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Carbonic Anhydrase-II Phenotypes in Peptic Ulcer and Ulcerated Cancers

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ABSTRACT With 12 different isozymes, Carbonic anhydrase plays a key role in acid-base balance, CO_2 and ion transport…etc. Any change in the enzymatic activity may cause disturbances in these processes leading to different disorders. The study focuses on the association of electromorphs of Carbonic anhydrase-II (CAII) with peptic ulcers and ulcerated cancers, which result due to an imbalance between the aggressive and defensive factors necessary for maintaining the pH of the gastric lumen. Endoscopically confirmed 210 duodenal ulcer, 50 gastric ulcer and 50 gastric cancer cases were considered along with 110 healthy individuals for comparative study. Since H.pylori infection is considered as primary risk factor, Rapid Urease Test (RUT) was performed to identify the infection status in both disease and control groups. Phenotyping of CA_{II} was carried out in both control and disease by subjecting the haemolysate to PAGE and detecting the bands based on esterase activity of CA_{II} using α or β-napthol acetate. Frequency distribution of different phenotypes with respect to various factors was compiled and relative risk estimates were obtained using Woolf's δ-method.The allelic frequencies of CA_{II} calculated, were tested for Hardy-Weinberg equilibrium. Frequency distribution of CA_{II} phenotypes showed increased number of heterozygotes (2-1) in controls, against higher number of homozygotes (2-2) in diseased group. Similarly, blood group O was predominant in disease group as against group B in controls. Most of the controls were negative for *H.pylori* infection and almost 100% individuals in disease group were positive. In conclusion, the allele $CA_{II}2$ was found to be associated with peptic ulcers and ulcerated cancers along with blood group O and positive *H.pylori* infectivity status, predisposing an individual to the disease condition.

BACKGROUND

Carbonic anhydrases (CAs) are zinc-containing metalloenzymes that are responsible for the reversible hydration of carbon dioxide.

Carbonic anhydrases are produced in a variety of tissues where they participate in several important biological processes such as acid-base balance, respiration, carbon dioxide and ion transport, bone resorption, ureagenesis, gluconeogenesis, lipogenesis and body fluid generation (Sly and Hu 1995; Parkkila and Parkkila 1996). The mammalian α-CA gene family includes at least twelve enzymatically active isoforms with different structural and catalytic properties. Carbonic Anhydrase I, II, III, VII and XIII are cytosolic enzymes (Sly and Hu 1995; Hewett-Emmett, Tashian 1996; Lehtonen et al. 2004). CarbonicAnhydrase(CA) isozymes **V**a and **V**b are mitochondrial proteins encoded by nuclear DNA (Nagao et al. 1993;Fujikawa-Adachi etal.1999) whereas Carbonic Anhydrase(CA) VI is the only secretory form being present in saliva and milk (Karhumaa 2001). The cluster of membrane-bound CAs includes four isozymes: CA IV(Zhu and Sly1990), IX, XII(Tureci et al. 1998), and XIV. The other members of the CA gene family (CA VIII, X and XI) are inactive isoforms whose functions have not yet been described. The present study focuses on CA_{II} electro-morphs and their association with peptic ulcers, which is one of the polymorphic markers expressed by RBCs and gastric mucosal cells as a cytosolic enzyme.

The polymorphism in CAII was first described by Moore et al. (1971).Hopkinson et al. (1974) developed a method to visualize enzyme activity using flourogenic substrate following the separation of CA on starch gel electrophoresis.

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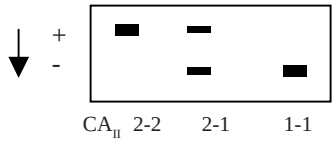
The three phenotypes distinguished are CA_{II}1-1, CA_{II} 2-1, CA_{II}2-2 encoded by co-dominant alleles.



Since peptic ulcers are the result of imbalance between aggressive factors like acid and pepsin and defensive factors like mucus secretions, bicarbonate etc, the present study examines the electromorphic variation of Carbonic anhydrase which is known to play a key role in maintaining the pH of the gastric lumen, deficiency of which may cause duodenal ulcers, gastric ulcers and gastric cancers.

METHODS

Preliminary diagnosis of peptic ulceration and gastric cancer was based on clinical symptoms and final confirmatory diagnosis was based on endoscopic findings. Endoscopically confirmed 210 duodenal ulcer cases, 50 gastric ulcer cases, 50 gastric cancer cases, apart from 110 healthy individuals between the ages of 18 to 65 were considered for the present study. Additionally 75 random control subjects, who were voluntary blood donors of OGH (Osmania General Hospital), were considered for the present study. The normal subjects included in the comparative study were hospital based subjects diagnosed endoscopically as normal individuals with no history of ulcer or cancer apart from voluntary blood donors.

