ROLE OF c-H-ras p21 EXPRESSION IN

HELIcobacter pylori MEDIATED

CARCINOGENESIS

5.1 INTRODUCTION

Over the last 20 years, considerable information has been gathered on regulation of cell growth and proliferation leading to the identification of proto-oncogenes and tumor suppressor genes. But still, the interacted process involved in carcinogenesis is not completely understood because of the conflicting reports in various malignancies. Current tumor research has demonstrated that during the process of carcinogenesis some oncogenes will be altered. Oncogenes can be classified into at least two groups according to their functions (1) proto-oncogenes, such as ras, c-myc, and c-erbB-2, which stimulate cell proliferation and promote malignant changes; and (2) suppressor genes, such as wild-type p53 and RB genes, which could suppress malignant changes.

5.1.1 Association of c-H-ras p21 expression in cancer

The ras gene was originally identified as an oncogene of a rat sarcoma virus (The Harvey and Kristen strains). Later, it was found that many human cancer cells contained mutated alleles of the cellular ras gene,
which changes the transformation of NIH3T3 cells after transfection compared to its normal counterpart. The substitution of a certain amino acid residue was found in constitutively active ras oncoprotein. Ras protein binds 1 mol of GDP or GTP per mol of protein and hydrolyses the bound GTP to GDP and inorganic phosphate. In the mutated transforming Ras, its GTPase activity is very often impaired. The significant structural similarity with G protein α subunits suggests that Ras functions as a signal transducer in a manner similar to other signal-transducing GTP-binding proteins (Barbacid 1987; Kaziro et al., 1991). The ras gene is conserved through evolution. It is widely distributed among eukaryotes, including mammals, Drosophila, slime molds, nematodes and yeasts.

Ras has been implicated in controlling cell proliferation, differentiation and apoptosis (Bos, 1989). The Ras loci have been shown to undergo multiple genetic alterations, including mutational activation, gene amplification and loss of the normal allele in a variety of human tumors (Fearon and Volgelstein, 1990). Ras family proteins constitute one of the three major branches of the Ras super family. Members of the Ras family, namely, H-ras, K-ras and N-ras, have been implicated in human cancers. The association of mutated ras (H-,K- and N-ras) genes with up to 30% of all human cancers suggests an important contribution of constitutively active ras function to the development of human cancers (Marshall, et al., 1998).

Ras mutations are found in a wide variety of human malignancies, with the highest incidence observed in adenocarcinomas of the pancreas.
(90%), the colon (50%) and the lung (30%) (Bos, 1989). Even though most of the cancers are related to the ras mutations, some cancers exhibit higher levels of ras protein which are lacking mutations. In ovarian cancer, the frequency of ras mutation is low (<5%), but the levels of activated ras are elevated.

5.1.2 Expression and Role of c-H-ras p21 in gastric carcinogenesis

Gastric adenocarcinoma is the second leading cause of cancer deaths world wide. The causative factors and molecular mechanism of gastric carcinogenesis have not yet been fully determined. Though multiple, heterogenous genes have been found to be correlated with these carcinogenic processes, no dominant gene having significant role in carcinogenesis has been documented in gastric cancer (Ranzani et al., 1990). Several research efforts were focussed on ras gene for the last few years and many dietary factors and H.pylori infection have been implicated as gastrointestinal carcinogens (Zarbl et al., 1985; McMahon et al., 1986; Wang et al., 2002) which can induce the expression of ras gene. Mutated ras p21 protein or abnormally high-expressed normal p21 have been reported to induce transformation of NIH3T3 cells in vitro (Chang et al., 1982; Feramisco et al., 1984; Theodoresu et al., 1990). In addition, enhanced expression of c-H-ras p21 has been commonly demonstrated in gastric cancers and the level of ras p21 was known to be correlated with tumor progression (Tahara et al., 1986; Ohuchi et al., 1987). Employing the RNA-RNA insitu hybridization technique, Ohuchi et al., (1987) have
demonstrated that the expressed ras p21 protein in gastric cancers and adjacent dysplasia might be a product of the c-H-ras gene.

5.2 WORKING HYPOTHESIS

Since there is a strong association between cytokines and modification in the expression of cell adhesion molecules in gastric carcinogenesis, it is necessary to determine the factors involved in the disruption of homeostatic control in cancer, and this is of fundamental importance in understanding the process of oncogenic transformation. It has been hypothesized that, gastric carcinoma and its precursor lesions are known to express high levels of activated ras gene (Ohuchi et al., 1987). The present study was based on this hypothesis to analyse the relationship between ras oncogene expression and H. pylori infection in malignant and premalignant lesions of the stomach. The purpose of this investigation was to obtain information for a better understanding of the genesis of gastric carcinoma induced by H. pylori in order to improve the diagnosis of premalignant and early malignant gastric lesions.

