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EFFECTS OF INFLAMMATION AND *H. PYLORI* INFECTION ON EXPRESSION OF CD44 VARIANT EXONS IN GASTRIC TISSUE

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Abstract

Helicobacter pylori (*H. pylori*) infection, which is always accompanied by inflammation, and increased expression of the cell adhesion molecule CD44 have both been purported to be correlated with gastric cancer. Specifically, altered expression of alternatively spliced CD44 transcripts has been found in many cancers, including cancer of the stomach. Considering the association between *H. pylori* infection and inflammation, it is possible that the correlation between CD44 alternative splicing and gastric cancer is due to inflammation. To test this, we compared the expression of all CD44 variant exons V2-V10 in non-cancerous individuals with and without inflammation. To ascertain the extent to which the effect of *H. pylori* is mediated by inflammation, CD44 variant exon expression in inflamed individuals with and without *H. pylori* infection was compared.CD44 variant exon expression was assessed in gastric biopsy samples of 43 *H. pylori* infected (*H. pylori*$^+$) and 46 non-infected (*H. pylori*$^-$) individuals using nested RT-PCR. Thirteen of the non-infected individuals were without inflammation. The results suggest that inflammation alone does not change CD44 expression. There was a significant correlation between the expression of CD44 variant exons V8, V9 and V10 and *H. pylori* infection. Further investigations will be necessary to address the possibility that infection and inflammation have synergistic roles in the increased expression of V8, V9 and V10. These exons code for parts of the extracellular portion of the protein and changes in their expression may affect oncogenic processes because of changed affinities for the extracellular matrix.

Keywords: *Helicobacter pylori*; CD44; Adhesion molecule; Inflammation; Stomach; Cancer

Introduction

CD44 is a transmembrane glycoprotein and an adhesion molecule expressed in a variety of cells including epithelial cells [1,2]. Many isoforms of CD44 ranging in size from 85 to 200 kDa exist, all of which are coded by a single copy gene containing 20 exons on chromosome 11 of the human genome [3-5]. The

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different forms are products of alternative splicing of at least nine variant exons (V2-V10) and post translational modifications [3]. The variant exons when expressed, create extracellular domains of the protein. The CD44 isoform in which variant exons V2-V10 are not expressed is called the standard form (CD44S).

CD44 binds multiple ligands including hyaluronic acid, fibronectin and collagen [6,7]. It has been suggested to have a role in a multitude of functions, many of which involve cell migration, for example lymphocyte homing, other immune related functions and metastasis [6,8-13]. CD44 expression in gastric epithelial cells has been demonstrated [1,14-16]. Studies on gastric tissue have suggested a correlation between the expression of standard and variant forms of CD44 and differentiation, tumor progression and metastasis [14-20]. It has even been suggested that CD44 may be useful for prognosis and diagnosis [15,17,20,21].

Helicobacter pylori, a flagellated gram negative bacterium, thrives in the antral region of the stomach and is a major cause of gastritis and duodenal and gastric ulcerations [22]. Epidemiological studies indicate that *H. pylori* infection may be a predisposing factor for development of cancer of the stomach [23,24]. The bacterium is commonly found in samples of early gastric cancer [25]. It has been shown to upgrade the expression of CD44 protein in a gastric epithelial cell line [26]. Furthermore, its presence was correlated with increased CD44 expression, specifically the variant exon V9 in several infected individuals [27]. As inflammation usually accompanies *H. pylori* infection and some inflammatory mediators such as interferon γ may increase the expression of CD44, an interplay between *H. pylori* infection and immune responses in causing increased CD44 expression has been considered [2,26,27].

We set forth to examine a possible correlation between the transcription of nine CD44 variant exons and inflammation. We also tried to assess the effect which *H. pylori* infection may exert on CD44 variant exon expression.