The samples were collected during the period 1998-2001 by visiting the hospital twice a week (Mondays and Thursdays) on endoscopy days for confirmatory diagnosis. Random control samples were exclusively collected once in a week.

Apart from the epidemiological and familial data, blood samples were also collected for the analysis of the CA phenotypes.

Helicobacter pylori (*H.pylori*) infection is also considered as primary risk factor hence, Rapid Urease Test (RUT) is the primary protocol adopted to identify the status of infection in both disease and control groups.

The procedure for PAGE (Davies,1964) includes preparation of 7% gel using separating gel solutions in the ratio of 1: 2: 4: 1 with 2 ml tris hydroxymethyl amine, 4ml acrylamide solution, 8ml ammonium persulphate and 2ml distilled water. After polymerization, the glass plates were fixed to the vertical slab gel electrophoretic unit and the 10X tank buffer of tris glycine (pH 8.3) was added to the upper and lower tanks. The upper tank electrode was connected to the cathodic end and the lower tank electrode to the anodic end of the DC power supply.

Hemolysate was prepared by taking 0.2 ml whole blood in an eppendorf tube, washed 3-4 times with 0.9% normal saline, frozen and thawed twice to get RBC without plasma and later to the pellet 60µl of ice cold water was added to lyse the RBC.

Hemolysate of 20µl, with 10µl of bromophenol blue indicator was loaded into sample wells and electrophoresis was conducted at 20mA constant current at 4°C for 2hrs.

After electrophoresis, the gel was placed in distilled water for quick rinse and then in staining solution, containing 2ml of 1% α- or β- naphthyl acetate in acetone,10ml 0.4 M tris buffer (pH 7.0) and 100mg Fast Blue RR salt and made up to 100ml with distilled water.

The zones of esterase activity were detected as blue-red bands on a colorless background with CA I being anodal and CA II cathodal from the origin.

The phenotypic frequencies of CA_{II} were computed with respect to epidemiological factors like variation of sex, *H.pylori* infectivity status and blood groups, in both control and disease groups. The percentage distribution of Carbonic anhydrase phenotype was obtained and the relative risk estimates were calculated following the Woolf's method (1955).

To determine the statistical significance of an association of specific electromorphs, the method most widely used is that of Woolf (1955), where the relative incidence(x) is calculated by

$$x = hK/Hk$$

h $\rightarrow$ number of patients with a marker.

H $\rightarrow$ number of controls with a marker.

k $\rightarrow$ number of patients with out a marker.

K $\rightarrow$ number of controls with out a particular marker.

Then $\log_e x = y$; Total $\chi^2 = \Sigma w y^2$.

Where $w = \frac{1}{1/h+1/H+1/k+1/K}$; the significance of the calculated value was related to the tabulated value at 5% LOS.

The observed and the expected allelic frequencies were calculated for polymorphic CA_{II} and tested for Hardy Weinberg equilibrium (Cavalli-Sfroza and Bodmer 1971).

RESULTS AND DISCUSSION

Peptic ulcers, comprising of duodenal and gastric ulcers are mucosal lesions in the gastric mucosa. A gastric ulcer undergoing malignant transformation is an ulcerated cancer. These disorders may result due to an imbalance between aggressive (acid and pepsin) and defensive (mucus and bicarbonate) mechanisms. The prevalence of peptic ulcers and ulcerated cancers is influenced by environmental and genetic factors. The hereditary basis of peptic ulcer has been suggested based on family studies, twin studies and studies on ABO blood groups and pepsinogen, as genetic markers (Rotter, 1983).

Since ulcers are quite common in all sections of Indian population, an attempt was made to identify the electromorphic distribution of CA_{II} in controls and disease groups and to identify the epidemiological factors in association with the specific electromorph of CA_{II}, thereby delineating the role of epidemiological factors and the underlying genetic heterogeneity of the condition.

The parameters considered were sex, familial status, presence of consanguinity, associations with *H.pylori* infection and ABO blood groups.

Table 1 gives the frequency distribution of CA phenotypes in control and disease groups. Among the controls, 51% of cases exhibited 2-1 phenotype. The frequency of 2-1 decreased to 39% in duodenal ulcer cases, 14% in gastric ulcer and 10% in gastric cancer cases, showing an increased frequency of heterozygotes in control population as against two homozygote phenotypes (2-2 and 1-1). In contrast, the frequency of individuals with 2-2 phenotype has increased from 48% in controls to 55% in duodenal ulcer cases, 86% in gastric ulcer and 90% in gastric cancer patients. Also, the frequency of other homozygous phenotype (1-1) had increased in duodenal ulcer cases, with controls being 1% and duodenal ulcer patients being 6 %.

Table 1: Frequency distribution of CA phenotypes in control and disease groups.

