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Farnesylcysteine Methyltransferase Activity and Ras Protein Expression in Human Stomach Tumor Tissue

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The processing pathway of G-proteins and Ras family proteins includes the isoprenylation of the cysteine residue, followed by proteolysis of three terminal residues and α-carboxyl methyl esterification of the cysteine residue. Farnesylcysteine methyltransferase (FCMT) activity is responsible for the methylation reaction which play a role in the membrane attachment of a variety of cellular proteins. Four kinds of Ras protein (c-Ha-ras, c-N-Ras, c-Ki-Ras, pan-Ras) expression were detected in adenocarcinoma of human tissue by immunohistochemical method, and hematoxylin and eosin staining. The level of Ras protein in human stomach tumor tissues was much higher than in normal and peritumoral regions of the same biopsy samples. The FCMT activities of each cellular fractions were high in mitochondrial fraction followed by microsomal fraction, whole homogenate and cytosolic fraction. The inhibitory effect on FCMT activity on stomach tumor tissue was determined after treatment with 0.25 μM of S-adenosyl-L-homocysteine. S-adenosyl-L-homocysteine inhibited FCMT activity from 11.2% to 30.5%. These results suggested that FCMT might be involved in Ras proteins activity.

Key words: Human stomach tumor tissue, Ras, Farnesylcysteine methyltransferase

INTRODUCTION

Farnesylcysteine methyltransferase (FCMT) methylates the free carboxyl group of the farnesylated cysteine residue present in the carboxy terminus of a large number of proteins. Farnesylated proteins such as Ras (Clarke *et al.*, 1988; Hancock *et al.*, 1991), lamin B and transducin are methylated by FCMT in a typical carboxyl methylation reaction. The enzyme transfers a methyl group from the methyl donor S-adenosyl-L-methionine to the cysteine's free carboxyl group (Ota and Clarke, 1989). This enzyme arouses scientist's interest in view of the relatively high incidence of ras gene expressions in human tumors.

Activating mutations in ras oncogenes occur in a wide variety of human tumors. The overall incidence is only between 10% and 15% but is as high as 95% in pancreatic carcinomas (Bos, 1988). Individual *ras* genes are commonly associated with specific tumors, for example, Ki-*ras*2 with cancers of the lung (Rodenhuis and Slebos, 1992), colon or pancreas, and N-*ras* with acute myelogenous leukemia (AML).

However, there is no specificity in thyroid tumors and thyroid adenomas and carcinomas, mutations in all three genes (Ha-*ras*, Ki-*ras*2, N-*ras*) may occur in the same tumor. Simultaneous mutations in Ki-*ras*2 and N-*ras* have also been detected in multiple myeloma (Portier *et al.*, 1992). Mutant alleles of the Ha-*ras*1 minisatellite locus represent a major risk factor for common types of cancer (~10% of breast, colorectum, and bladder cancers) (Krontiris *et al.*, 1993). The expression of N-*ras*, *v*-Ha-*ras* or human Ha- *ras*1 causes non-neoplastic proliferation and malignant tumors in a tissue-specific manner. Co-expression of *v*-Ha-ras and myc causes a synergistic increase in the initiation of tumors, indicating that the expression of an activated ras gene alone is not sufficient to transform differentiated cells *in vivo* (Sinn *et al.*, 1987).

Three Ras proteins Ha-, N-, and Ki-Ras undergo a complex series of posttranslational modifications. It comprises three tightly coupled modifications of a CAAX motif (C=cysteine, A=aliphatic, X=any amino acid), which is found at the C-terminus of all newly synthesized Ras proteins. First, the cysteine residue (Cys-186) is alkylated by a polyisoprenoid, probably C15

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farnesyl; second, the -AAX amino acids are removed by proteolysis; and third, methylesterification takes place probably at the α-carboxyl group of the new C-terminal cysteine (Gutierrez *et al.*, 1989; Hancock *et al.*, 1989; Deng *et al.*, 1994).

