Original Article

Altered Expression of Lewis Antigen on Tissue and Erythrocytes in Gastric Cancer Patients

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To elucidate the clinical significance of phenotypic alterations of Lewis antigen in gastric cancer patients, we investigated Lewis antigens by analyzing the genotypes of the Le and Se genes and by comparing the results obtained with the phenotypic expression of Lewis antigen in gastric cancer tissue and blood cells. One hundred and twenty gastric cancer patients were examined and compared with respect to Lewis blood phenotype and genotype. The expression of Le^a, Le^b, sialylated Le^a, and sialylated Le^x antigens was immunohistochemically examined in uninvolved gastric mucosa, intestinal metaplasia, and cancerous tissue. We also analyzed the significance of Lewis antigen expression by analyzing patient survival. The frequencies of the Lewis phenotypes of RBCs corresponding to Le(a+b-), Le(a-b+), and Le(a-b-) were 16%, 58%, and 26%, respectively. The Le and le allele gene frequencies calculated from genotyping in gastric cancer patients were 0.623 and 0.377, respectively. The frequency for Le(a-b-) of the RBC phenotype had a tendency to be higher in cancer patients than in normal healthy Koreans. However, no difference in the Lewis gene frequency was found between these gastric cancer patients and healthy persons. The phenotype of Le(a-b+) was most prevalent in uninvolved gastric mucosal tissue, whereas the most prevalent form in tumor tissue was Le(a-b-). Sialyl-Le^a and sialyl-Le^x antigens were hardly detectable in uninvolved gastric mucosa, whereas the two antigens were expressed highly in intestinal metaplastic mucosa and tumor cells. In conclusion, the loss of Lewis antigen expression in tissue and on RBCs in gastric cancer patients is not a result of genetic influences, but rather a result of sialylation in tissue. We also confirm that poor prognosis is associated with dimeric sialyl-Le^x and vascular spread.

Key Words: Lewis antigen, Lewis genotype, gastric cancer, sialylation

INTRODUCTION

Lewis antigens, found in the serum secretions of various tissue types, have the capability of binding to the surface of erythrocytes, and are thereby presented as erythrocyte antigens. Lewis antigens are regulated by the Le and Se genes, which have varied expression patterns that are dependent on the particular cell structure and cell type. The Lewis phenotype of erythrocytes has been reported to demonstrate heterogeneity in certain physiologic and pathologic conditions, such as pregnancy,[1] alcoholic pancreatitis,[2] hepatic cirrhosis,[2] and hydatid cyst.[3] Interestingly, the differential tissue expression of Lewis blood antigen presentation has also been reported in a subset of cancer patients.[4] Lewis antigen is derived from the Lewis (FUT3) and Secretor (FUT2) genes in the 19th chromosomes, and it is expressed in the form of the antigens Le^a, Le^b, Le^x, and Le^y.[5-7] If the Lewis antigen is derived from both the Le and Se alleles, Le^a and Le^b will coexist in tissue. However, Le^b binds onto the erythrocyte surface preferentially, and thus, the resulting RBC phenotype will be Le(a-b+), which is the secretory form of the Lewis antigen. However, if Lewis antigen has the Le allele and not the Se allele, the antigenic form of Le^a will be expressed, giving rise to the Le(a+b-) phenotype, which is the non-

secretory form of the Lewis antigen. If Lewis antigen does not carry the *Le* allele, the erythrocyte phenotype will be Le(a-b-) regardless of the presence or absence of the *Se* allele. The antigenic forms of Le^x and Le^y are derived from the type 2 Lewis precursor, and their expression and regulation is similar to that of Le^a and Le^b. However, Le^x and Le^y are expressed only in tissue and they lack the ability to bind to erythrocytes.[7]

Interest in Lewis expression in normal tissue and in tumors has increased in recent years because sialylated derivatives of the Lewis antigens, such as sialylated Le^a or sialylated Le^x, may act as tumor associated markers in certain alimentary tract cancers.[8] Sialylated Lewis antigen, as an E-selectin ligand, also plays a significant role in binding to the endothelium of blood and lymphatic vessels, an important step in metastasis.[9,10]

In this study, we investigated the phenotypic alterations of the Lewis antigen on erythrocytes by analyzing genotypes of *Le* and *Se* genes, and by comparing results with the phenotypic expression of Lewis antigen in gastric cancer tissue and blood. We also investigated the clinical significance of sialylated Lewis antigen expression in cancer tissues by analyzing patient survival.