Type	*2-2*		*2-1*		*1-1*	
	N	*%*	*N*	*%*	*N*	*%*
Control	88	48	95	51	2	1
Duodenal ulcer	116	55.0	81	39.0	13	6.0
Gastric Ulcer	42	86.0	7	14.0	-	-
Gastric cancer	45	90.0	5	10.0	-	-

This shows an increased preponderance of homozygous genotypes (2-2 and 1-1) in duodenal ulcer cases. Very high frequency of gastric ulcer and gastric cancer cases showing 2-2 phenotype gives an indication of association.

The relative risk estimates presented in table 2 also show significant association of 2-2 phenotype with peptic ulcers and ulcerated cancers. The relative risk of 2-2 Vs 2-1 was 1.55 ($\chi^{2:}$ 4.4), showing increased propensity of 2-2 towards duodenal ulcer when compared to 2-1. This trend was also observed in gastric ulcers and gastric cancers with 2-2 phenotype showing relative risks (RR) of 6.6 (χ^2: 19.14), and 9.9 (χ^2 = 21.7) respectively. In general, it was observed that, the presence of $CA_{II}2$ allele genetically predisposes an individual to peptic ulcers and ulcerated cancers.

Table 3 gives the allelic frequencies of $CA_{II}1$ and $CA_{II}2$ in the control and disease groups. The allelic frequency of $CA_{II}2$ was found to be 0.73 and $CA_{II}1$ to be 0.27 in control subjects ($\chi 2$ =11.1),

Table 2: Relative risk estimates of carbonic anhydrase phenotype (RR) in disease and control groups.

Phenotype	*Control*	*Duodenal Ulcer*			*Gastric Ulcer*			*Gastric Cancer*		
	N	*n*	*RR*	*÷2*	*n*	*RR*	*÷2*	*n*	*RR*	*÷2*
2-2	88	116	-	-	42	-	-	45	-	-
2-2 Vs 2-1	95	81	1.55	4.4	7	6.5	18.6	5	9.7	21.3
1-1	2	13	-	-	-	-	-	-	-	-
1-1 Vs 2-2	88	116	4.9	4.35	-	-	-	-	-	-
2-1	95	81	-	-	7	-	-	5	-	-
2-1 Vs 1-1	2	13	0.13	6.67	-	-	-	-	-	-
2-2 Vs others	97	94	1.36	2.2	7	6.6	19.14	5	9.9	21.7
1-1 Vs others	183	197	6.04	5..45	-	-	-	-	-	-
2-1 Vs others	100	129	0.66	4.2	42	0.15	18.65	45	0.1	21.3

Table 3: Distribution of carbonic anhydrase allelic frequencies in duodenal ulcer, gastric ulcer, gastric cancer and control group.

	Allele frequencies	
	Dominant allele p	*Recessive allele q*
Control	0.732	0.267
Duodenal Ulcers	0.74	0.255
Gastric Ulcer	0.928	0.071
Gastric Cancer	0.95	0.05

Test of HWE in controls ÷2 =11.1
Test of HWE in DU: ÷2=0.076
Test of HWE in GU: ÷2 =0.295
Test of HWE in GC: ÷2 =0.14

0.74 and 0.26 in duodenal ulcer patients (χ2=0.076), 0.928 and 0.071 in gastric ulcer (χ2 =0.295) and 0.95 and 0.05 in ulcerated cancer patients (χ2 =0.14) with no deviation from Hardy Weinberg equilibrium .

Table 4 gives the phenotypic distribution of various epidemiological parameters with respect to controls, duodenal ulcer, gastric ulcer and gastric cancer patients.

The phenotypic distribution of CA_{II} with respect to sex, when examined, revealed increased frequency of CA_{II} 2-2 phenotype in females (53.6%), while CA_{II} 2-1 was predominant in males (52.8%), of the control group. In disease condition, no such sex variation was observed, though in general, preponderance of 2-1 phenotype was noted. 84.4% of male and 100% female gastric ulcer cases and 91.5% male and 66.7% female gastric cancer cases exhibited CA_{II}2-2 phenotype. An interaction between the sex linked or influenced genes, apart from differential action of hormones resulting in predilection of males to the disease, may support the above observation of male predilection.

Among control subjects,46.4% of 2-2 phenotypic individuals and 41% of 2-1 individuals showed blood group B. In general, increased frequency of blood group B was observed irrespective to the phenotype of Carbonic anhydrase.

Unlike the control subjects, where large proportion of individuals belonged to blood group B, in duodenal ulcer patients showing 2-2 phenotype, 55.4% were of blood groupO. This trend of increased frequency of blood group O individuals was also observed in other phenotypes, in 2-1 , 51.8% and in 1-1, 46.1% of individuals belonging to group O.

Therefore, larger proportion of individuals belonged to blood group O in duodenal ulcer patients, irrespective to the phenotype of CA_{II}. This was also observed to be the case with gastric ulcer cases, where large number of cases of 2-2 and 2-1 phenotypes showed association with blood group O i.e...54.8% and 42.8% respectively.

