• GASTRIC CANCER •

Carbonic anhydrase isozymes IX and XII in gastric tumors

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Abstract

AIM: To systematically study the expression of carbonic anhydrase (CA) isozymes IX and XII in gastric tumors.

METHODS: We analyzed a representative series of specimens from non-neoplastic gastric mucosa and from various dysplastic and neoplastic gastric lesions for the expression of CA IX and XII. Immunohistochemical staining was performed using isozyme-specific antibodies and biotin-streptavidin complex method.

RESULTS: CA IX was highly expressed in the normal gastric mucosa and remained positive in many gastric tumors. In adenomas, CA IX expression significantly decreased towards the high grade dysplasia. However, the expression resumed back to the normal level in well differentiated adenocarcinomas, while it again declined in carcinomas with less differentiation. In comparison, CA XII showed no or weak immunoreaction in the normal gastric mucosa and was slightly increased in tumors.

CONCLUSION: These results demonstrate that CA IX expression is sustained in several types of gastric tumors. The variations observed in the CA IX levels support the concept that gastric adenomas and carcinomas are distinct entities and do not represent progressive steps of a single pathway.

INTRODUCTION

The carbonic anhydrases (CAs) catalyze the reversible hydration of carbon dioxide, $CO_2 + H_2O \Leftrightarrow HCO_3^- + H^+$, and participate in various physiological processes, including respiration, bone resorption, renal acidification, gluconeogenesis, and formation of cerebrospinal fluid and gastric acid. At present, 11 functionally active CA isozymes, differing in their tissue distribution and enzymatic activity, have been identified in mammals[1-3].

CA IX was initially described as a tumor-associated integral plasma membrane antigen (MN)[4]. It has been reported to contain an extracellular domain with the essential structural features and high activity of CAs[5-7]. CA IX has been detected in the normal gastric, intestinal, and biliary mucosa[8]. Because CA IX is more strongly expressed in the proliferating cryptal epithelium than in the upper part of the mucosa, it may play a role in the control of proliferation and differentiation of intestinal epithelial cells[9]. Cell proliferation is abnormally increased in premalignant and malignant lesions, and therefore CA IX has been considered as a potential biomarker for tumor progression[10]. Previous studies have shown that CA IX is expressed in a high percentage of human epithelial tumors, including carcinomas of uterine cervix, lung, kidney, biliary tract, colon, and breast[11-20]. Most of these tumors arise from tissues with no or low CA IX expression. On the other hand, it has been proposed that CA IX is absent or reduced in most tumors originating from CA IX-positive tissues[8].

CA XII is another transmembrane CA isozyme, whose expression has been demonstrated in normal human kidney, colon, prostate, pancreas, ovary, testis, lung, and brain[21, 22]. By immunohistochemistry, CA XII has been detected at the basolateral plasma membrane of the superficial epithelial cells of the colon and rectum, while the small intestine has remained negative[23]. CA XII protein has also been located in the epithelial cells of endometrium[24], renal tubules[25], and efferent ducts[26]. CA XII shows a clear association with some tumors, which was first reported by Türeci et al.[27]. They showed that in 10 % of patients with renal cell cancer, the CA XII transcript was expressed at much higher levels in the tumor than in the surrounding normal kidney tissue. These results have been recently confirmed by immunohistochemical staining showing CA XII expression in most cases of clear cell carcinomas and oncocytomas[25]. In addition to renal tumors, CA XII is expressed in a number of colorectal tumors[23] and in ductal carcinomas of the breast[19].

CA IX and XII seem to be regulated by similar mechanisms. First, Ivanov et al.[21] identified the *CA9* and *CA12* genes as von Hippel-Lindau (VHL) target genes. They observed that the wild-type VHL protein down-regulated the transcription of CA IX and XII mRNA, indicating that these isozymes may have a potential role in VHL-mediated carcinogenesis. Second, both isozymes have been induced under hypoxic conditions in tumors and cultured tumor cells[22,28]. Hypoxia activates transcription through hypoxia inducible factor-1 (HIF-1), which is composed of two subunits (HIF-1α and HIF-1α)[29]. It is also known that VHL gene product (pVHL) interacts with HIF-1α and is required for the destruction of HIF-1α under normoxic conditions[30]. Taken together, it seems plausible that

CA IX and XII are functionally connected to neoplastic processes controlled by HIF-1 and pVHL[22]. Furthermore, high expression of CA IX and XII in tumors has suggested that they may functionally participate in the invasion process, which is facilitated by acidification of extracellular space. In favor of this hypothesis, it has been shown *in vitro* that CA inhibitors can reduce the invasion capacity and/or proliferation of cancer cells[31-34].