5.3 STUDY DESIGN

This study was carried out in two hundred gastric biopsy specimens as given in earlier chapters. The c-H-ras p21 expression was analysed in the same biopsy tissues in which TNF-α, CD44 isoforms and H. pylori status were analysed. The data obtained were subject to correlation
analysis with cancer progression and *H. pylori* status. The details of the cases are also given in earlier chapters.

### 5.4 METHODOLOGY

Gastric biopsy samples were processed as given in Chapter-II. From each case, one biopsy sample was used to prepare frozen sections for histology and immunohistochemistry. 2 biopsies were used for western blotting analysis and one was used for RNA extraction and subsequent RT-PCR.

#### 5.4.1 RT-PCR analysis of c-H-ras p21 expression

A fresh biopsy sample was snap frozen in liquid nitrogen and RNA was extracted using standard TRIZOL protocol. The extracted total RNA was reverse transcribed to cDNA by using reverse transcriptase. PCR was performed on the cDNA using standard primers published already.

**Primers**

Forward 5'-CAA GAG TGC GCT GAC CAT CC-3’
Reverse 5'-CCC GAT CTC ACG CAC CAA C-3’

(For detailed protocols see Appendix).

#### 5.4.2 Immunoprecipitation and Immuno blotting

The tissue lysate prepared from the biopsies was immunoprecipitated with c-H-ras p21 mouse monoclonal antibody
(Oncogene research products, USA) using protein A sepharose. The immunocomplex was then resolved in 12% SDS-PAGE and transferred to nitrocellulose membrane. The transferred protein was then hybridized using c-H-ras p21 mouse monoclonal antibody and the expression was identified using antimouse secondary antibody tagged with HRP (Bangalore Genei, India). DAB substrate was used as a chromogen (see Appendix for detailed protocol).

5.4.3 Immunohistochemistry and immunofluorescence

The c-H-ras p21 protein expression was semiquantitatively analysed by immunohistochemistry and further confirmed by immunofluorescence analysis. The cryostat sections were used for the analysis. After airdrying and fixation in acetone, the tissue sections were treated with 1% NP 40 for membrane lysis and treated with c-H-ras p21 mouse monoclonal antibody. After appropriate incubation and washing, the sections were treated with HRP conjugated antimouse secondary antibody (or) Rhodamine tagged antimouse secondary antibody in case of immunofluorescence. DAB chromogen was used in immunohistochemistry, counter stained with haemotoxylin and examined under light microscope. In case of immunofluorescence, the sections were directly analysed under Axioscope-II fluorescence microscope using appropriate filters (for detailed protocol see Appendix).
Sections of normal skin tissue were used as positive control. Sections without primary antibody treatment were used as negative control.

5.4.4 Assessment of immunoreactivity

Immunoreactivity of c-H-ras p21 was graded based on the intensity of the brown DAB colour developed and the cells showing positivity. At least 10% cells in the field showing positivity were taken as significant c-H-ras p21 expression, 10-25% cells mild expression, 26-50% moderate, 51% and more were taken as intense expression.

5.4.5 Data analysis

$\chi^2$ analysis were used to identify the association of c-H-ras p21 with histopathological grade and with *H. pylori* status. The expression of c-H-ras p21 was analysed using three techniques, RT-RCR and immunoblotting for qualitative and immunohistochemistry for semiquantification. The patients were considered to express c-H-ras p21, if they were detected positive for both RT-PCR and western blot.

5.5 RESULTS

RT-PCR experiments were done to characterize the presence of c-H-ras p21 mRNA transcript in gastric biopsy samples. A representative RT-PCR result is shown in Plate 5.1a. The presence of 530 bp transcript determines the expression.
PLATE 5.1

A  RT-PCR analysis of c-H-ras p21 expression. *H. pylori* positive specimens showing transcript 530 bp, in contrast to normal showing no transcript

B  The quality and abundance of the cDNA were confirmed using β-actin specific PCR.

C  Proteins were electrophoresed on SDS - PAGE (5% and 10%) and visualised by CBB staining.

