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## ORIGINAL RESEARCH

# Expression pattern of ERBB2 and CYCLIND1 proteins in pre-neoplastic and neoplastic human gastric tissues

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## ABSTRACT

Cancer is a disease in which the cell grows and divides abnormally without respect to normal limit, invade and destroy and other tissue and in its most matured state metastasizes to other tissues in the various part of the body. In general the chance of getting a cancer tends to increase with age. Cancer results from the breakdown of regulator mechanisms that govern proliferation, differentiation and survival of individual cell. As a result of the loss of regulation, cancer cell grow and divide in an uncontrolled manner. They ultimately spread throughout the body, interfering with the functions of normal and organs. The generalized loss of growth control shown by cancer cells resulting in continual unregulated proliferation is the result of accumulated abnormalities. Gastric adenocarcinoma is a progressive disease that involves various distinct but overlapping stages. As gastric niches are highly proliferate marked with constant cellular turnover, tumors arising in this environment can be due to the accumulation of mutations in the progenitor or residual stem cells. Our study reveals expression pattern of ERBB2 and cyclin D1 in Normal, chronic gastritis, intestinal metaplasia, dysplasia and adenocarcinomatous tissues of the stomach.

**KEY WORDS:** *Adenocarcinoma, regulation, ERBB2 and Cyclin D1*

## Introduction

Cancer is of primary interest in medical research. In the past century several mechanism were proposed. It was hypothesized that cancer arises out from a single cell that loses its differentiated state through sequential mutations latter, this hypothesis led to the mutagenic and recently the oncogenic theories which hypotheisze that defects in tumor suppressor genes are responsible for the development of cancer (Trosko *et al.*, 1993). The impairment of cell to cell communication as a cause of cancer has also been postulated (Trosko *et al.*, 1983). Mutation and other genetic abnormalities observed in cancer cell could also be caused by or life style factor such alcohol or tobacco consumption or drug abuse (Wongen *et al.*, 2004).The discovery of stem cell (Passegue *et al.*, 2003; Sing *et al.*, 2003; Dick 2003) lent support to the theory cell and raised the question of cancer

stem cells arising from normal stem cells. Indeed, if normal stem cells could undergo the type of mutation observed in tumor cells, this would genetically compromise the genetic stability of the organism. Therefore the likelihood the normal stem cells are extremely well protected is demonstrated by their resistance to radiation and toxins. Cancer stem cells are defined as the unique subpopulation in the tumors that possess the ability to initiate tumor growth and sustain self-renewal as well as metastatic potential. Accumulating evidence in recent years strongly indicate the existence of cancer stem cells in solid tumors of a wide variety of organs. The specific dietary deficiencies also mimic the effects of chemical or radiation damage to DNA, which we propose plays an important role in human carcinogenesis and tumorigenesis. This allows considering cancer as a single disease, possibly developing from a single cancer stem cell.

Based on this, could assume that the observed genomic abnormalities in cancer cell are an effect rather than the cause of the disease. This idea also points to the direction of upstream events preceding the development of the malignant cell. Alterations in cell-cycle genes constitute the most common genetic abnormality in tumor cells. Even though disruption of the cell cycle is most probably one of the earliest events in tumorigenesis, little is known about in vivo cell-cycle gene alterations in pre-malignant or pre-invasive cells. Recent studies indicate that loss of function of the Ink4 cyclin dependent kinase inhibitor p16, through hypermethylation of the promoter region, may be a possible early event in tumorigenesis (Baylin S, Herman J 2000; Wong D *et al.*, 1999).

### **Anatomy of Stomach**

The stomach is a sac-like organ located between the esophagus and small intestine. The stomach aids in digestion and stores food. Anatomically, the stomach has been broken down into 5 different regions: cardia, fundus, body, antrum, and pylorus (see below). The stomach is also composed of 5 different tissue layers: mucosa, submucosa, muscularis, subserosa, and serosa. The more layers a cancer invades, the worse its prognosis.