The present study was designed to examine the expression of CA IX and XII in gastric tumors. Even though gastric mucosa is known to be the predominant site of CA IX expression, tumors originating from this area have not been comprehensively studied for the expression of these isozymes.

MATERIALS AND METHODS

Antibodies
The polyclonal rabbit antibodies against human CA XII have been produced earlier[24]. The monoclonal antibody M75 against human CA IX has also been described earlier[4]. Both antibodies have been characterized for specificity and they have shown no cross-reactivity with other CAs[9,24].

Immunocytochemistry
The tissue samples from the non-neoplastic gastric mucosa and the corresponding benign and/or malignant neoplastic samples were obtained alongside routine histopathological specimens collected at Oulu University Hospital (Oulu, Finland). The numbers of samples in each histological category are shown in Table 1. The study was approved by the Ethics Committee of Oulu University Hospital and performed according to the guidelines of the Declaration of Helsinki.

Table 1 Number of gastric specimens analyzed for CA IX and XII expression

Diagnosis	CA IX (n)	CA XII (n)
Nonneoplastic cardia	7	5
Nonneoplastic corpus	21	15
Nonneoplastic antrum	26	17
Hyperplasia	13	3
Adenoma, slight dysplasia	11	4
Adenoma, moderate dysplasia	8	3
Adenoma, severe dysplasia	3	1
Adenocarcinoma, grade I	11	8
Adenocarcinoma, grade II	16	15
Adenocarcinoma, grade III	16	15
Diffuse carcinoma	18	15
Metastasis of gastric carcinoma	13	13
Intestinal metaplasia	33	20

The specimens were fixed in 4 % neutral-buffered formaldehyde for 24-48 h. Then they were dehydrated, embedded in paraffin in a vacuum oven at 58 ℃, and 5 μm sections were placed on Superfrostä microscope slides (Menzel Gläser, Braunschweig, Germany). The CA isozymes were immunostained by the biotin-steptavidin complex method, employing the following steps: (a) Pretreatment of sections with undiluted cow colostrum (Biotop Ltd, Oulu, Finland) for 30 min and rinsing in phosphate-buffered saline (PBS). (b) Incubation for 1 h with anti-CA XII serum (1:100), normal rabbit serum (1:100) or hybridoma medium with M75 antibody (1:10) in 1 % BSA-PBS. (c) Incubation for 1 h with biotinylated swine anti-rabbit IgG (Dakoplatts, Glostrup, Denmark) or goat anti-mouse IgG (Dakopatts) diluted 1:300 in 1 % BSA-PBS. (d) Incubation for 30 min with peroxidase-conjugated steptavidin (Dakoplatts) diluted 1:500 in PBS and (e) incubation for 2 min in DAB solution containing 9 mg 3,3'-diaminobenzidine tetrahydrochloride (Fluka, Buchs, Switzerland) in 15 ml PBS and 5 μl 30 % H_2O_2. The sections were washed 3 times for 10 min in PBS after incubation steps b and c, and 4 times for 5 min after step d. All the incubations and washings were carried out at room temperature. After the immunostaining, the tumor sections were counterstained with hematoxylin. The stained sections were examined and photographed with Zeiss Axioplan 2 microscope (Zeiss, Göttingen, Germany).

