrieved, IHC for P21 was done and its expression was correlated with histopathological parameters. **CONCLUSION:** P21 IHC marker was positive in most of the well differentiated gastric adenocarcinomas and loss of P21 was noted in most of the poorly differentiated gastric adenocarcinoma and those tumours that have muscle invasion, lymph node deposits, peritoneal deposits and liver metastasis showed loss of P21 expression.

KEYWORDS

Gastric adenocarcinoma, Histopathology, Immunohistochemistry, P21 marker.

INTRODUCTION:

Gastric carcinoma is the second most common cancer and one of the leading causes of death worldwide(1). The symptoms and signs of gastric carcinoma are vague and nonspecific in early stage and hence most of the gastric carcinomas are diagnosed late when the disease is already in advanced stages with deep muscle invasion (2).

The major cause of mortality in gastric carcinoma patients are recurrence and metastasis even after a curative surgery. Gastric carcinoma is not very sensitive to chemotherapy. Despite knowing these facts, surgical resection and chemotherapy are considered as palliative treatment to give symptomatic relief and to decrease the tumor burden (2).

At molecular level, there are numerous epigenetic as well as genetic changes are known to occur in various genes such as increased expression of oncoproteins, dysfunction in regulators of cell cycle, loss of adhesion protein molecules in the cell and also loss of tumor suppressor proteins and genes (3). The molecular changes in regulators of cell cycle are one of important change involved in the carcinogenic process of human gastric carcinoma.

Hence we need molecular markers involved in pathogenesis of gastric cancer to develop primary prevention strategies. In this study P21 expression was analyzed by immunohistochemistry for its tumor suppressor activity and prognostic significance in gastric carcinoma.

AIM AND OBJECTIVES:

To analyze the P21 expression and its correlation with histopathological parameters like histological type, tumour grade and depth of tumour invasion in gastric adenocarcinoma.

To correlate the P21 expression in relation to lymph node, peritoneal and liver deposits in gastric adenocarcinoma.

To evaluate the prognostic value of the immunohistochemical expression of P21 in gastric adenocarcinoma.

REVIEW OF LITERATURE:

Gastric neoplasm is usually diagnosed in advance stage and has a very poor prognosis and those patients also have poor survival. Advanced gastric neoplasms usually do not respond to even newer chemotherapeutic drugs. So when the gastric cancer patients are diagnosed at an early stage using newer molecular biomarkers, it can be curable to some extent. Thus in view of better management of the patients with gastric adenocarcinoma, development of new molecular biomarkers is necessary to assess the outcome.

WHO and Laurens classification system are the most important system widely followed for gastric carcinoma. Lauren classified gastric cancer into two subtypes, intestinal and diffuse type. 86% of gastric cancers fit into these two subtypes. Other subtypes are mixed intestinal and diffuse and uncommon variants(4). WHO assigns the grades to adenocarcinoma based on the degree of differentiation and its similarity to metaplastic intestinal tissue into Well differentiated, Moderately differentiated & Poorly differentiated grades(5).

Gastric carcinoma can spread by direct extension, hematogenous dissemination, lymphatic spread and even transperitoneal dissemination. The most widely followed staging system is the TNM system proposed by the American Joint Committee on cancer(6).
The 5-year survival rate for patients with gastric adenocarcinoma is less than 20% in developing countries(7). The better management of the patients with gastric adenocarcinoma is possible with new molecular biomarkers which help to assess the outcome of those patients.

Cyclin dependent inhibitors are one of the molecular biomarker which can be used to assess the aggressive behaviour of the tumour. CDK inhibitors are grouped into CIP/KIP and INK family. P15, P16, P18 and P19 are the cell cycle inhibitors which come under INK category and P21, P57, P27 proteins comes under CIP/KIP group (8).

Cyclin dependent kinase phosphorylates RB gene protein & progress the cell cycle from G1 to S phase. Hence CDK inhibitors act as cell cycle inhibitors by inhibiting cyclin dependent kinases (9) (10).

P21 gene protein is one of the CDK inhibitor and p21 gene is located in short arm of chromosome 6(11). P21 function as a cell cycle inhibitor and antiproliferative effector in normal cells and is dysregulated in some cancers(12).