### **Epidemiology**

Although falling in incidence, gastric cancer is the second commonest cancer in the world; 60% of deaths from gastric cancer occur in 'developing' countries. The highest incidences are in eastern Asia, South America and Eastern Europe. It is extremely rare under the age of 30, but increases rapidly thereafter, with the highest rates in the oldest age groups in males and females. The intestinal type is more common in males, while the diffuse type tends to affect younger age groups and is more common in females. Gastric cancer remains a major public health issue as the fourth most common cancer and the second leading cause of cancer death worldwide. Demographic trends differ by tumor location and histology. The following chart shows the incidence of various types of cancers.

While there has been a marked decline in distal, intestinal type gastric cancers, the incidence of proximal, diffuse type

adenocarcinomas of the gastric cardia has been increasing, particularly in the Western countries. Incidence by tumor sub-site also varies widely based on geographic location, race, and socioeconomic status. Distal gastric cancer predominates in developing countries, among blacks, and in lower socioeconomic groups, whereas proximal tumors are more common in developed countries, among whites, and in higher socio-economic classes. Diverging trends in the incidence of gastric cancer by tumor location suggest that they may represent two diseases with different etiologies. The following figure indicates the global ranking of gastric cancer in men and women.

### **ERBB2**

The gene ERBB2, localized in chromosome region 17q11.2–q12, encodes a transmembrane glycoprotein that is a member of the epidermal growth factor receptor family. The protein ERBB2 (also known as p185, p185neu, c-erbB-2, and Her-2/neu) acts as a tyrosine kinase receptor, stimulating cell proliferation; it is potentially involved in the growth and progression of malignant cells. All four human receptors (EGFR (ERBB1), ERBB2 (HER2/neu), ERBB3 (HER3) and ERBB4 (HER4)) share four extracellular domains with high structural homology, a single transmembrane spanning helix, and a cytoplasmic portion that contains a conserved but not equally functional tyrosine kinase domain. Only the epidermal growth factor receptor (EGFR, ERBB1) and ERBB4 are fully functional in terms of ligand binding and kinase activity. ERBB3 has impaired kinase activity and relies on the kinase activity of its heterodimerization partners for activation. ERBB2 fails to bind any of the known ERBB ligands but contributes its potent kinase activity to all possible heterodimers. In heterodimers, ERBB2 acts as a general signal amplifier, and heterodimers of ERBB2 and ERBB3 are the most potent ERBB pair in mitogenic signaling (Carmen and Ralf, 2006). Over expression of the ERBB2 gene has been shown in several cancer tissues, including breast and colon carcinoma, and its over-expression has been linked to poorer prognosis of the patients. Only 20–30% of all breast cancer patients, however, show over-expression of Her2, making the determination of the state of the Her2 expression of a cancer patient an important diagnostic step in assessing

the suitability of anti-Her2 therapy for the patient (Christian et al., 2007).

ERBB2 (HER2) upon activation it forms homo or heterodimers (Yarden and Sliwkowski, 2001). ERBB2 has no specific direct ligand (Thompson et al., 2007), but ligand binding and activation other EGFR family members activate ERBB2 leading to autophosphorylation of specific tyrosine residues, and downstream signaling cascades like PI3K and MAP kinases ultimately leading to the initiation of biological processes such as proliferation (Schlessinger, 2000). ERBB2 can be activated by constitutive autophosphorylation when overexpressed or via heterodimerization and transphosphorylation with ligand-bound EGFR, ErbB3, and ErbB4. ERBB2-containing heterodimeric receptor combinations have been found to be more mitogenic and transforming. The receptor complexes have a higher ligand affinity and signaling potency by virtue of the potent latent kinase activity of ERBB2. The heterodimer ERBB2/ErbB3 in particular appears to have marked oncogenic potential in breast cancer (Holbro et al., 2003).