The immunohistochemical results were semiquantitative based on the percentage of the positive cells and on the intensity of the epithelial staining evaluated in a total field of a single section. The extent of staining (EXT) was scored by four investigators (M.L., J.S., T.J.K., and S.Par.) as 1 when 1-10 % of the cells stained, 2 when 11-50 % of the cells stained, and 3 when 51-100 % of the cells stained. A negative score (0) was given to tissue sections which had no evidence of specific immunostaining. The intensity of staining (INT) was scored on a scale of 0 to 3 as follows: 0, no reaction; 1, weak reaction; 2, moderate reaction; 3, strong reaction. In the normal and hyperplastic gastric mucosa, the scores were first counted in the luminal surface, proliferative zone and glands, and relative staining indices (on the scale 0-3) were calculated separately for each histological layer using the following formula: $\sqrt{(EXT \times INT)}$. In the adenomas and metaplasias, the EXT and INT scores were counted separately in the deep and superficial parts of the mucosa, and the relative staining indices were calculated accordingly for both regions. Finally, the staining indices obtained in each region were used to calculate the mean values for each normal sample or lesion. The principle of these calculations is shown in Figure 1.
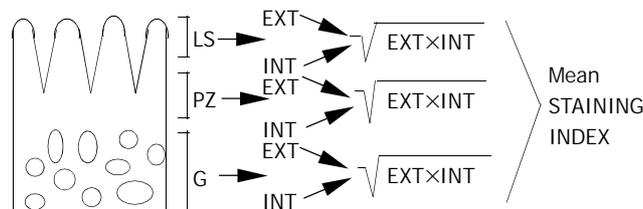

Figure 1 The principle for calculation of the relative staining index in one normal gastric sample (LS=luminal surface, PZ=proliferative zone, G=gastric glands).

Statistical analysis
Statistical analysis of the results was performed using SPSS for Windows software (SPSS Inc.). One-way analysis of variance was used to compare the staining for CA isozymes in different lesions. The pairwise comparisons between group means were performed using multiple comparison tests: Bonferroni, Tukey's honestly significant difference test, Sidak, Gabriel, Hochberg, and LSD (least significant difference).

RESULTS

Expression of CA IX
One intriguing finding of the present study was that the CA IX expression was sustained at relatively high level in many gastric tumors. Figure 2 shows some examples of CA IX immunostaining in the normal gastric mucosa (A) and different

gastric lesions (B-F). In the normal mucosa, CA IX was localized in all major cell types including parietal cells, chief cells, and mucus producing surface epithelial cells as described previously by Pastoreková et al.[8]. The positive staining covered all epithelial types of the gastric mucosa including glands, proliferative zone and the superficial epithelium of the mucosa. The staining was observed to be slightly weaker in the proliferative zone (data not shown) that contrasted with previous staining results in intestine where CA IX was mainly confined to the proliferative enterocytes[9]. Figure 2B demonstrates a typical distribution pattern of CA IX in intestinal metaplasia. It was notable that it was expressed in the crypts of metaplastic epithelium which was in line with its high expression in Lieberkühn crypts of the normal gut. Figure 3 demonstrates the mean staining indices for CA IX in the samples of normal gastric mucosa, hyperplasia, adenoma, adenocarcinoma, diffuse carcinoma, metastasis of gastric cancer, and metaplasia. The results demonstrated that the average staining reactions were quite similar in normal and hyperplastic gastric mucosa, while they became significantly weaker in dysplastic and malignant lesions. Figure 4 shows CA IX staining indices in gastric lesions grouped according to the stages of dysplasia and grades of malignancy. This analysis surprisingly revealed that adenomas and carcinomas formed two distinct entities based on the CA IX immunostaining. In adenomas, CA IX staining indices declined from 1.3-1.4 in lesions with slight or moderate dysplasia to 0.3 in those with severe dysplasia. In grade I adenocarcinomas, the staining index remained at the same level with normal and hyperplastic mucosa, while it again declined in carcinomas with higher malignancy grades.