Methods

Biopsy specimens of 89 patients none of them received previous medical therapy were studied. Two specimens were taken from each individual. They were taken during upper gasrtrointestinal endoscopy from the gastric antrum. The specimens of 43 of patients were *H. pylori* positive and those of 46 were *H. pylori* negative. *H. pylori* infection was assessed by microscopic examination of haematoxylin and eosin stained sections and by the CLO test. All *H. pylori*$^+$ and 71.7% of the *H. pylori*$^-$ individuals showed chronic inflammation of the gastric mucosa (Table 1). The mean age of the *H. pylori*$^+$ patients was 48 and of the *H. pylori*$^-$ patients with inflammation and also without inflammation was 44. Approximately half of the patients of each group were female.

Biopsy samples were immediately transferred to a solution containing 4 M guanidinium thiocyanate, 25 mM sodium citrate, pH 7 and 0.5% sarkosyl. They were stored at −70°C until RNA extraction which was done by standard protocols [28]. Single strand cDNA was synthesized in 25 µl reaction volumes using oligo-dT primers and Avian Myeloblastosis Virus (AMV) reverse transcriptase (Boehringer Manneheim) according to the company's instructions.

The expression of each of the variant exons was assessed by a nested PCR protocol using primers whose sequences have been published [29, Fig. 1]. PCR reactions were performed in a volume of 25 µl using 25 pm of each of the primers and 0.5 U Taq DNA polymerase (Cinagen Co., Tehran, Iran). The first PCR reaction contained 2 µl of the cDNA reaction mixture and outer primers C1 and C3 complementary to sequences present in all CD44 transcripts. 0.5 µl of the first PCR reaction was used as template in ten second PCR reactions, all of which contained the C2 primer and nine of which contained one of the nine variant exon specific primers, V2-V10. The tenth reaction was a negative control, which did not contain a variant exon specific primer. The C2 complementary sequence is present in all CD44 transcripts. After a denaturation step of 3 min in 93°C, PCR was performed using 35 cycles of denaturation (45 S at 93°C), annealing (45 s at 60°C) and extension (1 min at 72°C). A final extension time of 5min at 72°C was then performed.

Seven microliters of each of the second PCR reaction was electrophoresed on 8% acrylamide or 1.5% agarose gels. A Taq I digest of pBR322 was used as size markers. DNA bands were visualized after staining with ethidium bromide.

Statistical analysis of results was done with the Chi square and Fischer's exact tests, using the EPI Info software.

Table 1. Expression of CD44 variant exons V8, V9 and V10 in *H. pylori*$^+$ and *H. pylori*$^-$ samples with and without inflammation

CD44 Exon	*H. pylori*$^+$	*H. pylori*$^-$	
	Inflamed*	Non-inflamed	Inflamed
V8	16/43 (37%)	2/13 (15%)	6/33 (18%)
V9	19/43 (44%)	2/13 (15%)	8/33 (24%)
V10	20/43 (46%)	3/13 (23%)	6/33 (18%)

* All *H. pylori*$^+$ samples were inflamed.

Table 2. Comparison of expression of CD44 variant exons V8, V9 and V10 between different groups. Data are shown as p value

Comparison	p Value		
	V8	V9	V10
H. pylori$^-$ inflamed *vs.* *H. pylori*$^-$ non-inflamed	0.59	0.41	0.49
H. pylori$^+$ *vs.* *H. pylori*$^-$	0.03	0.02	0.006
H. pylori$^+$ inflamed *vs.* *H. pylori*$^-$ inflamed	0.06	0.07	0.009

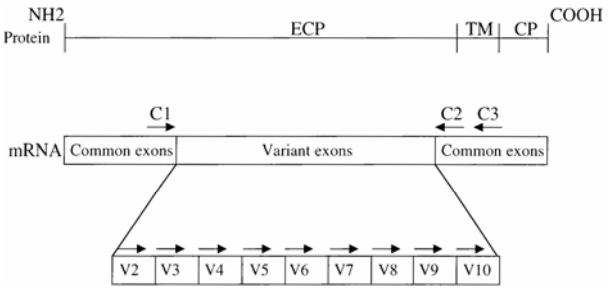

Figure 1. Schematic presentation of the position (⟶ and ⟵) of primers use in PCR reactions with respect to CD44 exons. Corresponding portions of the protein coded by the exons are also shown. TM, transmembrane region; CP, cytoplasmic portion; ECP, extracellular portion.