D  The proteins were transfered to nitrocellulose membrane and incubated with anti c-H-ras p21 MAb followed by a second antismouse peroxidase coupled antibody, visualised by diaminobenzidine staining
Figure 5.1 Expression of c-H-ras p21 in the study population
Figure 5.2 Association of ras p21 expression with H. pylori infection
Immuno blotting was done to characterize the presence of c-H-ras p21 protein in gastric biopsy samples. A representative western blot is shown in Plate 5.1D. The detection of 21kDa protein band showed the presence of c-H-ras p21.

5.5.1  c-H-ras p21 Oncogene Expression In The Study Population

Out of 200 cases analysed for c-H-ras p21 expression by RT-PCR and WB, only 57 cases (28.5%) were negatives for ras p21 while the remaining 143 cases (71.5%) were expressing c-H-ras p21. In that, out of 20 normal patients, 17 (85%) were not expressing c-H-ras p21, only 3(15%) were expressing c-H-ras p21. The expression in gastric preneoplastic lesions, chronic gastritis, intestinal metaplasia were 50/63 (79.4%), 17/20 (85%) and 9/11 (81.8%) respectively. In gastric adenocarcinoma, out of 86 patients 74.4% (64/86) showed c-H-ras p21 expression. These results indicate the steady increase in the expression of c-H-ras p21 from normal to intestinal metaplasia. When the data was analysed for association with progression a significant association between c-H-ras p21 and histology was observed p<0.001 $\chi^2$=35.96. Fig.5.1.

5.5.2  Association of H. Pylori infection and c-H-ras p21 Expression

Out of the 57 H.pylori negative patients, 20 cases 20/53 (35%) were positive for c-H-ras p21 expression. 37 cases 37/57 (65%) were not expressing c-H-ras p21. Out of 143 H.pylori positive cases, only 20 cases 20/143 (14%) were negative for c-H-ras p21, while the remaining 123/143
(86%) were expressing c-H-ras p21. A highly significant association was found between *H. pylori* infection and c-H-ras p21. P<0.001 and $\chi^2 = (51.87)$ Fig. 5.2. These results suggest that c-H-ras p21 expression plays a crucial role in the *H. pylori* mediated gastric transformation.

**5.5.3 Immunohistochemical localization of c-H-ras p21 in the study population**

A distinct membranous or cytoplasmic immunoreactivity for c-H-ras p21 was judged as positive. The expression of c-H-ras p21 was represented in 5 groups. The immuno reactivity assessment was carried out as mentioned earlier in the methodology.

The histopathological grades was assigned into 5 groups as mentioned earlier, and the c-H-ras p21 expression was correlated with these histopathological stages in the same 200 study population. The expression of c-H-ras p21 in relation to the histopathological stages are shown in plate 5.2 and 5.3. The significant expression of c-H-ras p21 increases in the precancerous condition from chronic gastritis to intestinal metaplasia.

The data for c-H-ras p21 immunoreactivity among the 5 histopathological stages are given in Table 5.3. Out of 20 normal samples, 19 were negative for c-H-ras p21 expression (95%). In chronic gastritis cases, only 11 (17.5%) were negative for c-H-ras p21 expression 50 (79.4%) were stained mild and 2(3.2%) were stained moderately. No intense staining was noticed in this group. In atrophy, only one case was observed as
PLATE 5.2

Immunohistochemical analysis of c-H-ras p21

(A) Normal stomach mucosa (20 x magnification)

(B) Chronic gastritis (20 x magnification)

(C) Gastric Atrophy (20 x magnification)

(D) Intestinal Metaplasia and (20 x magnification)

(E) Adenocarcinoma (20 x magnification)
PLATE-5.2
Immunohistochemical Expression of c-H-ras p21 in various stages of Helicobacter pylori Mediated Gastric Carcinogenesis
PLATE 5.3

Immunofluorescence analysis of c-H-ras p21

(A) Normal stomach mucosa (20 x magnification)

(B) Chronic gastritis (20 x magnification)

(C) Gastric Atrophy (20 x magnification)

(D) Intestinal Metaplasia (20 x magnification)

(E) Adenocarcinoma (20 x magnification)
PLATE-5.3
Immunofluorescent Analysis of c-H-ras p21
in various stages of Helicobacter pylori
Mediated Gastric Carcinogenesis
Figure 5.3 Immunohistochemical expression of c-H-ras p21 in different Histopathological Stages
negative, 5/20 (25%) stained mild, 13/20 (65%) were stained moderately and one case showed intense staining. In intestinal metaplasia no negative staining was observed, 1/11 (9%) was mild, 4/11 (36%) were moderate, and 6/11 (54%) were intensely stained. In gastric cancer patients 22 cases were negative (25.6%) 28/86 (32.6%) were mild, 22/86 (25.6%) were moderate and 14/86 (16%) were intensely stained for c-H-ras p21. When the data were analysed for the association between expression and histopathological stages, highly significant association was observed \( p<0.0001 \chi^2 = 14.43 \). Fig.5.3.