Expression of CA XII

Figure 5 shows examples of CA XII immunostaining in the normal gastric mucosa and different lesions. Figures 6 and 7 show the staining indices for CA XII. In the non-neoplastic gastric mucosa, CA XII showed no or weak immunoreactions. The staining indices appeared to be significantly increased in hyperplastic and adenomatous lesions as well as in grades I and II adenocarcinomas and metastases, although they did not reach the values observed with CA IX. The indices were only very slightly increased in grade III adenocarcinomas and diffuse carcinomas, but the difference was not statistically significant.
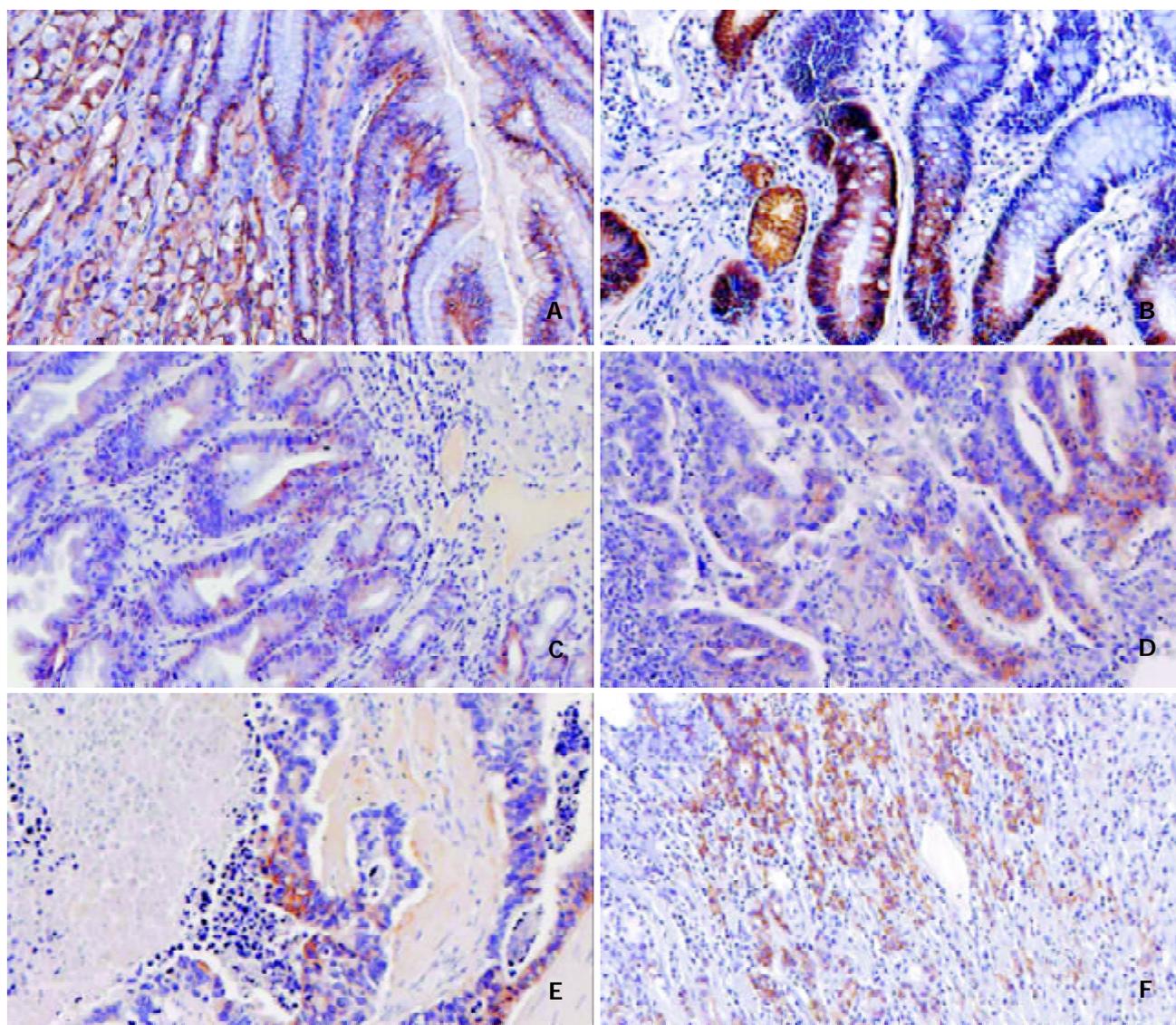

Figure 2 Immunohistochemical staining of CA IX in normal gastric mucosa (A), metaplasia (B), adenoma with moderate dysplasia (C), adenocarcinoma grade II (D), adenocarcinoma grade III (E), and diffuse carcinoma (F). In the normal mucosa, CA IX was expressed in all histological layers covering the luminal surface (LS), proliferative zone (PZ), and gastric glands (G). It was notable that CA IX was confined to deep crypts in metaplastic epithelium. The adenoma sample and all malignant lesions shown in this figure were positive for CA IX. (Original magnifications ×200).