This study was performed to investigate the role of FCMT involved in Ras protein activity, and to suggest the possibility as a new anti-cancer agent of FCMT inhibitor. FCMT activity was examined in human normal and tumor stomach tissues in order to determine the differences of FCMT activity between normal and tumor cells. And the expression of Ras protein was observed in human normal stomach tissues and tumor tissues. The inhibitory effect of S-adenosyl-L-homocysteine on FCMT activity was examined in human stomach tumor tissues.

MATERIALS AND METHODS

Materials

S-Adenosyl-L-[methyl-^{14}C]methionine (52 mCi/mmol) was purchased from Amersham (Buckinghamshire, U. K.), S-Adenosyl-L-homocysteine, guanosine 5'-O-(3-thiotriphosphate) (GTPγS), collagen type I and 3,3'-diaminobenzidine tetrahydro-chloride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Monoclonal antibodies [c-Ha-ras, c-N-ras, c-Ki-ras, pan-ras (Ab-1)] were purchased from Oncogene Science (San Diego, CA, U.S.A.). ABC kit was purchased from Vector Co. (Burlingame, CA, U.S.A.).

Preparation of human stomach tissue

Normal, tumor and peritumoral human stomach tissues (14 cases, 30~70 years old) were obtained from the Department of General Surgery of Yonsei University Hospital (Seoul, Korea). Frozen tissues were homogenized in 7 volumes of 0.25 M sucrose buffer (pH 7.4). The homogenate was centrifuged for mitochondrial fraction (14,000×g, 12 min), with high speed centrifuge (Beckman, U.S.A), and then the supernatant was centrifuged again for cytosolic fraction (4°C, 120,000×g, 1 h, Ultra speed centrifuge, Kontron Instrument, Zurich, Switzerland). The microsomal fraction was obtained by suspended pellets in 0.25 M sucrose buffer (pH 7.4).

Synthesis of N-acetyl-trans,trans-farnesylcysteine

N-acetyl-trans,trans-farnesylcysteine (AFC) has been synthesized as a FCMT substrate according to the method as described (Tan *et al.*, 1991). Acetylcysteine 0.1 g was depressed in nitrogen gas. Triethylamine and 0.183 ml farnesyl bromide was added on ice bath after acetylcysteine was dissolved in 5 ml ethanol. The solution was checked by TLC (developing solvent; benzene:tetrahydrofuran:formic acid=20:3:1). Ether and distilled water was added after evaporation. Water layers were collected after extract with ether, and dried with $MgSO_4$ anhydrous. Sample was purified with silicag el column, butanol:acetic acid (100:1, v/v), and purified with HPLC (Waters Associates, U.S.A), Shodex C18 column. AFC was identified with 300 MHz proton nuclear magnetic resonance Spectrometer (Bruker, Germany).

Assay for farnesylcysteine methyltransferase

Farnesylcysteine methyltransferase activity was measured as described (Park *et al.*, 1994) with a slight modification. A total reaction mixture containing 50 μM AFC, 10 mM HEPES buffer (pH 7.4), 0.8 mM GTPγS, 5 mM $MgCl_2$, 0.2 mM S-adenosyl-L-[^{14}C-methyl] methionine (52 mCi/mmol) and 200 mg of enzyme preparation in a total volume of 100 μl was incubated at 37°C for 60 min. The reaction was stopped by addition of 1 ml of cold ethylacetate. The ethylacetate layer was withdrawn after centrifuge at 7,500 rpm for 5 min (Heraeus Sepatech, U.S.A), and then mixed with 500 μl of distilled water. This process of extraction was repeated three more times. All ethylacetate extracts were combined and an aliquot of the mixture was counted for radioactivity by a liquid scintillation counter (Hewlett Packard, U.S.A).

Histopathologic analysis of human stomach tissue

Before tissue preparation, slides were rinsed in 95% ethanol and distilled water, and then dried at 60°C and soaked in 10% poly-L-lysine solution. Each tissue was fixed in 10% neutral formalin solution for 24 h. Tissue was dehydrated in ethanol solutions of increasing concentration (70% → 80% → 85% → 90% → 95% → 100%) for 2 h each, and soaked in xylene for 2 h. Tissue was fixed with paraffin for 30 min, and the paraffinized tissues were cut 4~5 μm by ultramicrotome. Then the slide was stained with hematoxylin and eosin (Munoz *et al.*, 1968) and observed under an optical microscope.