The EGFR proteins phosphorylate MUC1 and increase the binding of MUC1 with  $\beta$ -catenin (Li et al., 2001). ERBB2 is an important integrator of transmembrane signaling by the EGFR family. It is probably for this reason that ERBB2 is clustered on the surface of breast tumor cells. Cooperative signaling between ErbB proteins and integrins is a common feature of invasive cancer cells and association of b1-integrin and ERBB2 proteins could provide a framework in which tumor cell metastasis could be better understood (Pinliang et al., 2006).

Despite the identification of the essential transcriptional factor, AP-2 which binds to the ERBB2 promoter, the cellular signaling pathway for the induction of ERBB2 has not been clearly defined. It has been reported that the activation of two distinct MAP kinases, ERK and p38 kinase is involved in the process of AP-2 activation. Inhibition of ERK or p38 kinase did not reduce the AP-2-mediated ERBB2 expression in p53D5,6 MEC. Hence, these kinases are unlikely to be responsible for the induction of ERBB2 by p53 inactivation. Inhibition of PKA by H-89 led to simultaneous blocking of AP-2a and ERBB2 protein increase. Thus, signaling cascades involving the PKA activation in p53-inactivated MEC may serve as an essential mechanism for the induction of ERBB2 gene.

Thus altered ERBB2 and its signaling mechanism express characteristics that bestow the cells with malignant potential. Hence we sought to study the importance of ERBB2 in the progressive stages of Gastric adenocarcinoma formation.

### Cyclin D1

Close attention has been paid to the role of cell-cycle regulators associated with cell proliferation in oncogenesis. Under the stimulation of proliferation signals, cells could produce early stage transcription factors such as c-fos, c-jun. These factors could bind to DNA specifically in nuclei and initiate cell cycles (Shaulian and Karin, 2002). During the cell cycle, the progression from G1 phase to S phase (DNA synthesis phase) is essential for initiation of the cell cycle (Blagosklonny and Pardee, 2002).

Cyclin D1 is a proto-oncogene is located in the 11q13 region and plays a positive-regulation role in the progression of cell cycle (Ortega, Malumbres and Barbacid, 2002). Cyclin D1 protein consists of 295 amino acids; its function in normal cells is to regulate the progression through G1 phase of the cell cycle in combination with cyclin dependent kinases (Cdk) by phosphorylation of pRb. The expression of cyclin D1 is an early event that is stimulated by growth factors or other mitogens. The major targets of cyclin D1-Cdk complexes are the retinoblastoma family of protein Rb (Rafferty, Fenton and Jones, 2001). Phosphorylation of Rb in mid-G1 leads to the release of active forms of the E2F family of transcription factors. Free E2F mediates transcription of E2f-dependent genes, including DNA polymerase, thymidine kinase (Coqueret, 2002). Genetic alteration of cyclin D1 was observed in several kinds of human tumors such as breast carcinoma (Steeg and Zhou Q, 1998), squamous cell carcinoma of the lung (Qiuling, 2003), endometrial carcinoma (Moreno-Bueno, 2003) and malignant gliomas (Buschges, 1999). Yang et al. (1994) reported that a characteristic translocation, t (11:14) (q13; q32) involving rearrangement of cyclin D1 gene, was detected in centrocytic lymphoma. Independent of its CDK4 activity, Cyclin D1 also functions as transcriptional modulator by regulating the activity of several transcription factors and histone deacetylase (HDAC3) (Coqueret, 2002).