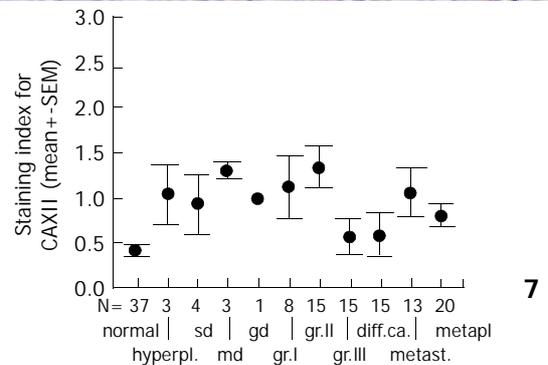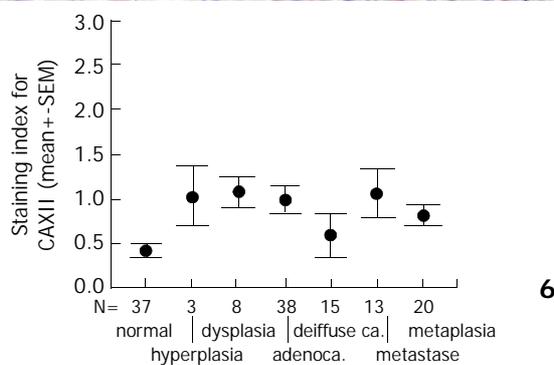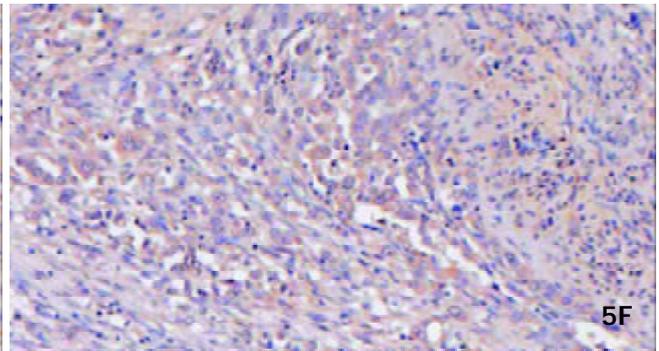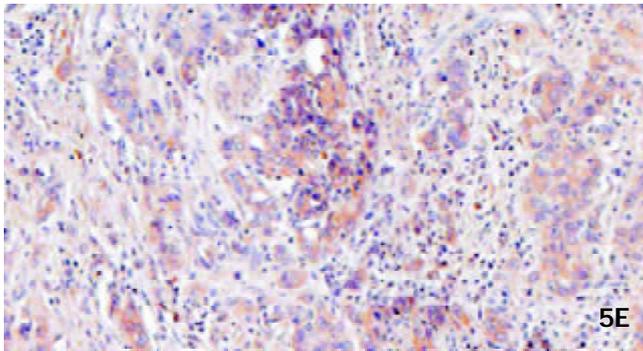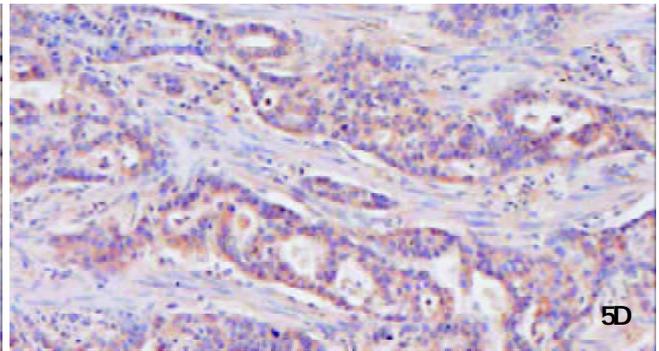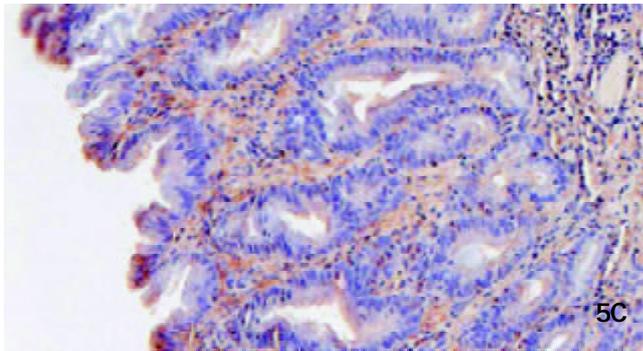**Figure 3** The mean staining indices for CA IX in the normal gastric mucosa and different gastric lesions (*P*=0.002, normal *vs* dysplasia, adenocarcinoma, and metaplasia).
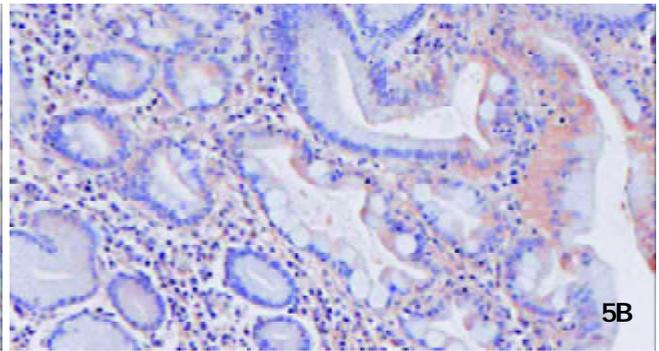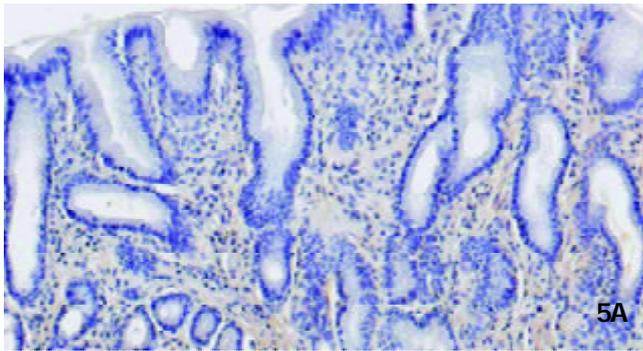
Figure 4 The mean staining indices for CA IX in the normal gastric mucosa and lesions grouped according to the stages of dysplasia and grades of malignancy (*P*<0.05, normal *vs* gd, gr. II and gr. III).