Results

The electrophoretic patterns of the RT-PCR products of three representative individuals are presented in Figure 2. It can be seen that variant exons V8, V9 and V10 were expressed in the biopsy samples of all three individuals. V3 was additionally expressed in one (Fig. 2b) and two V8 containing transcripts were expressed in another (Fig. 2c). The expression of the CD44S isoform could sometimes be detected because of the effect of carryover primers from the first PCR reaction. At least one variant exon corresponding to at least one CD44 transcript was detected in 51% of the *H. pylori*$^+$ samples and 26% of the *H. pylori*$^-$ samples, 28% in inflamed samples and 23% in non-inflamed samples. The difference in the expression of a CD44 variant exon between *H. pylori*$^+$ and *H. pylori*$^-$ samples was statistically significant (p=0.02), but that between the inflamed and non-inflamed samples of the *H. pylori*$^-$ group was not (p=0.50).

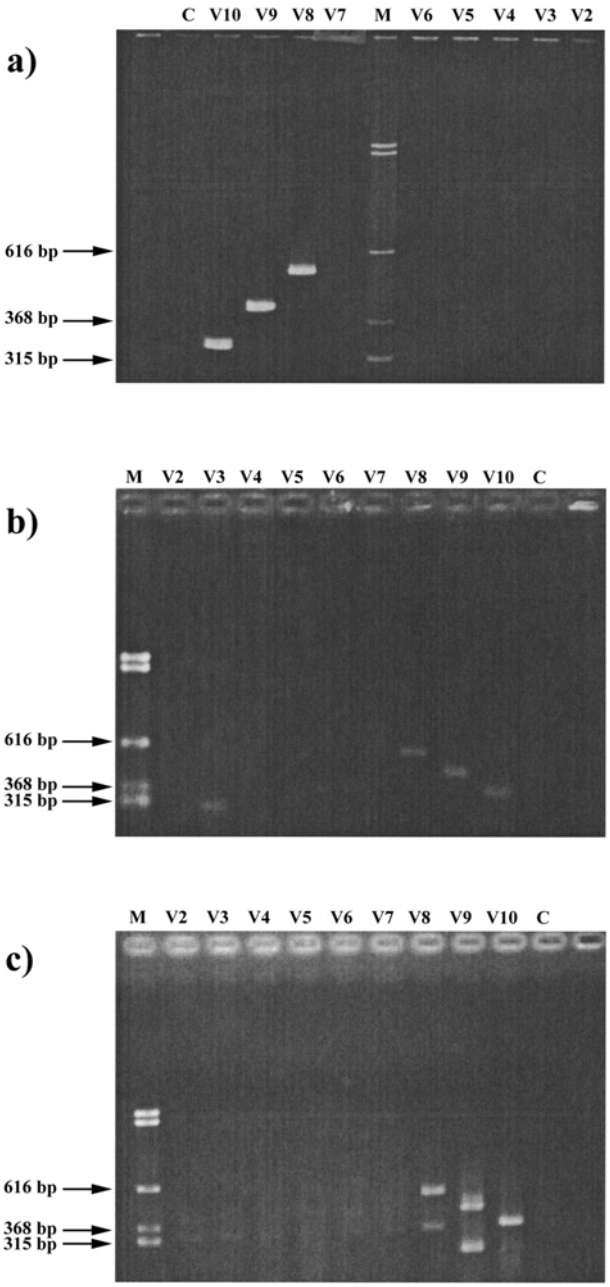

Figure 2. Electrophoretic pattern of nested PCR products of three individuals. The variant exon specific primer used in the reactions is indicated on top of the respective lanes, a) acrylamide gel; b and c) agarose gels.