The analysis of P21 gene protein expression has been studied in various cancers like prostate, bladder, breast, colon, esophagus and lung for its prognostic significance and reported to have tumour

suppressor activity by inhibiting the cell cycle. The regulator of cell cycle like p21 has been studied in gastric carcinoma patients who showed a positive expression in early stage (13). Hence P21 can be used as one of the molecular markers for early gastric carcinoma. The histotechniques which help to evaluate the presence of P21 expression are immunohistochemistry and reverse transcriptase polymerase chain reactions.

INCLUSION CRITERIA

Fifty specimens both total and partial gastrectomy operated for primary gastric adenocarcinoma from April 2018 to March 2020 were included.

EXCLUSION CRITERIA

1) Patients who were treated with preoperative chemotherapy and radiotherapy.
2) Patients with gastro intestinal stromal tumors (GIST).

MATERIALS AND METHODS

A total of 50 specimens both subtotal and total gastrectomy which were diagnosed as gastric adenocarcinoma were taken up for this study. This retrospective study was conducted at Department of Pathology, Government Vellore Medical college and Hospital, Vellore, Tamil Nadu, India, during the period from April 2018 to March 2020. Tumours were subtyped according to Lauren's classification system and graded according to WHO grading system. Paraffin wax blocks of corresponding specimens with tumour were evaluated for P21 expression by Immunohistochemical technique based on Ultra vision One HRP polymer technique along with positive and negative controls. The positive control was normal skin and the negative control was obtained by omitting primary antibody.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is defined as a method for localizing specific antigens in cells or tissue by means of antigen-antibody interaction. The main factor in immunohistochemistry is the specificity of antibodies for particular antigens. IHC uses either polyclonal or monoclonal antibody to identify the particular antigen. The monoclonal antibody recognizes only a single type of epitope on an antigen. Hence monoclonal antibody is widely used (14).

The fixative used was 10% neutral buffer formalin for a duration of 24 hours for 3mm thick tissue slice. The tissue antigens are usually masked during formalin fixation and paraffin processing due to chemical reactions. Hence the antigens are first unmasked before staining sections by; Proteolytic enzyme digestion method, heat mediated antigen retrieval methods or combined of these two methods(15). In this study heat mediated antigen retrieval method was used.

Hydrogen Peroxidase block was applied to reduce the background staining due to endogenous peroxidase in the tissue. After TRIS buffer wash , Ultra V Power block was applied to block non specific antigen antibody reaction. Then Primary antibody (P21) was applied followed by super enhancer and Secondary antibody. Again after TRIS buffer wash, DAB substrate solution was added followed by counterstaining with haematoxylin. The sections were dried and mounted with DPX and IHC analysis done.

P21 IHC scoring was done using a criteria cited by Jang et al(16). A total of ten high power fields were selected and counted for the percentage of cancer cells with intense nuclear staining.

SCORE 0 : less than 5% of the neoplastic cells express intense nuclear staining.

SCORE 1 : 5-25% of the neoplastic cells express intense nuclear staining.

SCORE 2 :26-50% of the neoplastic cells express intense nuclear staining.

SCORE 3 : 51-75% of the neoplastic cells express intense nuclear staining.

SCORE 4 : more than 75% of the neoplastic cells express intense nuclear staining.

Tumours with score 0 & 1 was considered as negative for P21 expression whereas score 2, 3 & 4 was considered as positive for P21 expression. Statistical analysis was performed to correlate between the P21 expression status and histopathological parameters like histological type, tumour grade, depth of tumour invasion, lymph node metastasis, omental deposit and liver deposits. Results were tabulated and statistical significance was analysed.

OBSERVATION AND RESULTS:

Based on Laurens classification system, among the 50 cases studied, 36 cases (72%) were intestinal type and 14 cases (28%) were diffuse type. Hence the predominant growth pattern of adenocarcinoma in this study was intestinal type (Chart:1).

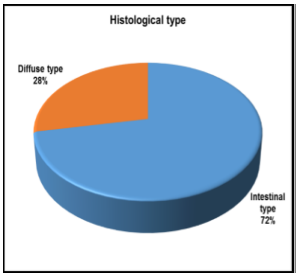

Chart 1: Laurens Histological type in 50 cases of gastric carcinoma

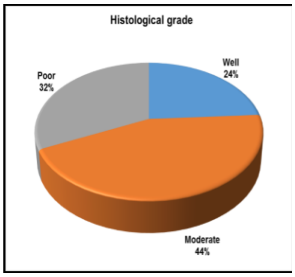

Chart 2: Histological Grade in 50 cases of Gastric Adenocarcinoma

Among the 50 cases studied, 22 cases (44%) were moderately differentiated adenocarcinoma(Figure:1), followed by poorly differentiated tumors(Figure:2) accounting to 16 cases (32%) and only 12cases (24%) were well differentiated tumors(Chart:2).