MATERIALS AND METHODS

Patients

Fifty seven gastric cancer patients were selected for genotyping of the Lewis antigen, Lewis phenotyping of RBCs and immunohistochemical staining for the expressions of Le^a, Le^b, silalylated Le^a, and sialylated Le^x antigens. Patients who had undergone gastric resection were randomly selected at Yonsei University Medical Center between November 1998 and March 1999. An additional 63 gastric cancer patients had undergone gastric resection at the same hospital between September 1992 and November 1993 were included for immunohistochemical and survival analysis (Table 1). Data of the patients' clinical courses, histological tumor type, and recurrence were obtained from inpatient chart records, and patient survival was confirmed from clinician outpatient records.

Lewis genotyping by the PCR-RFLP method

DNA was extracted using a DNA Wizard kit (Promega, Madison, WI, USA) from EDTA collected whole blood. In order to amplify the entire *Se* allele, we used primer sets tk1 and tk2, and to detect A385T mutations in the *Se* genotyping, nested PCR was performed with primer sets tk7 and tk8 as previously described.[11-13] To detect T59G mutations in the *Le* genotype, primer sets 59A and 59AS were used. The amplified product of *le* alleles (missense mutation, T59G) was cleaved into two fragments, 97 and 23 bp by *Msp I* (Takara biotechnology Co., Shiga, Japan) digestion. The *se* allele (A385T) was cut to size, corresponding to 51 and 29 bp, by *Alu I* (Takara Biotechnology Co., Shiga, Japan) digestion.

Immunohistochemical staining

Deparaffinized 4-μm sections of cancer tissue and adjacent uninvolved normal tissue specimens were carefully selected so that both tumor containing tissue and uninvolved tissue could be compared. The paraffin was removed by tissue treatment in xylene for 5 minutes, 3 times. The tissue was washed with 100% alcohol for 3 minutes, 90% alcohol for 3 minutes, and 70% alcohol for 3 minutes, and then rinsed with PBS (phosphate buffered saline, pH 7.5). Antigen detection was performed by applying primary monoclonal antibody, followed by biotinylated sheep anti-mouse IgG or anti-mouse IgM and then avidin-peroxidase complex (Dako LSAB2 system, Dako Co., Carpinteria, CA, USA). Glass slides were washed with PBS between each step, and 3-amino-9-ethylcarbazole (AEC, Dako Co., Carpinteria, CA, USA) or diaminobenzidine was used for the peroxidase reaction. Monoclonal antibodies in this study were as follows: a 50-fold dilution of murine monoclonal antibody BG-5 for anti-Le^a (Signet Laboratories Inc., Dedham, MA, USA); a 40-fold dilution of the murine monoclonal antibody BG-6 for anti-Le^b (Signet Laboratories Inc., Dedham, MA, USA); a 150-fold dilution of CA19-9 for anti-sialyl Le^a (provided by Dr. Narimatsu H, Soka University, Tokyo, Japan); a 100-fold dilution of FH-6 for anti-sialyl Le^x (also provided by Dr. Narimatsu H, Soka University,

Table 1. Clinical and Pathologic Characteristics of 63 Gastric Carcinoma Patients Examined for the Survival Analysis

Categories	Variables	
Clinical features		
Age (year, mean ± SD)		55.6 ± 12.4
Sex (male/female)		35/28
Patient outcome		
	Alive with no evidence of disease	9
	Alive with disease	3
	Dead of disease	30
	Dead of other causes	21
Pathological features		
Tumor size (cm, mean ± SD)		5.5 ± 4.2
Histologic differentiation		
	Well differentiated	21
	Poorly differentiated	42
Depth of invasion (serosa exposed)		
	Negative	4
	Positive	59
Lymph node metastasis		
	Negative	13
	Positive	50
Lymph node invasion		
	Negative	25
	Positive	38
Venous invasion		
	Negative	44
	Positive	19
Tumor stage	Stage I and II	12
	Stage III and IV	51

Tokyo, Japan). Slides were counterstained with Meyer's hematoxylin. The degree of staining for intracellular antigen expression was as follows: +, more than 10% positive staining of the tumor cells; -, absence of staining or less than 10% staining of the tumor cells (Fig. 1).

RBC phenotyping for Lewis antigen

Determination of RBC phenotypes for Lea and Leb were carried out using a standard hemagglutination test, which uses a microtube technique (DiaMed, Murten, Switzerland).