Among the 48 control subjects with 2-2 phenotype tested for presence or absence of *H.pylori* infection, 73% were *H.pylori* –ve. Sixty controls with 2-1 phenotype had 70% of *H.pylori* –ve status. Generally, most of the controls were *H.pylori* –ve.

But in the case of duodenal ulcers, 88% of 2-2 phenotypes recorded were *H.pylori* +ve and

Table 4: Frequency distribution of various epidemiological parameters in controls, duodenal ulcer patients, gastric ulcer patients and Gastric cancer patients.

Epidemiological variables	*Controls Phenotypes*			*Duodenal ulcer Phenotypes*			*Gastric ulcer Phenotypes*			*Gastric Cancer Phenotypes*		
	2-2	*2-1*	*1-1*	*2-2*	*2-1*	*1-1*	*2-2*	*2-1*	*1-1*	*2-2*	*2-1*	*1-1*
1. No. of cases reported	88	95	2	116	81	13	42	7	-	45	5	-
2. Blood groups												
A	15	24	-	6	6	3	-	-	-	-	-	-
B	41	39	-	37	26	3	14	2	-	40	4	-
AB	9	6	1	9	7	1	5	2	-	3	1	-
O	23	26	1	64	42	6	23	3	-	2	-	-
Total	88	95	2	116	81	13	42	7	-	45	5	-
3. H.pylori infection status												
+ve	13	18	-	102	75	11	42	6	-	45	5	-
-ve	35	42	2	14	6	2	-	1	-	-	-	-
Familial	-	-	-	8	6	1	-	-	-	-	-	-
Non-Familial	-	-	-	108	75	12	-	-	-	-	-	-
Total	-	-	-	116	81	13		-	-	-	-	-
Consanguinous	-	-	-	14	10	1	-	-	-	-	-	-
Non-consanguinous	-	-	-	102	71	12	-	-	-	-	-	-

92.6% of 2-1 phenotypes were *H.pylori* +ve. This increase in *H.pylori* +ve cases in duodenal ulcer was also seen among 1-1 phenotype, with 84.62% of them being *H.pylori* +ve.

It can be concluded from the above observations that, there is an increased association of *H.pylori* infection status with duodenal ulcer cases.

Frequency distribution in gastric ulcer and gastric cancer cases show that almost 100% cases were associated with +ve *H.pylori* infection status.

No variation in carbonic anhydrase phenotype distribution was observed with respect to consanguineous and non-consanguineous groups , familial and non-familial status. With respect to familial status though, 2-2 phenotype predominated the groups with 53.3% of cases being familial .

DISCUSSION

Peptic ulcers are acute lesions on the gastric mucosa extending into the muscularis propria surrounded by an acute and chronic inflammatory cell infiltrate. A chronic ulcer undergoing malignancy is ulcerated cancer. An interaction of various epidemiological, genetic and biochemical factors may result in ulcerogenesis and ulcerated cancers. Hence the present study was envisaged and aimed to investigate the contributory roles of these factors in ulcerogenesis and carcinogenesis.

Phenotype distribution when examined revealed significant predisposition of carbonic anhydrase 2-2 phenotypic individuals to duodenal ulcer, gastric ulcer and gastric cancers.

Carbonic anhydrase, is secreted by surface epithelial cells, functions mainly to maintain the mucus – bicarbonate barrier by back diffusing the H^+ ions from the cells. From the present study, it can be proposed that CA_{II}2-2 phenotype may confer decreased protection to gastric mucosa due to retarded back diffusion of H^+ ions, which is commonly associated with gastric lesions. This particular allele may encode a carbonic anhydrase which retards the back diffusion of H^+ ions leading to decreased protection to gastric mucosa. Therefore, the increased role of CA_{II}2 allele in predisposing an individual to the disorder can be established.

Further, an interesting finding is that CA_{II}2-2 individuals are also at increased risk to gastric ulcers and ulcerated cancers. The presence of this particular allele in double dose may lead to the production of highly unstable carbonic anhydrase, which can be considered as an indicator of the benign to malignant progression of the condition.

Increased association of peptic ulcer and ulcerated cancers with blood group O and also occurrence in large numbers in these diseased individuals, the *H.pylori* infection, indicates the possible interaction of these genes/gene products with that of the proteins secreted by *H.pylori* establishing the host pathogen interactions and genetic predisposition.

In conclusion, the mutation which shows the phenotype CA_{II}2-2 may produce an enzyme that has reduced activity, resulting in decreased protection to gastric mucosa. The association of specific phenotypes of CA_{II} with the different epidemiological parameters gives a clue regarding the complexity of the pathways, in the etiology of the peptic ulcers.

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