

# ***Helicobacter pylori* Alters the Tight Junction-Regulating Adhesion Protein BVES and Promotes Epithelial-Mesenchymal Transition in a Nontumorigenic Murine Gastric Epithelial Cell Line**

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BRIEF. *Helicobacter pylori* infection reduces levels of BVES and promotes carcinogenesis through epithelial-mesenchymal transition.

**ABSTRACT.** *Helicobacter pylori* is the strongest risk factor for developing gastric adenocarcinoma; however, the molecular pathways are incompletely defined. Epithelial-mesenchymal transition (EMT), a change in cell morphology mediated by *H. pylori*-induced translocation of tight junction proteins, is critical for cancer metastasis. An inverse relationship between BVES expression, a protein implicated in tight junction formation, and EMT has been observed in colorectal cancer; therefore, this study examined *H. pylori* regulation of BVES. mRNA expression was analyzed using real-time RT-PCR in *H. pylori*-infected and uninfected MGEC cells and AGS and MKN28 human gastric cancer cells. Immunofluorescence was used to determine BVES localization. BVES transcription was detected in non-cancerous cells but undetectable in gastric cancer cells. *H. pylori* co-culture of MGEC cells decreased BVES expression independent of the *cag* pathogenicity island, a virulence constituent linked to gastric cancer. Translocation of BVES from membrane to cytosol was observed, and BVES protein levels were substantially reduced upon infection. Taken together, these results suggest that manipulation of this regulatory protein may exert oncogenic effects within the context of *H. pylori* infection.

## INTRODUCTION.

Every year, approximately 700,000 people develop gastric cancer, the second leading cause of cancer-related deaths worldwide [1]. Gastric tumors can be traced back to a variety of genetic, racial, geographical, and life-style influences, but the strongest known risk factor for gastric cancer is infection by the bacterium *Helicobacter pylori*. *H. pylori* inhabits the stomach of over half of the world's population [2]. Classified as a Class 1 cancer-causing agent by the World Health Organization, this bacterium induces inflammation of the stomach in all infected individuals. While the majority of this infected population may never suffer clinical consequences, the percentage of individuals who develop severe disease, including ulceration and cancer, is substantial. In order to prevent disease progression, the factors leading to development of disease, specifically cancer, need to be identified. Identifying proteins involved in EMT is critical because they can be targeted to prevent the loss of cell polarity, adhesion, and subsequent cancer spread that occurs as the cell undergoes this pathologic alteration in shape. The discovery of genetic components that promote cancer development can allow for adequate response time for therapy prior to severe disease progression in gastric cancer cases among clinical populations.

With only a 94% commonality of genes between strains [3], the genetic composition of *H. pylori* is a fruitful area for further investigation. The variety of genes present in the remaining 6% may account for variability in disease progression and severity. Such studies have been previously conducted and several genes have been shown to be required for successful colonization and disease development of *H. pylori*.

One set of genes has been coined the *cag* pathogenicity island (PAI). A few genes within the *cag* PAI, such as *cagE*, encode for a bacterial type IV secretion system (TFSS), a structure resembling a hypodermic needle that translocates CagA into host cells [4]. Upon injection of CagA, the host cell undergoes important changes in shape [5]. CagA alters normal cell processes and is identified in the majority of *H. pylori* strains associated with gastric cancer [6].

Although the presence of CagA has been shown to increase the risk of gastric cancer, it is important to note that the progression to cancer is the result of multiple processes. One process of particular interest is epithelial-mesenchymal transition (EMT). Cells transform from their original epithelial shape to a mesenchymal morphology, which results in increased migratory capacity. The implications of increased migratory capacity include the spread of cancer, resistance to cellular suicide, and increased invasiveness [7].

The proteins responsible for maintaining cell-cell adhesion prior to EMT are located in the junctions between cells that are also responsible for communication and transportation of molecules. It is unclear, however, which proteins in these junctions are being displaced as a result of infection. One candidate is a protein involved in the formation of tight junctions, BVES (blood vessel epicaldial substance). Tight junction proteins are of extreme interest in the study of *H. pylori* infection as the bacteria specifically attaches to the tight junction region of the gastric epithelium in which the ability of cells to adhere to one another and communicate is a defining trait. BVES is a protein previously shown to regulate cell-cell adhesion and, subsequently, EMT, which occurs as the result of the loss of such adhesion. This research aims to relate *H. pylori* disruption of the tight junction protein BVES upon infection to tumor development via EMT in gastric epithelial cells.

The task of defining the relationship between *H. pylori* infection and BVES levels with induction of EMT was achieved through three sub-aims. The first was to compare transcriptional levels of BVES across cancerous and non-cancerous cell lines. The second sub-aim focused on observing *H. pylori* regulation of BVES levels over time and defining the importance of the *cag* PAI in this process. The third sub-aim was designed to visualize the localization of BVES prior to and following infection.