Gastrin the stomach hormone is found to increase the expression of cyclin D1 in vitro (Zhukova et al., 2001; Song

et al., 2003), In addition, *H. pylori* infection, which can lead to gastric cancer and is associated with hypergastrinemia also induces cyclin D1 transcription (Hirata *et al.*, 2001). The cyclin D1 protein has been shown to be unstable with a short half-life (~24 min) (Diehl *et al.*, 1998) and is degraded mainly via the 26S proteasome in a ubiquitin-dependent manner (Diehl *et al.*, 1997). Early studies suggested that the Skp2 F-box protein might be involved in cyclin D1 degradation (Yu, Gervais and Zhang, 1998). Recently, two further F-box proteins were identified in separate studies as playing major roles in targeting the cyclin for degradation (Lin *et al.*, 2006 and Okabe *et al.*, 2006).

Activation of the ERBB2/Neu tyrosine kinase has been found to increase cyclin D1 expression in breast adenocarcinoma cell lines by the transactivation of p27, E2F, SP1 and SP3 (Lee, 2000). In order to understand the alteration of cyclin D1 in gastric carcinoma, the protein expression of cyclin D1 in normal mucosa, gastritis, intestinal metaplasia, dysplasia and adenocarcinoma were detected by immunohistochemistry.

## Materials and Methods

### Samples

35 antral biopsy samples were collected from patients who underwent upper gastrointestinal endoscopy at the Department of Medical Gastroenterology Government Stanley Medical College and Hospital, Chennai after informed consent. The work was approved by the hospital ethical committee.

In all 10 Normal, 11 Antral Gastritis, 2 Intestinal Metaplasia and 5 dysplasia and 7 adenocarcinoma tissues were collected. Of the three bits taken one was processed for immunofluorescence while the other two were processed for blotting.

### Protein Analysis

#### Extraction of protein

The human gastric cancer biopsies samples were homogenized by using Iris buffer in 135mM NaCl, 20mM Tris, 2mM EDTA and 1mM PMSF, pH 7.4 (pH 7.4). The homogenates were centrifuged (15 min, 10,000 rpm at 4°C) and the protein content of the supernatant determined by Lowry method (1951). The Determination of Protein content

is done by the Lowry's method by keeping Bovine serum albumin as a Standard for protein estimation.

### Estimation of Protein

The protein sample to be estimated was taken in various test tubes with different concentrations and made up to 1ml using double distilled water. Simultaneously the blank was also prepared without sample. 4.5ml of reagent 'C' was added to each test tube and mixed thoroughly by using cyclomixer and incubated at room temperature for 10 minutes. After 10 minutes, 0.5ml of Folin Phenol reagent was added to each test tube and mixed well. The setup was incubated at room temperature for 20 minutes. After incubation, the sample was measured in the Hitachi 320 spectrophotometer at 620nm. The measurements were recorded. Comparison with the standard gave us the amount of protein present in the sample.

### SDS PAGE

In this analysis, the proteins are separated based on their net charge mass. Electrophoretic separation is one of the most commonly used methods and is performed on polyacrylamide gel which is casted between a pair of glass plates. When an electric current is applied forcing the various size of proteins present in the sample to migrate along the gel. Smaller proteins migrate faster than larger proteins, hence they will move a greater distance along the plate. The SDS (Sodium dodecyl sulphate), which is added before during gel electrophoresis is to denature proteins. SDS treatment eliminates the effect of differences in shape, such that protein length is role determinant of the migration rate of proteins.

The samples were analyzed on SDS-PAGE in discontinuous buffer system of Laemmli (1970) to determine its molecular weight. This was performed using a 5% stacking gel (pH 6.8) and a 15% separating gel (pH 8.8) in Tris - glycine buffer (pH 8.3). Electrophoresis was performed by using 15% resolving gel and was prepared by adding the following composition.

The solutions were brought to room temperature before polymerization. Glass plate measuring the size of 12 x 14 cms were used for this study. Glass plate sandwich was assembled using two clear plates and 1-2mm thick spacer by sealing spacers with the glass plates using vacuum grease.