Immunohistochemical analysis of human stomach tissue

The paraffinized tissue on the poly-L-lysine coated slide was deparaffinized with xylene and hydrated with ethanol solutions of decreasing concentration (100% → 95% → 90% → 85% → 80% → 70%) for 2~3 min each.

Immunohistochemical analysis was performed (Hsu *et al.*, 1981) with monoclonal antibodies [c-Ha-ras, c-N-ras, c-Ki-ras, pan-ras (Ab-1)]. Slide was rinsed with PBS for 10 min, then fixed with methanol containing 0.3% H_2O_2. Slide was rinsed again with PBS for 20

min, then blocked non-specific bound with 1.5% horse normal serum solution in 10 mM sodium phosphate (pH 7.5), 150 mM PBS for 25 min. After washing with PBS for 10 min, the slide was incubated at 4°C for 12 h with 100 (g/ml of monoclonal antibodies (1 : 250 dilution in 0.05 M sodium phosphate containing 0.1% sodium azide, 0.2% gelatin). The slide was washed with cold PBS for 15 min, and with water for 5 min and then treated with 0.5% periodic acid for 5 min. After washing with cold PBS for 10 min, slide was incubated with biotinylated anti-mouse IgG diluted to 1 : 100 in 10 mM sodium phosphate (pH 7.5), 150 mM PBS for 2.5 h. After washing with cold PBS buffer for 10 min, slide was incubated with avidin-biotin complex (ABC) solution. And slide was washed with PBS and developed with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB), hydrogen peroxide and nickel solution for 2 min. The slide was washed with cold PBS, water, 70%, 90%, 95%, 100% ethanol for 5 min each and then washed with xylene for 10 min. 3 times. The slide was observed under an optical microscope (× 100).

RESULTS

Activity of farnesylcysteine methyltransferase in human stomach tissue

The FCMT activities of each cellular fractions of the normal, peritumoral and tumor human stomach tissues were observed. The mitochondrial fraction of stomach tumor tissue showed the highest activity of FCMT, 1.41 units (Unit: pmole/min/protein mg), while FCMT activities of whole homogenate, cytosolic fraction and microsomal fraction were 0.77, 0.16 and 1.02 units, respectively. The FCMT activities of mitochondrial fraction of normal, peritumoral and tumor stomach tissues were 0.72, 0.28 and 1.41 units, respectively. FCMT activity in tumor stomach tissue was higher than those of normal and peritumoral tissues. FCMT activity in various fractions was high in mitochondrial fraction, followed by the activities of microsomal fraction, whole homogenate and cytosolic fraction. The overall results of FCMT activity in human stomach tissue were shown in Table I.