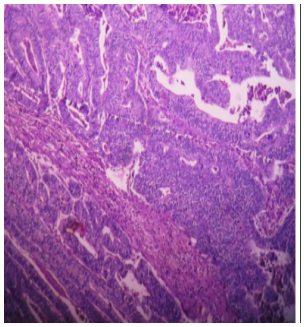

Figure:1. Moderately differentiated adenocarcinoma of stomach. 10X (H&E)-Neoplastic cells arranged in solid sheets along with areas of gland formation.

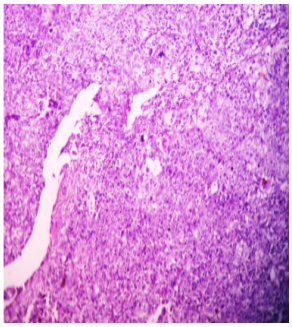

Figure:2. Poorly differentiated adenocarcinoma of stomach. 10X (H&E)-Neoplastic cells arranged in solid sheets

Among the 50 cases studied, 18 cases(36%) showed tumour invasion beyond muscularis propria into the subserosa, 14 cases (28%) showed invasion up to muscularis propria, 11 cases (22%) had invaded serosa and adjacent structures. Only 7cases (14%) were limited to mucosa and submucosa(Table:1)

Table 1: Depth of invasion in 50 cases of Gastric Adenocarcinoma

Depth of invasion	Number of cases	%
Mucosa & sub mucosa (T1)	7	14%
Muscularis propria (T2)	14	28%
Subserosal tissue (T3)	18	36%
Serosa & adjacent structures (T4)	11	22%

IHC analysis was done for P21 expression with normal gastric mucosa (Figure:3) or skin epithelium as positive control. P21 IHC scoring was done using a criteria cited by Jang et al(19). P21 IHC Score 4 with 95% of the cancer cells showing intense nuclear staining was observed in most of the well differentiated gastric adenocarcinoma (Figure:4), moderately differentiated adenocarcinoma showed variable P21 expression, with score of 0 to 4 (Figure: 5&6). Most of the poorly differentiated tumors showed P21 negative expression.

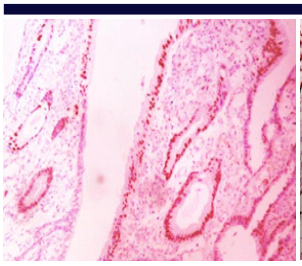

Figure:3. Normal gastric mucosal epithelium. Positive for P21 expression, (Internal control).

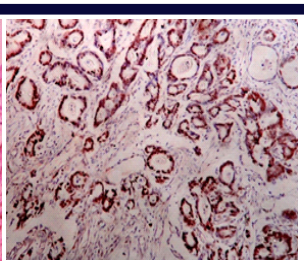

Figure: 4.Well differentiated adenocarcinoma of stomach. P21 IHC Score 4 :95% of the cancer cells showing intense nuclear staining.

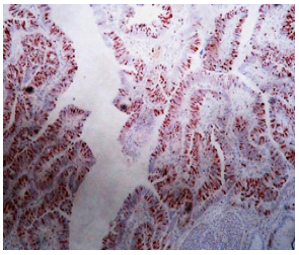

Figure: 5. Moderately differentiated adenocarcinoma of stomach. P21 IHC Score 4 : 90% of the cancer cells showing intense nuclear staining.

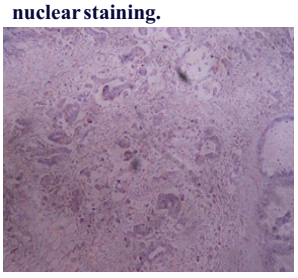

Figure: 6. Moderately differentiated adenocarcinoma of stomach. P21 IHC Score 0 : Negative

Most of the well differentiated tumours, 9 out of 12 cases were positive for P21 whereas most of the poorly differentiated tumors, 13 out of 16 cases showed negative for P21expression. Percentage of P21 positive cases decreased and percentage of P21 negative cases increased as grade of the tumor increased and was statistically significant (p value 0.003),(Table:2 & 3).