The plate was locked using casting stand metal clamp. The separating gel solution (15%) was prepared and transferred to the center of the sandwich along an edge of one of the spacers until the height of the solution in the sandwich was 14cm without air bubbles. The stacking gel solution (5%) was also prepared and it was poured over the separating gel. A teflon comb was inserted into the layer of stacking solution to form loading wells. It was allowed to completely polymerize for 5-10 min. at room temperature. After polymerization of gel, teflon comb was removed carefully from the gel. The wells were washed with tank buffer and half of the well was filled with the same buffer. The bottom spacer in the glass sandwich was removed and the grease at the bottom of the gel was wiped out using filter paper. The bottom reservoir of the electrophoretic unit was filled with tank buffer. Then the sandwich was inserted into the bottom tank without any air bubbles and it was fitted to the upper tank using the metal clamps. 20µg of sample was mixed with equal volume of sample buffer (pH 6.8). The mixture was boiled for 5 min. in water bath and loaded on to the well. The remaining space in the well was also filled with tank buffer (pH 8.3) and the buffer loaded into the whole tank. Power pack was connected to the tank and run was performed at room temperature with constant current 100V upto final stage of the run. After the bromophenol blue tracking dye had reached the bottom of separating gel, power supply was disconnected.

#### **Western Blot**

The western blotting has become one of the most common protein analysis technique used in this biomedical research. Western blotting provides a direct a method for identifying, monitoring, and determining the relative amount of specific protein in large numbers of different sample. The general technique of western transfer and immunodetection can be readily used with SDS-PAGE, native PAGE and 2 D PAGE. Variations on standard western blotting technique are numerous. In general, a complex protein mixture (such as cell lysate or extract or purified protein preparation) is fractionated on a gel by electrophoresis. After separation, proteins are transferred on to membrane, which can be nitrocellulose, polyvinylidene fluoride (PVDF), where they probed using antibodies specific to target proteins. 10% SDS-PAGE was run with regenerating limb tissue, X-

organ sinus gland complex and Y-organ of different molting stage of *Scylla serrata*. Transfer the gel into nitrocellulose membrane by semi dry blot apparatus. It was run 1.30 hours/25V/130mA at 4°C. Block the membrane by incubating it for overnight at room temperature with agitation. Incubate the membrane for 2 hours at room temperature with agitation in the appropriate primary antibody diluted in blocking buffer. Followed by washing the membrane 5 times [5minutes each] with washing buffer. Then membrane was incubated with corresponding secondary antibody HRP conjugate diluted in blocking buffer for 1 hour at room temperature. Wash the membrane 5 times [5minutes each] with wash buffer. The rinse the membrane 2 times with double distilled water.

#### **Chemiluminescent Detection**

For development add equal volume (each 1ml) of the substrate Solution A [lumiGLO working reagent] and Solution B and incubate 1 minutes at room temperature. Remove the membrane from the working reagent with forceps, drain excess reagent and place the membrane and X-ray film in the Film exposure Folder, and close the folder without air bubbles between the membrane and X-ray film. Exposure time may varied from a few seconds to few minutes depending upon the amount of antigen being detected Finally signal was detected on X-ray film by using developing solution.

#### **Histology**

The histological evaluation of Regenerating limb, eyestalk neural ganglia and Y-organ was performed in *Scylla serrata* according to the method of Presnell and Screibman (1997). Preparation of Slide for Sections: New slides were washed with chromic acid followed by rinsing with diluted NaOH and distilled water and dried in the hot air oven.

Gelatin/Glycerol Coating: 0.2 ml of 10% gelatin/glycerol solution was spread evenly an each slide and dried well. The slides were stored for 2-3 weeks at room temperature. Preparation of Paraffin Sections:

The tissue from different stage was surgically removed and fixed in Bouin's fixative for 24-48h. Paraffin embedding of tissues was done as for histological evaluation taking care not to over fix the tissues. 5 µm tissue sections were cut from the paraffin embedded blocks and floated on a hot water bath containing distilled water. The flattened sections

were collected on clean glass slides coated with Gelatin/Glycerol and dried overnight. For optimal adhesion the slides were placed in an oven at 60°C for 1h.