Statistical analysis

Gene frequencies were derived from the Hardy-Weinberg equilibrium for diallelic systems.[14] Survival analysis was calculated using the pro-

portional hazards regression method in the SAS software program (SAS Institute Inc., Cary, NC, USA). Parameters univariantly influencing survival ($p < 0.05$) were included in the multivariate analysis.

RESULTS

The frequencies of Lewis RBC phenotypes and genotypes in cancer patients (n=57) are shown in Table 2. The frequencies of Lewis phenotypes of RBCs corresponding to Le(a+b-), Le(a-b+), and Le(a-b-) were 16%, 58%, and 26%, respectively. The Le and le allele gene frequencies calculated from genotyping in gastric cancer patients were 0.623 and 0.377, respectively (Le=71/114, le=43/114).

When comparing the genotype with the RBC

Table 2. Comparison of RBC Phenotypes and Genotypes in the Lewis Systems of the 57 Gastric Cancer Patients

	RBC phenotype			Genotype*	
	No. of cases	Frequency (%)		No. of cases	Frequency (%)
Le(a+b-)	9	16	Le(a+b-)	6	11
Le(a-b+)	33	58	Le(a-b+)	39	68
Le(a-b-)	15	26	Le(a-b-)	12	21

*Genotyping was performed on DNA from gastric cancer patients.

Table 3. Loss of the Lewis Antigen in Erythrocytes of Gastric Cancer Patients with the *Le* Alleles

Genotype		Expected Lewis RBC phenotype	No. of cases	
Lewis	*Secretor*		No change	Le(a-b-)*
Le/Le	*se/se*	Le(a+b-)	4	2
	Se/Se	Le(a-b+)	9	0
	Se/se	Le(a-b+)	3	2
Le/le	*se/se*	Le(a+b-)	5	1
	Se/Se	Le(a-b+)	14	2
	Se/se	Le(a-b+)	7	2
le/le	*Se/Se*	Le(a-b-)	4	0
	Se/se	Le(a-b-)	2	0

*loss of Lewis RBC phenotype expression.

phenotype of the Lewis antigen in gastric cancer patients, 9 patients were found to have lost the Lewis antigen in erythrocytes. However, in 6 *Le* gene negative (*le*) gastric carcinoma patients, no anomalous Lewis antigen acquisition was observed (Table 3).

Among the 120 gastric carcinoma patients, immunohistochemical results were not available in 6 cases, because the small amount of tumor tissues. Of 114 patients who were stained with Lewis antibody, 92 patients (80.7%) had intestinal metaplasia in the nearby mucosa. In uninvolved gastric mucosa, Le^b antigen was detected in 104 of the 114 specimens (91.2%). In addition, the tissue phenotype forms of Le(a-b+) predominated while Le(a+b-) and Le(a-b-) were only observed occasionally. Le^a positive staining occurred more frequently in intestinal metaplasia than in uninvolved gastric mucosa. In gastric cancer tissue, the Le(a-b-) phenotype was the predominating form in 40.4% (Table 4).

Sialyl-Le^a antigen and sialyl-Le^x antigen ex-

pression was observed in 10% and 4% of uninvolved gastric mucosa, respectively. In intestinal metaplasia, the expressions of sialyl-Le^a and sialyl-Le^x were detectable in 76% and 82%, respectively. However, in gastric cancer tissue, sialyl-Le^a and sialyl-Le^x were expressed in 55% and 50%, respectively, demonstrating a moderate reduction versus intestinal metaplasia (Fig. 2).

In the 57 gastric carcinoma patients who were examined for Lewis phenotype in carcinoma tissue and for blood genotype, the concordance rate between the Lewis genotype and the immunohistochemistry of Lewis related antigens was 24.6% (14 patients). Of the 43 cases in which the genotype did not correspond to the Lewis tissue phenotype, 35 patients (61.4%) showed loss of the Lewis antigen and 8 cases (14.0%) expressed the anomalous Le^b antigen (Table 5).