Table 2:P21 Expression Vs Histological Grade in Gastric carcinoma

Grade	Number of Positive cases	Number of Negative cases	P21 Positive cases (%)	P21 negative cases (%)
Well (12)	9	3	75%	25%
Moderate(22)	7	15	31.8%	68.2%
Poor(16)	3	13	18.8%	81.2%

Table 3: P21 IHC Score with respect to Histological Grade in 50 Cases of Gastric Adenocarcinoma

P21 IHC score	Well Differentiated grade	Moderately Differentiated grade	Poorly Differentiated grade	P21expression
0	3	13	11	NEGATIVE
1	0	2	2	NEGATIVE
2	2	3	3	POSITIVE
3	2	2	0	POSITIVE
4	5	2	0	POSITIVE

P21 expression decreased as tumor invades deeper into muscle layers. Percentage of p21 negative tumors increased as depth of tumor increased. Loss of p21 expression was significantly correlated with the deeper muscle invasion in this study (p value 0.030)(Table:4).

Table 4: Depth of invasion vs. p21 expression in Gastric carcinoma

Depth of invasion	Number of Positive cases	Number of Negative cases	P21 Positive cases (%)	P21 negative cases (%)
T1(7)	5	2	71.4%	28.6%
T2 (14)	8	6	57.1%	42.9%
T3(18)	4	14	22.2%	77.8%
T4(11)	2	9	18.2%	81.8%

P21 expression was negative in most of the cases presented with lymphovascular invasion. 23 out of 29 cases that presented with lymphovascular invasion were p21 negative and was significantly correlated in this study (p value 0.003),(Table:5)

Table 5: Lymphovascular invasion vs. p21 expression in Gastric carcinoma

Lymphovascular invasion	Number of Positive cases	Number of Negative cases	P21Positive cases (%)	P21negative cases (%)
Absent(21cases)	13	8	61.9%	38.1%
Present(29cases)	6	23	20.7%	79.3%

P21 expression was negative in most of the cases presented with lymph node deposits. 20 out of 23cases that presented with lymph node deposits were p21 negative and was significantly correlated in this study (Table:6).

Table 6: Lymph node deposit vs p21expression in Gastric carcinoma

Lymph node deposit	P21 positive	P21 negative	P21Positive cases (%)	P21negative cases (%)
Absent(27cases)	16	11	59.3%	40.7%
Present(23cases)	3	20	13.1%	86.9%

P21 expression was negative in most of the cases presented with the omental deposits. 22 out of 24 cases presented with the omental deposits were p21 negative and was significantly correlated in this study (p value <0.001),(Table:7).

Table 7: Omental deposit vs. p21expression in Gastric carcinoma

Omental deposit	P21 Positive	P21 Negative	P21Positive cases (%)	P21negative cases (%)
Absent(26cases)	17	9	65.4%	34.6%
Present(24cases)	2	22	8.3%	91.7%

P21 expression was lost in all cases presented with tumor deposits in liver and was significantly correlated (p value 0.009) in this study (Table:8).

Table 8: Liver deposit vs. P21expression in Gastric carcinoma

Liver metastasis	P21 positive	P21 negative	P21Positive cases (%)	P21negative cases (%)
Absent(41cases)	19	22	46.3%	53.7%
Present(9cases)	0	9	0%	100%

DISCUSSION:

In normal gastric mucosa, when the gastric epithelial cells move near the mucosal surface it will start exit from the cell cycle. Normally P21 protein is expressed in superficial areas of gastric foveolar crypts. They typically display intense nuclear expression. Cells in the proliferative zone, epithelial cells of fundic gland and pyloric glands are P21negative. Intestinal metaplastic cells are p21 positive as demonstrated by El-Deiry et al (17).

P21 was one of the molecular markers which can be used to assess the behavior of the tumor. Its prognostic significance was studied in various solid tumors like prostate, bladder, breast, colon, esophagus, and lung. In the present study P21 expression was studied in 50 cases of gastric carcinoma for its tumor suppressor activity and prognostic significance.