When the expression of each of the various variant exons is considered independently, it becomes apparent that the differences noted above are largely due to the pattern of expression of exons V8, V9 and V10 (Tables 1 and 2). Each of the nine variant exons was expressed in a relatively low number of samples. V2, V4, V5, V6, and V7 were each expressed in less than 5 individuals

and V3 was expressed in 10 individuals. There was no significant difference in the expression of any of these exons between inflamed and non-inflamed samples nor between *H. pylori*$^+$ and *H. pylori*$^-$ samples. (Amongst these exons, the highest correlation was for V6 in *H. pylori*$^+$ samples; p=0.1). V8, V9, and V10 were expressed in 24, 29, and 29 individuals, respectively.

Among the *H. pylori* negative samples in which V8, V9 or V10 was expressed, there were no significant difference between inflamed and non-inflamed samples (p=0.59, 0.41 and 0.49, respectively). Furthermore, each of the exons V8, V9 and V10 was detected in a considerably larger fraction of *H. pylori* positive inflamed samples compared to *H. pylori*$^-$ inflamed samples (p=0.06, 0.07 and 0.009, respectively). Our data are summarized in Tables 1 and 2.

Discussion

The expression of CD44 has been studied in many normal and tumor tissues as well as in various cell lines. Most of the studies have been done using labeled antibodies and immunological procedures for detection of the protein [30-33]. A fewer number of investigations were performed by RT-PCR, sometimes along with immunohistochemical techniques [18,34-36]. We used a combined RT-PCR and nested PCR protocol for detection of CD44 transcripts containing variant exon sequences [29]. As adhesion molecules play a role in cell-cell interactions and such interactions are bound to be relevant in immunological processes, it was reasonable to consider the effect of inflammation on CD44 expression [11,37]. The results show no significant difference in the expression of any of the CD44 variant exons between *H. pylori*$^-$ inflamed and non-inflamed samples (p=0.28-0.64). Therefore in the absence of *H. pylori* infection, inflammation alone does not seem to be a determining factor in CD44 variant exon expression. On the other hand, the V8, V9 and V10 variant exons of CD44 were expressed in a significantly larger fraction of *H. pylori* infected as compared to non-infected gastric tissue biopsy samples (p=0.03, 0.02, and 0.006, respectively). This suggests that *H. pylori* may cause increased expression of these exons. This effect may be relevant to *H. pylori* associated disease, including carcinogenesis. Variant forms of CD44 show different affinities towards extracellular matrix ligands and this is expected to be pertinent to the metastatic potential of cells expressing different forms of the protein [38]. Finally there was a considerable difference in the expression of V8, V9 and V10 between inflamed *H. pylori*$^+$ and inflamed *H. pylori*$^-$ samples (p=0.06, 0.07 and 0.009, respectively). The effect of *H. pylori* infection on CD44 expression may reflect the combined effects of infection and the inflammation which almost always accompanies it. In this regard, inflammatory mediators probably play an important role. Fan *et al.* reached a similar conclusion based on analysis of CD44 protein expression in 15 *H. pylori* infected and 13 non-infected individuals [27]. A cytokine mediated effect on CD44 expression in myelomonocyte cells has also been previously reported [2,6].

In our study the combination of different variant exons expressed together in each sample amongst *H. pylori*$^+$ and also *H. pylori*$^-$ individuals was very variable. Variant exons V8, V9 and V10 were expressed more frequently than the other exons in both groups of individuals. Nevertheless there was a significant difference in the fraction of *H. pylori*$^+$ as compared to *H. pylori*$^-$ samples in which V8, V9 and V10 were expressed. Exons V8-V10 contribute to the synthesis of the epithelial form of CD44 [5]. Fan *et al.* had compared the expression of two variant exons V6 and V9 in infected and non-infected individuals and in accordance with our results, found a significant difference only in the expression of V9 [27].

In conclusion we have shown that inflammation alone is not associated with altered CD44 variant exon expression in gastric tissue. However, *H. pylori* infection is associated with increased expression of CD44 variant exons, specifically V8, V9 and V10. As CD44 expression has been associated with gastric cancer, this effect of *H. pylori* infection may be relevant to its association with the disease.

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