The expression of sialyl-Le^x antigen in gastric carcinoma tissue, age, stage, and venous invasion were found to be statistically significant ($p < 0.05$)
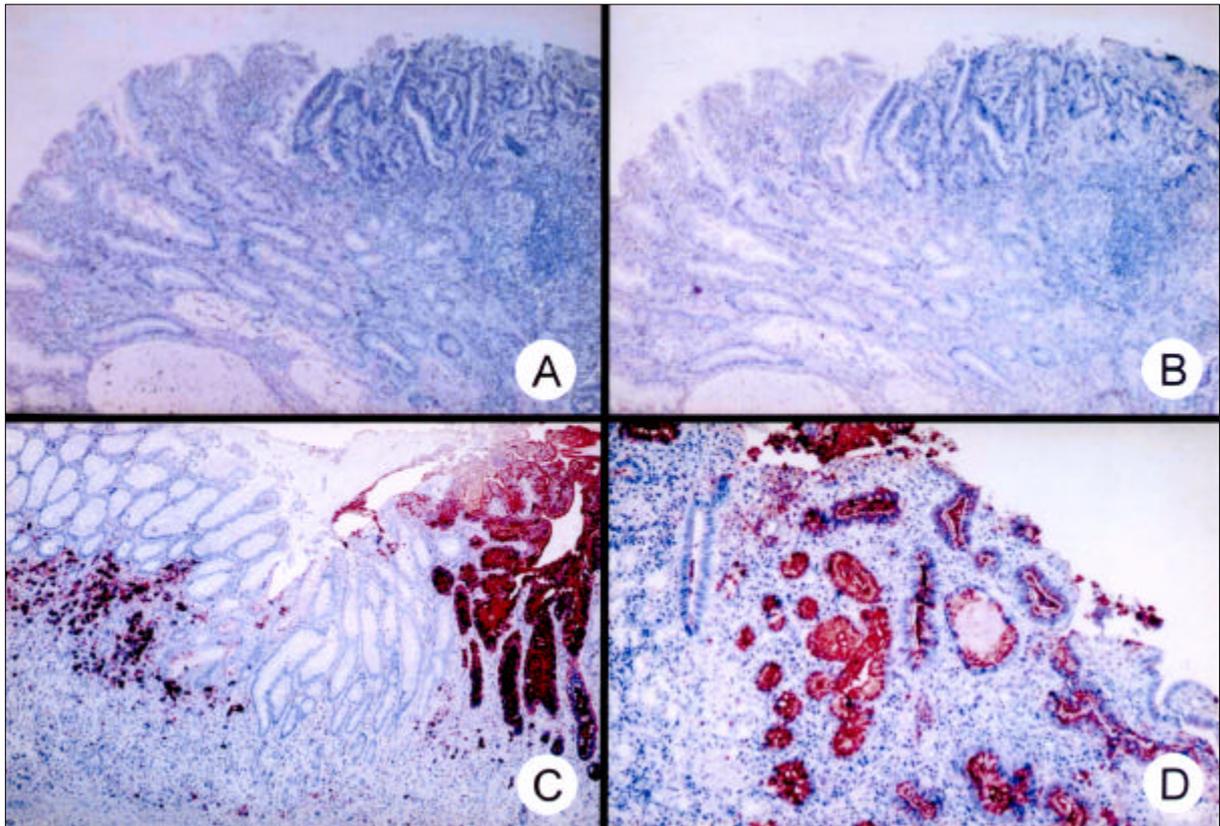
Fig. 1. Negative reaction of immunohistochemical staining on gastric tissue with anti-Le^a antibody (A) and anti-Le^b antibody (B). The genotype of this patient was *le/le, Se/se*. Positive reaction with anti-Le^a antibody (C) and anti-Le^b antibody (D). Using both antibodies, most of the cancer cells show the expressions of the Le^a and Le^b antigens. The genotype of this patient was *Le/le, Se/Se* ($\times$ 40).
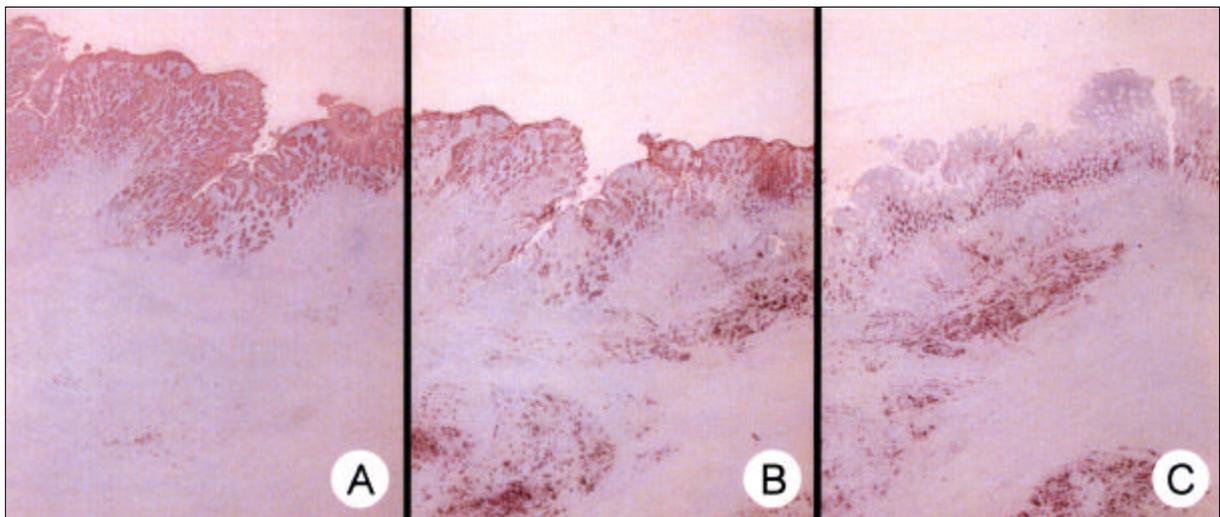