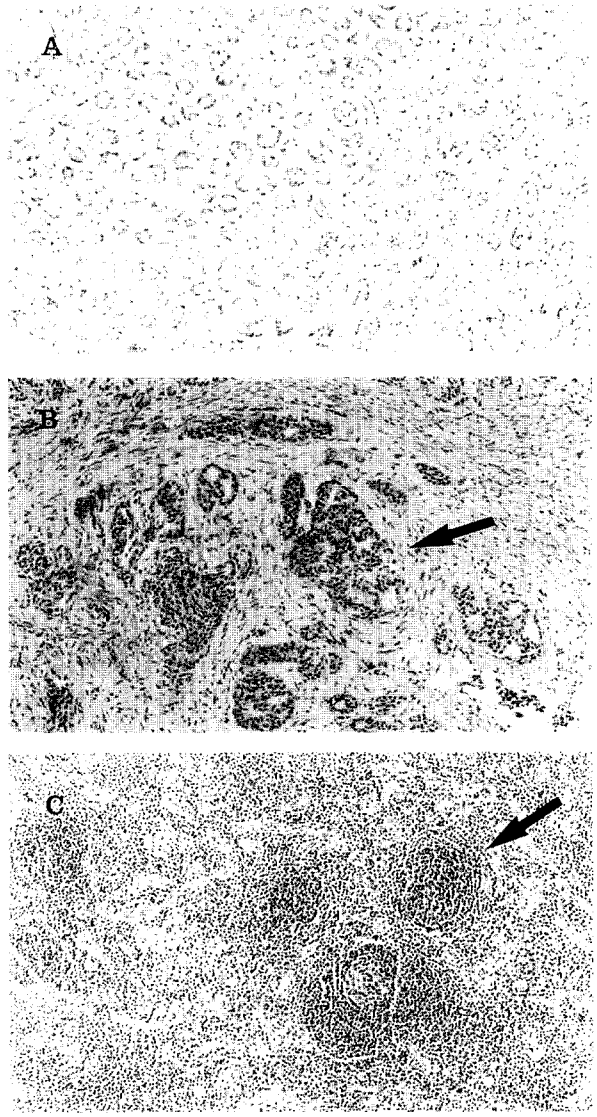

Fig. 1. Hematoxylin and eosin staining of human stomach tissues. Each tissue was fixed in 10% neutral formalin solution for 24 h. Tissue was dehydrated in ethanol solutions of increasing concentration (70% → 80% → 85% → 90% → 95% → 100%) for 2 h each, and soaked in xylene for 2 h. Tissue was fixed with paraffin for 30 min, and was cut 4~5 μm. Then (A) normal stomach mucosa (B) moderately differentiated stomach carcinoma (C) regional lymph node of stomach carcinoma was stained with hematoxylin and eosin and observed under an optical microscope (×100).

Table I. FCMT activity in human stomach tissues

Tissue	No. of case	whole homogenate	mitochondrial fraction	cytosolic fraction	Microsomal fraction
Normal	14	0.31±0.08	0.72±0.19	0.16±0.07	0.45±0.16
Tumor	14	0.77±0.18*	1.41±0.22*	0.17±0.06	1.02±0.20*
Peritumoral	14	0.18±0.07*	0.28±0.09*	0.16±0.05	0.34±0.11*

Values are mean±S.D. Unit of value is pmole/min/protein mg.
No. of case is the number of human patient. FCMT activity was measured using N-acetyl-*trans,trans*-farnesylcysteine as the methyl acceptor substrate. Each tissue was assayed twice, in duplicate.
* Significantly different from normal group ($P<0.05$).

Histopathological analysis of human stomach tissue

Histopathological analysis was performed in normal, peritumoral and tumor human stomach tissue using hematoxylin and eosin staining. As shown in Fig. 1, adenocarcinoma and tumor cells invaded to submucosa, muscularis and serosa were observed in tumor stomach tissue. But it was not observed that tumor cells were metastasized to close lymph nodes. The cells of tumor tissue had irregular cavities and relatively well-differentiated glandular structures. And the shape of nucleus was pleomorphic.

Expression of Ras protein in human stomach tissue

Immunohistochemical analysis was performed in normal stomach, adenocarcinoma and peritumoral tissues. Table II shows the staining intensity of the ras product of each stomach tissue. As shown in Fig. 2~4, the expression of N-ras, K-ras, H-ras and Pan-ras in normal and peritumoral tissues showed weak staining pattern on epithelial cells. In adenocarcinoma, the expression of N-ras, H-ras, Pan-ras showed moderative staining pattern on epithelial cells.

Table II. Immunohistochemical detection of *ras* oncogene expression in normal, adenocarcinoma and peritumoral tissue of human stomach

antibody	normal	adenocarcinoma	peritumoral
non-treatment	-	-	-
N-ras	+	++	+
Ki-ras	+	-	+
Ha-ras	+	++	+
Pan-ras	+	++	+

-: negative, +: weak positive, ++: moderately positive
Each human stomach tissue was fixed with methanol containing 0.3% H_2O_2. and were treated with horse normal serum for 25 min. Tissue was treated at 4°C for 12 h with 100 μg/ml of monoclonal antibodies for 12 h at 4°C. Tissue was treated with biotinylated anti-mouse IgG for 2.5 h and to avidin-biotin complex for 30 min. Tissue was observed under an optical microscope (×100) after treat with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB), hydrogen peroxide and nickel solution for 2 min.