#### Hematoxylin and Eosin Staining:

Of the numerous sections cut from Bouin's fixed paraffin-embedded specimens, one section was used for routine hematoxylin/eosin staining and others were used for immunofluorescence. Hematoxylin and eosin staining was done with Dewaxed using xylene and then treated with absolute, 90% and 70% grades of alcohol and then dipped in water. The slides were stained with hematoxylin for 10 min. followed by washing with running tap water until the sections became blue. Then the slides were stained in 1% eosin for 1 min. followed by washing in running water for 4-5 min. The sections were dehydrated in ascending grades of alcohol, cleared in xylene and the sections were mounted in DPX permanent mount. The sections were visualized under camera attached to bright field microscope and photographed.

#### Immunofluorescence

Immunofluorescence is a technique for localization and visualizing an antigen in a section by using an antibody specific for the target proteins. The immunofluorescence procedure consists of tissue preparation antibody incubation and series of detection reactions. The tissue are frozen or fixed, sectioned, and attached to slides. The section are then dewaxed, treated with a target retrieval solution, blocked with a protein based blocking solution and then incubated with a primary and corresponding fluochrome conjugated secondary antibody and covered with a mounting medium.

## Results and Discussion

The intensity of membranous ERBB2 expression was very low in normal and gastritis and increased gradually in intestinal metaplasia, dysplasia and adenocarcinoma (Plate – II) which is also evident from the western blotting results (Plate- IV). There was a decrease in the intensity of CyclinD1 expression in gastritis and intestinal metaplasia, while the expression was high in normal and dysplasia and very high in adenocarcinoma (Plate – III), which is also evident from the western blotting results.

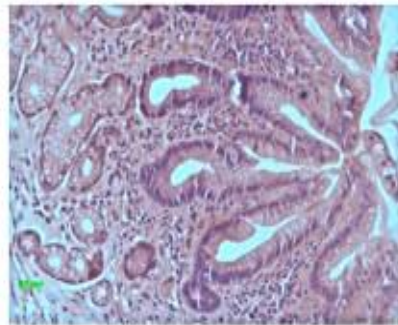
The very low or almost null expression of ERBB2 can be explained from the fact that ERBB2 does not control normal homeostasis and is mostly upregulated in response to various stimuli like gene amplification. Further the highly oxidative environment in gastritis stage could have caused the down regulation of critical signaling pathways that serve to upregulate ERBB2. Further the increase in expression of ERBB2 in the metaplastic tissues can be correlated from the fact that there is increased cellular rearrangement and transdifferentiation into the intestinal phenotype. As ERBB2 and its signaling mechanism are essential players in the Neoplastic transformation sequence of human gastric tissues, targeting it will have tremendous application in therapy. Further the modulation of pathways that govern the ERBB2 signaling in people with chronic *Helicobacter pylori* infection will serve to intervene in the progression of the adenocarcinoma cascade.

The high intensity of CyclinD1 expression in normal gastric tissues can be explained by the fact that the gastrointestinal epithelium witness high turnover of cells under normal circumstances ultimately causing higher rates of cell proliferation and hence increased Cyclin D1 expression. While the dip in intensity of CyclinD1 in Gastritis stages can be thought of as the tilting of the balance between cell proliferation and cell death in favour of the later, indicating a state of diminishing endogenous repopulating ability of the gastric epithelium or to be precise the degradation of the gastric stem cell niche. Gastrin has been found to maintain and increase the CyclinD1 expression and hence cell proliferation in the normal human stomach, since the G-cells which produce this enzyme is lost in intestinal metaplastic tissues, CyclinD1 levels were found to be decreased in such tissues. However the dysplastic and adenocarcinoma tissues showed a resurgence in CyclinD1 expression which can be explained as due to the possible resurgence of gastrin producing cells or altered gastrin signaling or the action of other signaling pathways that do play a role in CyclinD1 overexpression. ERBB2 has been found to modulate CyclinD1 expression in breast adenocarcinoma cell lines and hence such a kind of cyclinD1 upregulation in cancerous tissues could be possible in gastric adenocarcinoma formation, especially in the dysplastic and adenocarcinoma tissues.