Figure 5 Immunohistochemical staining of CA XII in normal gastric mucosa (A), metaplasia (B), adenoma with moderate dysplasia (C), adenocarcinoma grade II (D), adenocarcinoma grade III (E), and diffuse carcinoma (F). From these samples, CA XII was

not expressed in the normal epithelium and showed weak expression in metaplastic epithelium and diffuse carcinoma. The signal was stronger in adenoma and adenocarcinoma samples.
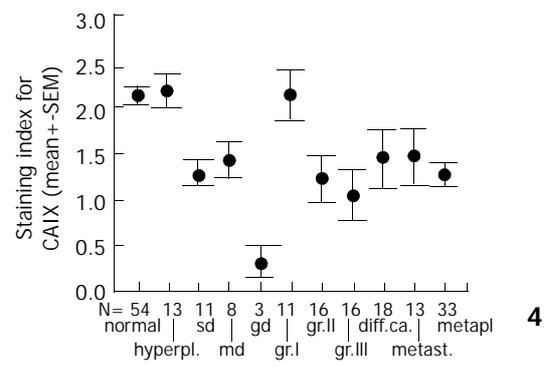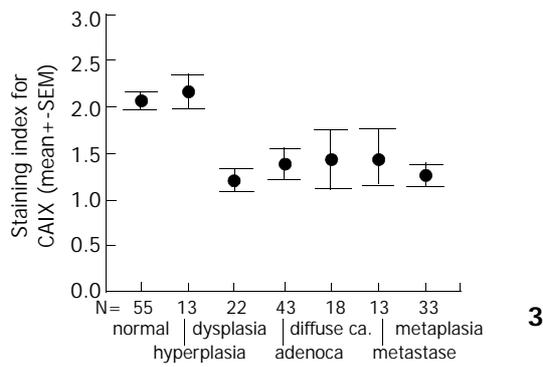
Figure 6 The mean staining indices for CA XII in the normal gastric mucosa and different gastric lesions (*P*<0.05, normal *vs* adenocarcinoma).