## MATERIALS AND METHODS.

### *Cell Culture and H. pylori Culture Conditions.*

MGEC (mouse gastric epithelial cells) were immortalized in RPMI+10% FBS media with INF- $\gamma$  at 33°C. Twenty-four hours prior to *H. pylori* co-culture, media was replaced with INF- $\gamma$ -free media and cells were placed in primary conditions at 37°C. MKN28 and AGS (human gastric cancer cell lines) were grown in RPMI+10% FBS media at 37°C.

The *cag*<sup>+</sup> *H. pylori* strains 7.13 and 60190 were grown in *Brucella* broth with 10% FBS for 16 hours, harvested by centrifugation, and co-cultured with MGECs at a multiplicity of infection of 100:1. Isogenic strains 60190 *cagE*<sup>-</sup> and 7.13 *cagE*<sup>-</sup> were selected using kanamycin as previously described [4].

### *Real-time Reverse Transcriptase PCR (RT-PCR).*

RT-PCR followed by quantitative PCR was performed to determine transcriptional regulation of BVES in *H. pylori*-infected and uninfected MGEC, AGS, and MKN28 cells normalized to levels of *GAPDH* (TaqMan<sup>®</sup>, Applied Biosystems).

### *Immunofluorescence.*

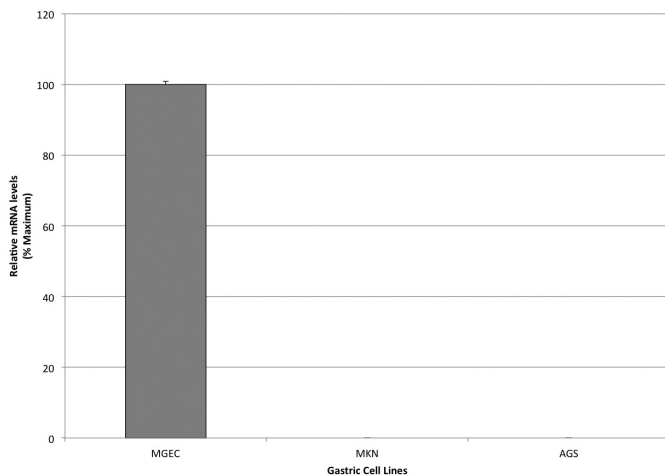
MGEC cells were cultured on 4-well chamber-slides, washed twice in 1X PBS, and formalin-fixed. Cells were permeabilized using 0.1% Triton X-100, and non-specific sites were blocked by incubation in 1% BSA. Formalin-fixed

MGEC cells were stained with rabbit anti-occludin antibody in combination with Alexa Fluor 488-conjugated anti-rabbit IgG antibody and with mouse anti-BVES gifted by Chris Williams (Vanderbilt) in combination with Alexa Fluor 546-conjugated anti-mouse IgG antibody. Nuclei were labeled with DAPI. Images were captured with a Leica DM-LB microscope (Solms) using NIS-Elements BR 2.30.

## RESULTS.

### *BVES transcription is reduced in human gastric cancer cell lines.*

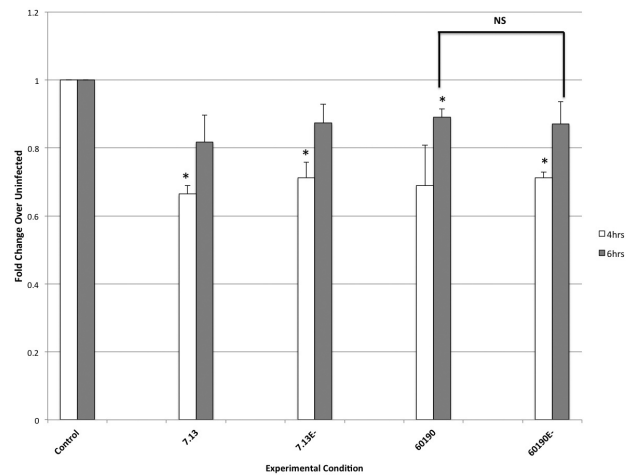
In order to determine the relative levels of BVES in uninfected cancerous and noncancerous gastric cell lines, real-time RT-PCR analysis was conducted. The level of BVES measured in the MGEC noncancerous cell line was set at 100%, and cancer cell lines MKN28 and AGS were recorded as a percent maximum. All values were reported as means  $\pm$  S.E.M. for four experiments. BVES transcriptional activity in uninfected MKN28 or AGS cultures was undetectable, and levels of BVES were significantly higher in MGEC versus MKN28 cells ( $p < .001$ ) and AGS cells ( $p < .005$ ) (Fig. 1).