Inhibitory effect of S-adenosyl-L-homocysteine on FCMT activity in human stomach tissue

Inhibitory effects of S-adenosyl-L-homocysteine on FCMT activity were examined in human stomach tis-

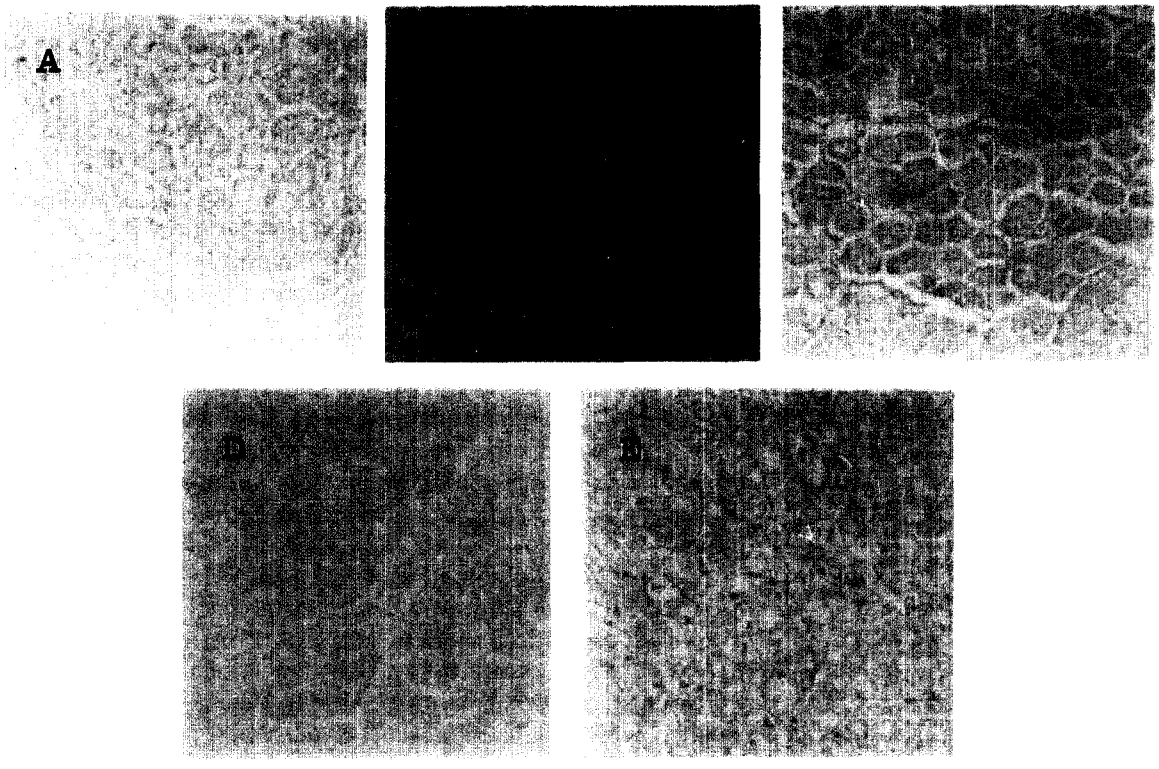

Fig. 2. Immunohistochemical staining of normal human stomach tissue with *ras* monoclonal antibodies. Normal human stomach tissue was fixed with methanol containing 0.3% H_2O_2. and were treated with horse normal serum for 25 min. Tissue was treated at 4°C for 12 h with 100 μg/ml of monoclonal antibodies (A) blank (B) anti-pan-Ras-antibody (C) anti-N-Ras-antibody (D) anti-Ha-Ras-antibody (E) anti-Ki-Ras-antibody. Tissue was treated with biotinylated anti-mouse IgG for 2.5 h and to avidin-biotin complex for 30 min. Tissue was observed under an optical microscope (×100) after treatment with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB), hydrogen peroxide and nickel solution for 2 min.