Fig. 2. Serial section of normal gastric epithelium and gastric adenocarcinoma demonstrating the expression of Le^a, sialyl Le^a, and sialyl-Le^x. Le^a is expressed in the normal gastric epithelium and rare in the cancer cells (A). Sialyl-Le^a is expressed in the normal gastric epithelium and some of the cancer cells (B). Silayl-Le^x is expressed in some of the cancer cells (C). Normal gastric epithelium in sialyl-Le^x is not detected in the gastric epithelium, however, sialyl-Le^x antigen is expressed in the cancer cells of the right lower corner ($\times$ 4).

Table 4. The Expression of Lewis Antigens in Uninvolved Gastric Mucosal Tissue, Intestinal Metaplasia, and Carcinoma Tissue of the 114 Cases

Tissue phenotype	No. of positive cases (%)		
	Uninvolved gastric mucosa	Intestinal metaplasia	Tumor
Le(a+b+)	24 (21.1)	54 (58.7)	42 (36.8)
Le(a+b-)	2 (1.8)	16 (17.4)	7 (6.1)
Le(a-b+)	80 (70.1)	10 (10.9)	19 (16.7)
Le(a-b-)	8 (7.0)	12 (13.0)	46 (40.4)

Table 5. Comparisons of the Lewis Genotype and Phenotype of Gastric Cancer Tissues from 57 Gastric Carcinomas

Tissue phenotype	Le genotype			Total (%)
	Le/-*, se/se	Le/-, Se/-†	le/le, -/-	
No change	3	8	3	14 (24.6)
Change				
Anomalous Le^b	5	0	3	8 (14.0)
Le^a loss	4	0	0	4 (7.0)
Le^b loss	0	31	0	31 (54.4)

*denotes any type of *Le* allele.
†denotes any type of *Se* allele.

Table 6. Multivariate Analysis of Factors Affecting Disease Recurrence in 63 Patients with Curative Resection for Gastric Cancer

Variable	Relative risk	95% confidence interval	p-value
Sialyl Le^a (negative vs. positive)	0.40	0.11 - 1.44	0.161
Sialyl Le^x (negative vs. positive)	4.22	1.05 - 17.04	0.043
Age (year, 60 < vs. 61≥)	0.40	0.21 - 0.77	0.006
Stage (I/II vs. III/IV)	2.16	1.03 - 4.52	0.040
Histologic type (well diff. vs. poorly undiff.)	0.85	0.27 - 2.73	0.786
Lymph node invasion (negative vs. positive)	1.10	0.37 - 3.34	0.862
Lymph node metastasis (negative vs. positive)	0.23	0.01 - 8.20	0.423
Venous invasion (negative vs. positive)	3.28	1.07 - 10.08	0.038

prognostic indicators of clinical outcome (Table 6).