In the present study, intestinal type of gastric adenocarcinoma (36 cases-72%) was more common than diffuse type (14 cases -28%). This study also showed increased occurrence of moderately differentiated adenocarcinoma 22 cases (44%) followed by poorly differentiated carcinoma (16 cases 32%) and least well differentiated grade(12cases 24%). Majority of the tumors showed invasion into the deep muscle layers with 29 cases which constitutes 58% (T3-18cases & T4-11cases) and most of the tumors were reported to be Stage III (16cases 32%).

In all 50 cases of gastric adenocarcinoma studied, histopathological parameters such as Laurens histological type, grade of the tumour, depth of tumour invasion, lymphovascular invasion, lymph node deposit, omental deposit and liver deposit were evaluated for p21 expression.

Huang J et al (18) studied the relationship between the expressions of tumour suppressor proteins P21 and p53 and malignant growth

of gastric carcinoma in 88 cases of gastric carcinoma with immunohistochemical method. The expression rate of P21 protein in undifferentiated carcinoma and poorly differentiated carcinoma were low which was similar to the observation made in this study.

In the present study, 19cases (38%) were p21 positive and 31cases (62%) were p21 negative. Intense nuclear staining was seen in more than 25% of cancer cells in P21 positive cases and less than 25% of cancer cells in negative cases. P21 expression was also observed in foveolar epithelial cells of adjacent normal gastric mucosa. P21 expression was found to be high in most of the well differentiated tumours (9 out of 12 cases were positive) and loss of expression was noted in most of the poorly differentiated tumors (13 out of 16cases showed negative expression), whereas moderately differentiated tumours showed variable P21 expression. Percentage of p21 positive cases decreased and percentage of p21 negative cases increased as grade of tumour increased and was statistically significant in this study, similar to the Ogawa et al(19) study.

In the present study P21 expression decreased as tumor invades deeper into muscle layers. Percentage of p21 positive cases decreased and percentage of p21 negative cases increased as depth of tumor increased. Loss of p21 expression was significantly correlated with the deeper muscle invasion in this study similar to the Ogawa et al(19) and Young Ho Seo et al(20) study.

P21 expression was negative in most of the cases presented with lymphovascular invasion in this study (23 out of 29cases with lymphovascular invasion were p21 negative) .Our study showed significant association between the loss of P21 expression and lymphovascular invasion in gastric carcinoma similar to the observation by Ogawa et al(19) and Ken Mizokami et al(21).

P21 expression was negative in most of the cases presented with lymph node deposits. 20 out of 23cases presented with nodal deposits were P21 negative and was significantly correlated in this study similar to Ogawa et al(19) and Ken Mizokami et al(21).

P21 expression was negative in most of the cases presented with the omental deposits. 22 out of 24cases presented with the omental deposits were p21 negative and was significantly correlated with in this study similar to the observation by Ogawa et al(19).

Out of 50 cases studied, only 9 cases showed liver deposits. P21 expression was lost in all cases presented with tumour deposits in liver and was significantly correlated in this study similar to the Ogawa et al study(19).

CONCLUSION

From this study it was observed that most of the tumors were of moderately differentiated grade and was found to invade beyond muscularis propria into the subserosa at the time of diagnosis.

Significant correlation was present between p21 expression and histological grade, P21 positivity was observed in most of the well differentiated gastric adenocarcinoma, whereas moderately differentiated adenocarcinoma showed variable P21 expression and most of the poorly differentiated tumors showed P21 negative expression.

Immunohistochemical expression of P21 was negative in most of the tumours that has invaded deep into muscular layers, those with lymphovascular invasion, lymph node, omental and liver deposits.

Hence P21 expression has shown a significant association with the grade and stage of the gastric adenocarcinoma. To conclude, P21 IHC marker can be used as an indicator of disease progression which has prognostic value in gastric adenocarcinoma patients.

ABBREVIATIONS:

CDK	-	Cyclin Dependent Kinase
CIP/KIP	-	Cyclin-Dependent Kinase Inhibitor Proteins
DAB	-	Diamino benzene
DPX	-	Dibutylphthalate Polystyrene Xylene
H & E	-	Heamotoxylin and Eosin
HRP	-	Horse Radish Peroxidase.
IHC	-	Immunohistochemistry.
P21	-	Cyclin Dependent Kinase Interacting Protein 1
RB	-	Retinoblastoma Gene Protein
TNM	-	Tumor Node and Metastasis.
WHO	-	World Health Organization

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