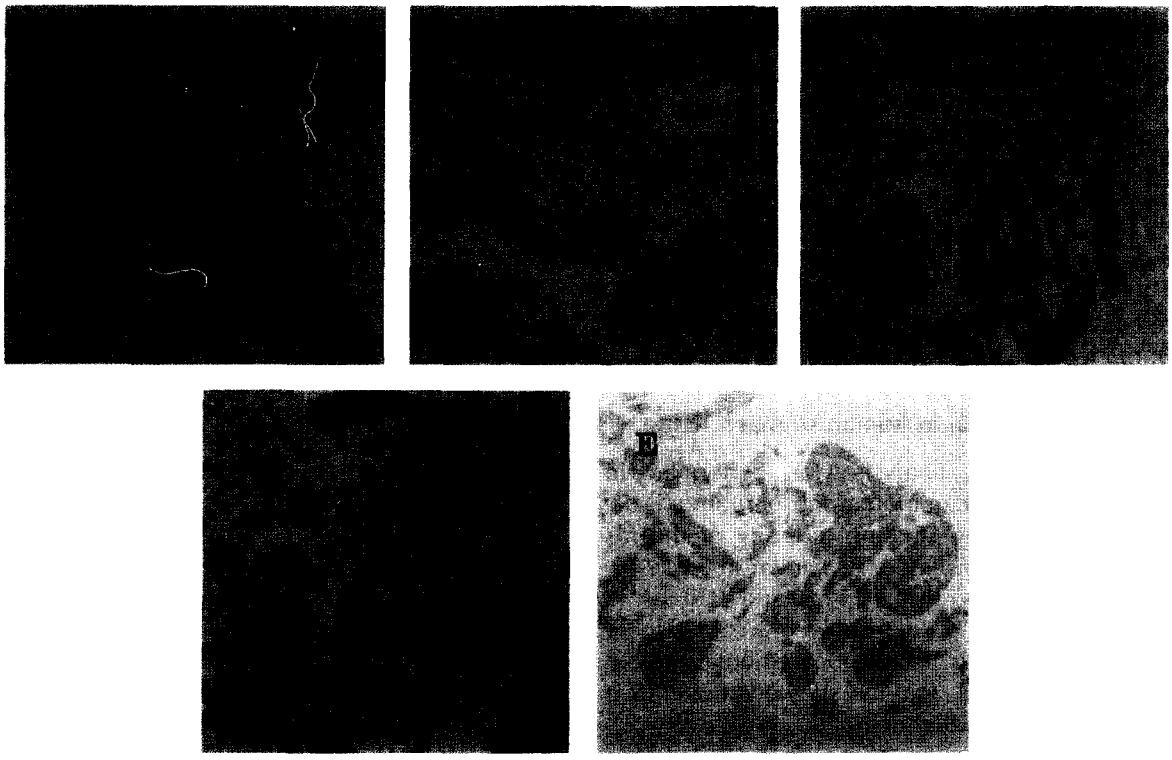

Fig. 3. Immunohistochemical staining of human stomach tumor tissue with anti-*ras* monoclonal antibodies. Human stomach tumor tissue was fixed with methanol containing 0.3% H_2O_2. and were treated with horse normal serum for 25 min. Tissue was treated at 4°C for 12 h with 100 μg/ml of monoclonal antibodies (A) blank (B) anti-pan-Ras-antibody (C) anti-N-Ras-antibody (D) anti-Ha-Ras-antibody (E) anti-Ki-Ras-antibody for 12 h at 4°C. Tissue was treated with biotinylated anti-mouse IgG for 2.5 h and to avidin-biotin complex for 30 min. Tissue was observed under an optical microscope (× 100) after treatment with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB), hydrogen peroxide and nickel solution for 2 min.

sues. The inhibition of FCMT activity was determined after treatment with 0.25 μM S-adenosyl-L-homocysteine. As shown in Table III, treatment with 0.25 μM of S-adenosyl-L-homocysteine resulted in 30.5% inhibition of FCMT activity in mitochondrial fraction of tumor stomach tissue. In cytosolic fraction of peritumoral tissue, inhibition of FCMT activity was 11.2 %. Although S-adenosyl-L-homocysteine did exert inhibitory effect on FCMT activity in various tissues and fractions, there was no significant difference among them.

DISCUSSION

Ras oncogene is found commonly in cancer tissues or in transformed human cell lines (Rodenhuis and Slebos, 1990) and three kind of *ras* genes, Harvey-ras (Ha-ras), Kirsten-ras (Ki-ras), and N-ras are related with human cancers. Ras proteins cause cancer on cells as a result of point mutation. The highest incidences are found in adenocarcinomas of the pancreas (90%), the colon (50%), and the lung (30%), in thyroid tumors (50%), and in myeloid leukemia (30%) (Bos, 1988).