DISCUSSION

The incidence of phenotype in Lewis antigen systems has been found to differ according to racial composition. In Koreans, the frequencies of the RBC phenotypes Le(a+b-), Le(a-b+), Le(a-b-) were reported to be 16.1%, 70.2%, and 13.7%,

respectively.[15] Lewis antigen expression in RBCs and tissue specimens has also been reported to be heterogenous, and to depend on the physiologic and/or pathologic status of the patient. Cases best demonstrating this phenomenon are found among pregnant women and cancer patients, in whom a change of biologic function results in the loss of Lewis antigen. In pregnancy, the production of steroid hormones affects the enzyme fucosyl-transferase, which contributes to the loss of Lewis

antigen.[16] However, a different mechanism is observed in cancer patients, in whom the Lewis antigen adsorption process is reported to be suppressed, thus preventing antigen expression.[4,17-19] In our experiment, we found a difference in the frequencies of RBC phenotypes Le(a-b-) in gastric cancer patients and normal healthy Korean subjects; these were 26% and 13.3%, respectively.[13,15] However, the *Le* and *le* allele gene frequencies have been reported to be 0.665 and 0.335, respectively in Koreans,[20] and we previously confirmed theses results.[13] The *Le* and *le* allele gene frequencies calculated from genotyping in gastric cancer patients were 0.623 and 0.377, respectively, and, the *Le* and *le* allele frequencies calculated from the RBC phenotyping of Lewis antigen in healthy controls were 0.635 and 0.365, respectively.[13] Moreover, these gene frequencies were similar in gastric cancer and normal subjects, which indicates that loss of Lewis blood antigen expression in gastric cancer is not a result of genetic differences.

In the present study, we investigated the correlation between the Lewis genotype and Lewis antigen expression in gastric cancer tissue. In uninvolved gastric mucosa, a paucity of Le^a expression was observed, and Le^b antigen expression was generally largely positive. However, in intestinal metaplasia, Le^a antigen expression was abundant. Gastric cancer patients also showed inconsistency between genotype and tissue phenotypes. Gastric cancer patients with phenotype Lewis(a-b-) exhibited loss of the Lewis antigen, which implies that the formation of Le antigen terminates after tumor formation. These findings can also be attributed to the excessive sialylation of the precursor chain in cancer tissue.

Therefore, sialyl-Le^a antigen is formed instead of the Le^a antigen. In both intestinal metaplasia and cancer, the sialyl-Lewis antigen was found to be expressed, while it was absent in uninvolved normal tissue. Therefore, sialylation seems to occur during tumorigenesis. However, although we found the loss of Lewis antigen in gastric cancer tissue and in the blood of patients, the relationship between these phenotypic alterations of cancer tissue and blood cannot be explained, because the non-tumorous mucosa of some gastric cancer patients retained the Lewis antigen.

As was done by a number of preceding reports,[21,22] we confirm that poor prognosis is associated with the dimeric sialyl-Le^x antigen and vascular spread. In conclusion, altered Lewis antigen expression on tissue and RBCs in gastric cancer patients is not a result of genetic influences, but rather a result of sialylation in tissue. However, in order to elucidate the biologic behavior of tumors containing the sialyl antigen expression, a study including a larger patient population will be required.

REFERENCES

1. Hammer L, Mansson S, Rohr T, Chester MA, Ginsburg V, Lundblad A, et al. Lewis phenotype of erythrocytes and Le^b actine glycolipid in serum of pregnant women. Vox Sang 1981;40:27-33.
2. Stigendal L, Olsson R, Rydberg L, Samuelsson BE. Blood group Lewis phenotype on erythrocytes and in saliva in alcoholic pancreatitis and chronic liver disease. J Clin Pathol 1984;37:778-86.
3. Makni S, Dalix AM, Caillard T, Compagnon B, Pendu JL, Ayed K, et al. Discordance between red cell and saliva Lewis phenotypes in patients with hydatid cysts. Exp Clin Immunogenet 1987;4:136-43.
4. Orntoft TF, Holmes EH, Johnson P, Hakomori S, Clausen H. Differential tissue expression of the Lewis blood antigens: enzymatic, immunohistologic, and immunochemical evidence for Lewis an a and b antigen expression in Le(a-b-) individuals. Blood 1991;77:1389- 96.
5. Couillin Ph, Mollicone R, Griscard MC, Gibuard A, Ravise N, Feingold J, et al. Chromosome 11q localization of one of the three expected genes for he human-3-fucosyltransferases by somatic hybridization. Cytogenet Cell Genet 1991;56:108-11.
6. Nishihara S, Nakazato M, Kudo T, Kimura H, Ando T, Narimatsu H. Human-1,3-fucosyltransferase (*FucT-VI*) gene is located at only 13 Kb 3' to the Lewis type fucosyltransferase (*FucT-III*) gene on chromosome 19. Biochem Biophys Res Commun 1993;190:42-6.
7. Reguigne-Arnould I, Couillin P, Mollicone R, Faure S, Fetcher A, Kelly RJ, et al. Relative positions of two clusters of human-L-fucosyltransferases in 19q (*FUT1-FUT2*) and 19p (*FUT6-FUT3-FUT5*) within the microsatellite genetic map of chromosome 1. Cytogenet Cell Genet 1995;71:158-62.
8. Nakagoe T, Sawai T, Tsuji T, Jibiki M, Nanashima A, Yamaguchi H, et al. Difference in prognostic value between sialyl Lewis^a and sialyl Lewis^x antigen levels in the preoperative serum of gastric cancer patients. J Clin Gastroenterol 2002;34:408-15.
9. Phillips ML, Nudelman E, Gaeta FCA, Perez M,