5.5.4 Association of Immunoreactivity between c-H-ras p21 and *H. pylori* infection

The immunoreactivity of c-H-ras p21 in relation to *H. pylori* status was analysed in the study population Table 5.4 explains the data analysis.

Among the 57 *H. pylori* negative cases, 34 (59.4%) were negative while the remaining 8/57 (14%) were mild, 11/57 (19%) were moderate and 4/57 (7.0%) were intensely stained. In the 153 positive cases, only 19 (13.3%) were negative, remaining 77/143 (53.8%) were mild, 30/143 (21%) were moderate and 17/143 (12%) were intensively stained for c-H-ras p21 expression. Highly significant association \( p<0.0001 \) and \( \chi^2 = 49.23 \) was observed (Fig.5.4). This indicates the close association between *H. pylori* infection and c-H-ras p21 oncogene expression in gastric carcinogenesis.
Figure 5.4 Immunohistochemical expression of c-H-ras p21 in relation to H. pylori
5.6 DISCUSSION

Ras is an oncogene product that is found on chromosome 11. It is found in normal cells, where it helps to relay signals by acting as a switch. When receptors on the cell surface are stimulated, Ras is switched on and transduces signals that inform the cell to grow. If the cell surface receptor is not stimulated, Ras is not activated and so the pathway that results in cell growth is inhibited. In about 30% of human cancers, Ras is mutated so that it is permanently switched on, initiating the cell to grow regardless of whether receptors on the cell surface are activated or not.

It has been reported that ras p21 overexpression is related to the early events of human gastric carcinogenesis. (Ierardi et al., 1997; Czerniak et al., 1989). The family of ras genes consists of three functional genes, H-ras, K-ras and N-ras, which are located on chromosomes 11, 12 and 1 respectively, and encode highly similar guanine nucleotide-binding proteins, p21 of 21-kDa. Ras p21 protein contains 158 amino acids, has GTPase activity (Lee, 1997), and participate in the regulation of normal cell growth and differentiation. The ras p21 proteins have also been reported to take part in signal transduction through cell membrane to the nucleus and in the process of carcinogenesis (Miturishi et al., 1998). Yu and Zhang (1994) have studied correlations among H.pylori infection, point mutation at codon 12 of the Ha-ras oncogene and have found that the overexpression and mutation rate of the H-ras oncogene was higher in the group with H.pylori infection
than in that without the infection. In our study, the rate of expression of ras p21 was higher in the *H. pylori* infected precursor lesions, chronic gastritis 49/56 (87.5%), atrophy 16/17 (94%) and intestinal metaplasia 9/11 (82%) whereas in the case of *H. pylori* negative cases these groups, show 12.5%, 5.9% and 18.2% respectively. In the case of normal mucosa, out of 20 cases, 3 expressed c-H-ras p21. The results are in agreement with the result of Karayianni et al., (1989). Our data suggest that the over expression of ras p21 oncogene in precursor lesions may be an early event in carcinogenesis and it is predisposing to carcinoma. Noguchi et al. (1986) reported an increased expression of the ras gene in about two thirds of gastric adenocarcinoma and in some non neoplastic gastric epithelial cells. Sugaluma et al. (2001) postulated a new carcinogenic mechanism of human stomach cancer development associated with *H. pylori*, wherein the HP-MP1 protein of *H. pylori* induced TNF-α expression in co-operation with viral Ras protein resulted in cell transformation in v-Ha-ras transferred BALB/3T3 mice model. Moreover, the expression of viral ras protein may be replaced by c-Ha-ras p21 expression in the stomach cancers of humans, based on evidence that levels of c-Ha-ras p21 in extracts of human stomach adenocarcinomas were higher than those from extracts of their normal counterparts and also higher than those of c-ki-ras and c-Na-ras (Ohuchi et al., 1987). These results clarified some aspects of the association between *H. pylori* infection and gastric carcinogenesis. The drugs targeting ras p21 expression may be useful in counteracting the *H. pylori* mediated gastric carcinogenesis.
Chapter VI