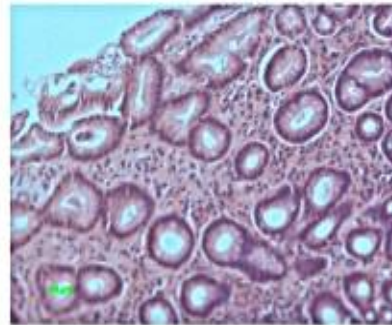
ERBB2 and CyclinD1 might serve as potential targets to intervene in the Neoplastic transformation cascade of human gastric tissues. Since ERBB2 has been found to alter

CyclinD1 expression targeting the signaling intermediates that favour CyclinD1 gene expression might serve to limit the adenomatous transformation of gastric epithelium.

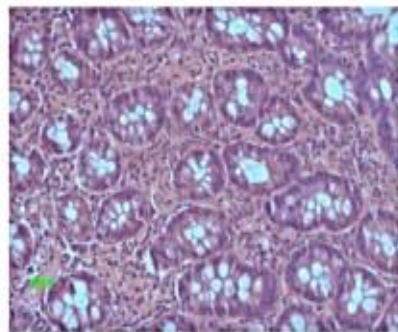
**PLATE - I**  
**HISTOLOGY OF THE PROGRESSIVE STAGES IN THE NEOPLASTIC TRANSFORMATION**  
**SEQUENCE OF GASTRIC ADENOCARCINOMA**



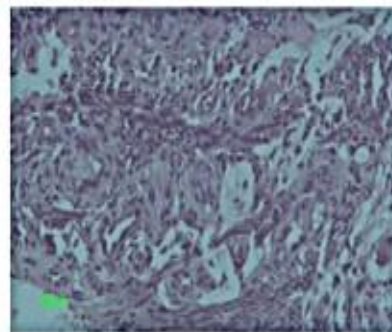
**Normal**



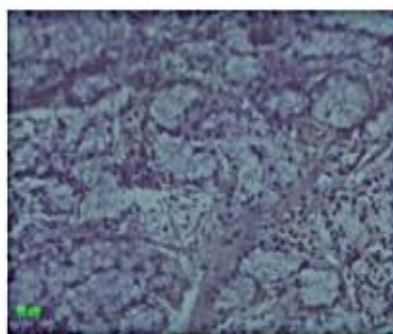
**Gastritis**



**Intestinal Metaplasia**



**Dysplasia**



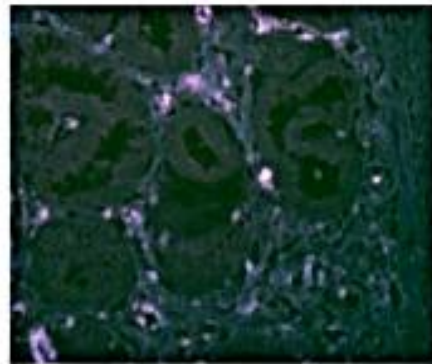
**Adenocarcinoma**

**PLATE- II**

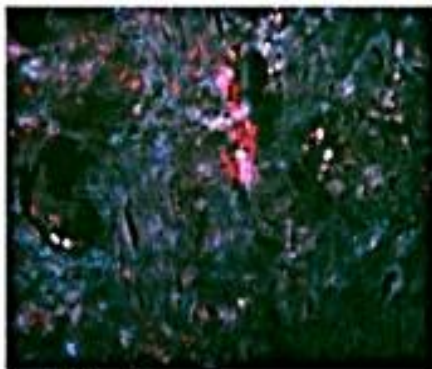
**ERBB2 Expression in the Progressive Stages of Gastric Adenocarcinoma Formation**