Figure 7 The mean staining indices for CA XII in the normal gastric mucosa and lesions grouped according to the stages of dysplasia and grades of malignancy (*P*=0.003, normal *vs* gr. II).

DISCUSSION

CA IX is present in several types of human tumors, whereas it is usually absent in the normal tissues from which these tumors originate. For example, it is expressed in neoplasias of uterine cervix and renal cell carcinomas, but not in the normal cervix or kidney[12,13,15,35]. On the other hand, there are only a few non-cancerous tissues expressing MN/CA IX, including epithelia of stomach, intestine, and gallbladder[8]. The present study was focused on the tumors arising from the gastric mucosa, which is normally the predominant site of CA IX expression. One interesting finding of the present study was that CA IX expression was sustained in most cases of gastric neoplasias, even though the expression indices declined compared to the normal and hyperplastic gastric mucosa.

The exact molecular mechanisms of gastric tumorigenesis are still under discussion. Some investigators have postulated that differentiated adenocarcinoma may arise from pre-existing adenoma, following a similar adenoma-carcinoma sequential axis as described for colorectal tumors[36]. However, when gastric adenomas and adenocarcinomas were examined for loss of heterozygosity using microsatellite markers or *MUC* gene expression, the results suggested that the adenoma-carcinoma sequence was not a major pathway in gastric carcinogenesis[37-41]. Since CA IX expression levels seemed to be very low in adenomas with severe dysplasia and normal in grade I adenocarcinoma, the present findings could support the concept that these tumor types are usually distinct entities and do not represent progressive steps from one to the other. In this respect, CA XII did not show any prominent changes in different tumor categories, although its expression was slightly increased in all pathological lesions compared to the nonneoplastic gastric mucosa.

CA IX differs from the other CA isozymes in that it has a proposed dual function as an efficient enzyme[7] and as an adhesion molecule[42,43]. This dual function appears to be related to the structure of CA IX molecule that consists of an extracellularly exposed N-terminal proteoglycan-like region and a CA domain. The CA domain may contribute to the regulation of acid-base homeostasis on the basolateral surfaces of the gastrointestinal epithelial cells. In tumors, CA activity has been suggested to participate in acidification of an extracellular microenvironment facilitating tumor invasion[21]. On the other hand, the adhesion properties of CA IX may be involved in the maintenance of mucosal integrity contributing to proper intercellular contacts and communication[42,43]. Recent studies in *CA9* knockout mice have indicated that CA IX is, indeed, an important factor in gastric morphogenesis and homeostasis of the gastric epithelium possibly acting through the control of cell differentiation and proliferation[44]. The proposed involvement of CA IX in cell differentiation agrees with our observation that high expression of CA IX was associated with a differentiated phenotype of gastric epithelial cells. However, this fact is in contrast to data from other types of tumors, e.g. breast carcinomas, where CA IX expression is increased in tumors with less differentiation, while the opposite is true for CA XII[19].

Even though the present results indicated that CA IX and XII were not specific biomarkers for any categories of gastric tumors, and thus their clinical significance may be limited in this area, they have already revolutionized the field of the CA research in terms of the proposed functions. CA IX and XII

are not only enzymatically active isoforms, but also play several important roles in biological processes such as malignant cell invasion[31], cell adhesion[42,43], and cell proliferation[9,10]. These "cancer-associated CAs" are also considered as potential targets in cancer therapy[21,35,45] and a number of promising anticancer therapeutic agents directed to inhibition of CA activity have already been developed and await further evaluation[32-34].

ACKNOWLEDGMENT

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