Motojima *et al.*, (1994) reported that the expression of p21 was higher in gastric carcinoma tissue than in normal tissue by Western blot analysis. Tahara *et al.*, (1986) reported that high levels of c-Ha-ras p21s were found in gastric carcinoma by Western blot analysis. And their immunohistochemistry result showed that c-Ha-ras p21 was detected in 3 of 27 (11.1%) early carcinomas and in 63 of 144 (43.8%) advanced carcinomas. Deng *et al.*, (1994) reported that mutation of the 12th codon of Ha-ras was found in Chinese stomach cancer patient by using polymerase chain reaction-restriction analysis. In this study, immunohistochemical analysis was performed in order to find out Ras protein expression in human normal, tumor and peritumoral stomach tissue. The high expression of Ras protein was found in stomach tumor tissue while it was found to less extent in normal and peritumoral tissue. These results are in accordance with reports of Motojima, *et al.*, (1994), Tahara, *et al.*, (1986) and Deng, *et al.*, (1994). The recent discovery of the widespread occurrence of protein modification reactions resulted from the recognition of the common features of several seemingly unrelated biological systems. Klein *et al.*, (1994) characterized prenylated protein methyltransferase of human endometrial carcinomas and compared it to the prenylated protein methyltransferase of normal human endometrium. They reported that this