Singhal AK, Hakomori S, et al. ELAM-1 mediates cell adhesion by recognition of a carbohydrate ligand, sialyl-Le^x. Science 1990;250:1130-2.

10. Takada A, Ohmori K, Takahashi N, Tsuyuoka K, Yago K, Zenita K, et al. Adhesion of human cancer cells to vascular endothelium mediated by a carbohydrate antigen, sialyl Lewis A. Biochem Biophys Res Commun 1991;179:713-6.

11. Kudo T, Iwasaki H, Nishihara S, Shinya N, Ando T, Narimatsu I, et al. Molecular genetic analysis of the human Lewis histo-blood group system. II. Secretor gene inactivation by a novel single missense mutation A385T in Japanese nonsecretor individuals. J Biol Chem 1996;271:9830-7.

12. Koda Y, Soejima M, Kimura H. Detection of T to G mutation at position 59 in the Lewis gene by mismatch polymerase chain reaction. Int J Legal Med 1995;107: 280-2.

13. Kim MJ, Kim HO, Kim HS, Song KS. Influence of Lewis phenotype and genotype on serum CA19-9 and DUPAN-2 level in gastric cancer patients. Korean J Blood Transfusion 2000;11:115-23.

14. Bodmer WF, Cavalli-Sforza LL. Genetics, evolution, and man. San Francisco: WH Freeman; 1976. p.232-58.

15. Choi S, Kim SI, Cho HI. Study on gene frequencies of blood groups in Koreans. Korean J Hematol 1984;19: 63-75.

16. Issit PD, Anstee DJ. Applied blood group serology. 4th

ed., Durham, North Carolina: Montgomery scientific publications; 1998. p.261.

17. Hirano K, Kawa S, Oguchi H, Kobayashi T, Tonekura H, Ogata H, et al. Loss of Lewis antigen expression on erythrocytes in some cancer patients with high serum CA19-9 levels. J Natl Cancer Inst 1987;79:1261-8.

18. Langkilde NC, Wolf H, Orntoft TF. Lewis antigen expression in benign and malignant tissues from RBC Le(a-b-) cancer patients. Br J Haematol 1991;79:493-9.

19. Orntoft TF, Vestergaard EM, Holmes E, Mortensen M, Johnson P, Bross P, et al. Influences of Lewis 1-3/4-L-fucosyltransferase (*FUT3*) gene mutations on en- zyme activity, erythrocyte phenotyping, and circulating tumor marker sialyl-Lewis a levels. J Biol Chem 1996; 271:32260-8.

20. Suh IB, Kim YK. Relationship of Helicobacter pylori IgG seroprevalence with Lewis (a,b) blood group phenotype/genotype. Korean J Blood Transfusion 2001; 12:35-45.

21. Amado M, Carneiro F, Seixas M, Clausen H, Sobrinho-Simoes M. Dimeric sialyl Le^x expression in gastric carcinoma correlates with venous invasion and poor outcome. Gastroenterology 1998;114:462-70.

22. Sakamoto J, Watanabe T, Tokumaru T, Takagi H, Nakazato H, Lloyd K. Expression of Lewis^a, Lewis^b, Lewis^x, Lewis^y, Sialyl-Lewis^a, and sialyl-Lewis^x Blood group antigens in human gastric carcinoma and in normal gastric tissue. Cancer Res 1989;49:745-52.