**Normal**



**Gastritis**



**Intestinal Metaplasia**



**Dysplasia**

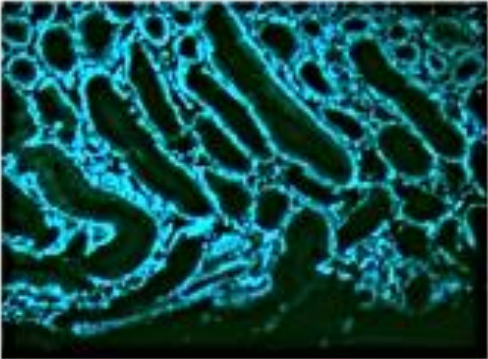


**Adenocarcinoma**

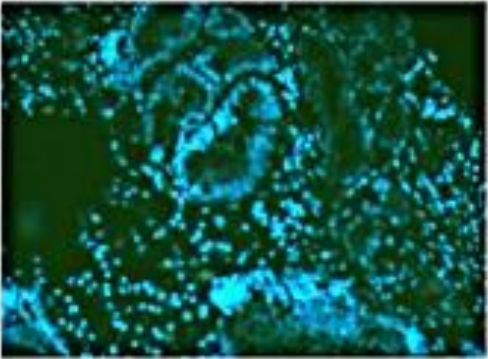


**PLATE - III**

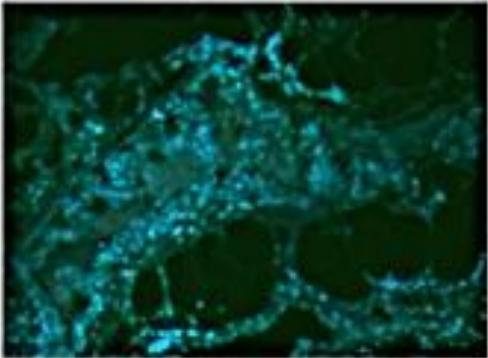
**CyclinD1 Expression in the Progressive Stages of Gastric Adenocarcinoma Formation**



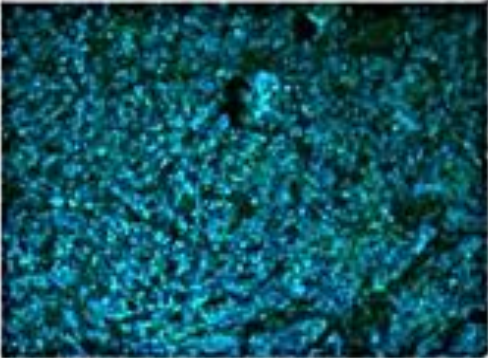
**Normal**



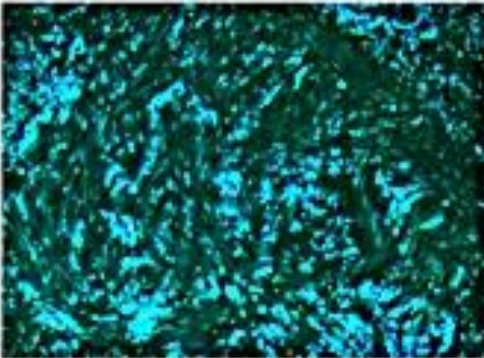
**Gastritis**



**Intestinal Metaplasia**



**Dysplasia**



**Adenocarcinoma**

**PLATE - IV**  
**Immunoblot of ERBB2 and CyclinD1 in the Progressive Stages of Gastric Adenocarcinoma Formation**



**LEGEND:**

- 1 - Normal
- 2 - Gastritis
- 3 - Intestinal Metaplasia
- 4 - Dysplasia
- 5 - Adenocarcinoma

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