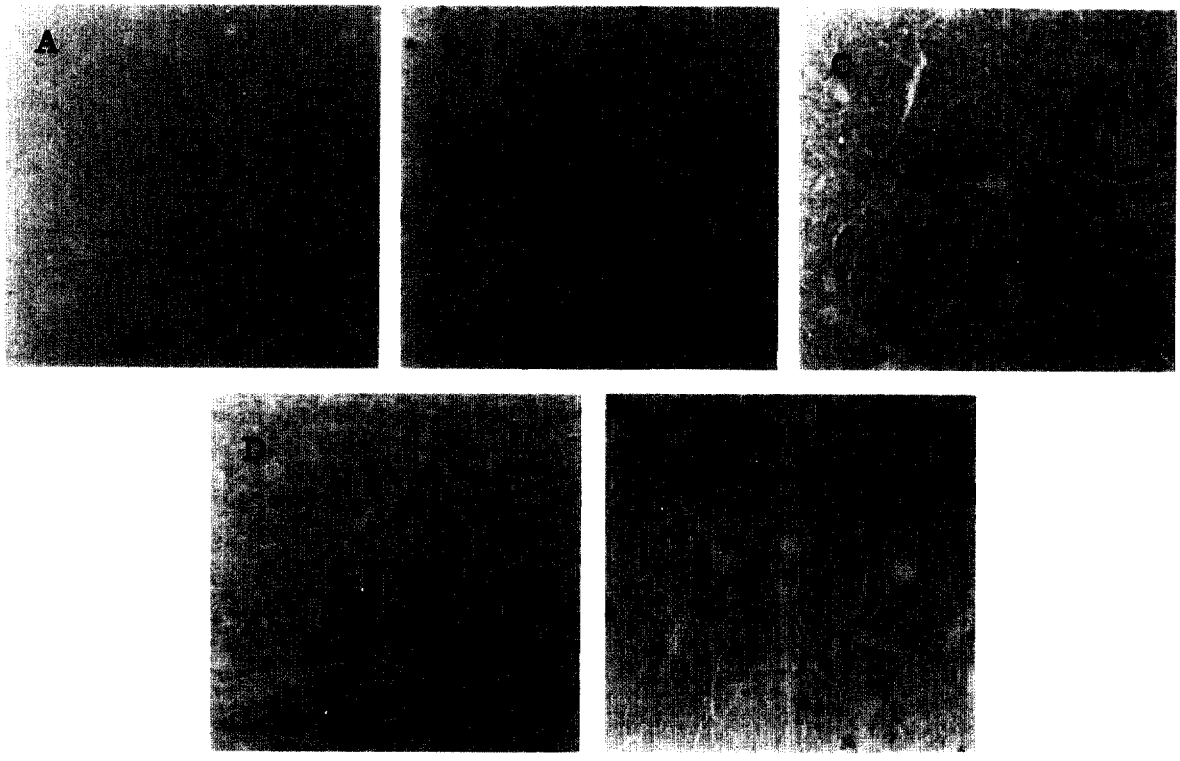

Fig. 4. Immunohistochemical staining of human stomach peritumoral tissue with anti-*ras* monoclonal antibodies. Human stomach peritumoral tissue was fixed with methanol containing 0.3% H_2O_2, and were treated with horse normal serum for 25 min. Tissue was treated at 4°C for 12 h with 100 μg/ml of monoclonal antibodies (A) blank (B) anti-pan-Ras-antibody (C) anti-N-Ras-antibody (D) anti-H-Ras-antibody (E) anti-K-Ras-antibody for 12 h at 4°C. Tissue was treated with biotinylated anti-mouse IgG for 2.5 h and to avidin-biotin complex for 30 min. Tissue was observed under an optical microscope (× 100) after treat with 3,3'-diaminobenzidine tetrahydrochloride substrate (DAB), hydrogen peroxide and nickel solution for 2 min.

Table III. Inhibitory effect of S-adenosyl-L-homocysteine on FCMT activity in human stomach tissues

Tissue	No. of case	Whole homogenate	Mitochondrial fraction	Cytosolic fraction	Microsomal fraction
Normal	14	15.7±4.36	18.6±4.52	11.7±3.58	13.3±4.01
Tumor	14	11.8±3.73*	30.5±5.29*	12.9±3.93	19.5±3.46*
Peritumoral	14	17.3±5.35	12.3±3.96*	11.2±3.57	14.5±3.22

Values are mean±S.D. Unit of value is percentage.
No. of case is the number of human patient. Inhibition effect (%) was measured using 0.25 μM SAH. SAH: S-adenosyl-L-homocysteine as synthetic FCMT inhibitor. Each tissue was assayed twice, in duplicate.
*Significantly different from normal group ($P<0.05$).

enzyme is involved in Ras protein methylation. It was suggested that carboxymethylation plays an important role in activation of G protein and functional response (Lederer, *et al.*, 1994). In this study, FCMT activity was measured in the human normal, tumor and peritumoral stomach tissues to observe difference between tumor and normal cell growth. As a result of measuring FCMT activity, stomach tumor tissue showed higher FCMT activity when compared with normal tissue. These results suggest the possibility that elevated FCMT activity in some types of human cancers may be an important factor for the expression of uncontrolled cell growth.

On the other hand, studies on transmethylation inhibitor have been performed with S-adenosyl-L-homocysteine which is a structural analogue of S-adenosyl-L-methionine and acts as a competitive antagonist. And several transmethylation inhibitors were extracted and purified from animal tissues. Briefly, Kim *et al.*, (1971) reported that protein methylase II inhibitor has been identified in rat liver particulate fractions. Hong *et al.*, (1986) purified an inhibitor for S-adenosyl-L-methionine-dependent methyltransferase from rat liver. Chiva *et al.*, (1984) reported that rat liver cytosol contains a heat-stable factor inhibiting phospholipid methylation by rat liver microsomes. In this study, human stomach normal, tumor and peritumoral tissues of human stomach were treated with 0.25 μM of S-adenosyl-L-

homocysteine in order to observe inhibitory effect to FCMT activity. The inhibitory effect of S-adenosyl-L-homocysteine on FCMT activity was shown from 11.2% to 30.5%.

From the result of experiment on each human stomach tissue, it is supposed that FCMT might be involved in the reactions of Ras protein activity related with growth and differentiation of cells. And we suggest that development of an inhibitor to FCMT can lead to anti-cancer agent, from the fact that FCMT activity was high in transformed tissues, and S-adenosyl-L-homocysteine inhibited FCMT activity. It will be interesting to examine FCMT in human tumors other than stomach tumor, particularly those in which the incidence of ras gene mutations is relatively high, and to determine whether selective FCMT inhibitors can attenuate or inhibit their growth.

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