**Figure 1.** Uninfected gastric cell lines were analyzed for BVES transcription. Values were normalized to *GAPDH*. Real-time RT-PCR analysis demonstrated reduction of BVES transcription in gastric cancer cell lines and is presented as a percentage of the maximum levels of BVES transcription in MGEC cell line. Data are expressed as means  $\pm$  S.E.M, MGEC n=13; MKN28 n=6; AGS n=2. In MKN28 and AGS cells, BVES was below the level of detection and, therefore, represented as 0. A one-way ANOVA with Newman-Keuls Multiple Comparison Post-Test was employed to determine statistical significance.

### *BVES transcription is reduced in MGEC after H. pylori infection.*

Genetically manipulated *cagE*- isogenic mutant strains were used since the absence of *cagE* confers an inability to form the type IV secretion system and, therefore, an inability to translocate CagA. These isogenic mutants were used to determine whether the effects on BVES were *cag* island-independent or -dependent. To detect changes in mRNA levels of BVES over time, a real-time time course analysis was used. BVES transcription levels were detected in uninfected MGECs, along with MGECs infected with *H. pylori* strains 60190 and 7.13 and their isogenic *cagE*- mutants. Values were recorded as fold changes over uninfected with controls represented as one. At four and six hours, all values for co-cultured populations were less than uninfected populations. Co-culture with *H. pylori* wild-type strains significantly decreased BVES expression compared with the uninfected control at four hours with strain 7.13 ( $p=0.005$ ) and with strain 60190 at six hours ( $p=0.010$ ). Transcription also significantly decreased at four hours upon co-culture with *cagE*- isogenic mutants of both strain 7.13 ( $p=0.024$ ) and of strain 60190 ( $p=0.003$ ) compared to the uninfected control (Fig. 2).



**Figure 2.** MGEC cells were infected with *H. pylori* strains 7.13, 60190, and their corresponding *cagE*- mutants. Analysis of real-time RT-PCR data shows a statistically significant decrease in 7.13, 7.13 *cagE*- mutant, and 60190 *cagE*- mutant at 4 hours. A significant decrease in wild-type strain 60190 was also observed at 6 hours; however, it is not significantly different from the decrease observed in its *cagE*- isogenic mutant (NS). There is no statistical significance between wild-type strain decreases and the decreases demonstrated by respective isogenic mutants. Recorded values are shown as fold changes over uninfected cells. Asterisks represent statistical significance. Data are expressed as means  $\pm$  S.E.M, n=2 at 4 hours and n=3 at 6 hours.

### *BVES translocates in MGEC cells after H. pylori infection.*

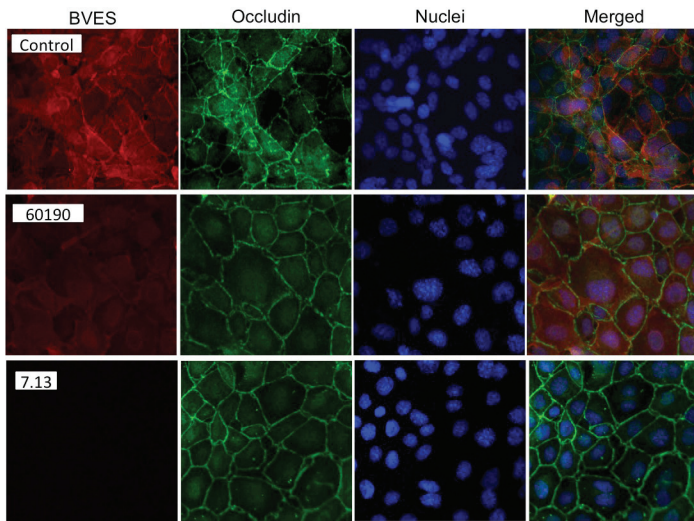
In order to visualize localization of BVES in cells prior to and following infection, Occludin and BVES were tagged with antibodies that could be detected using immunofluorescence microscopy and cellular nuclei were labeled with DAPI. BVES was primarily localized to the cell membrane in uninfected cells. BVES expression was reduced when cells were co-cultured with *H. pylori* strain 60190 and staining was virtually nonexistent in cells co-cultured with strain 7.13. Translocation of BVES from the membrane to the cytosol was also observed in cells infected with strain 60190 (Fig. 3).

## DISCUSSION.

Before beginning to define relationships between BVES, *H. pylori* infection, and EMT, it was important to note that the relevance of BVES to gastric cancer was as yet undetermined. Similar to the previous findings recorded in colon cancer cell lines [8], it was shown in this study that BVES expression was reduced in the human gastric cancer cell lines AGS and MKN28 compared to a noncancerous gastric cell line. This result confirms the relationship between reduced tight junction components and improved migratory capacity observed in cancer cells.

With previous studies suggesting changes in BVES at the transcriptional level [8], it was determined that the effects of *H. pylori* infection on BVES could be established through analysis of BVES transcription. Furthermore, through the use of *cagE*- isogenic mutants of *H. pylori* strains 7.13 and 60190, the ability to define the significance of the *cag* pathogenicity island was available. Real-time RT-PCR analysis of BVES transcription showed decreased levels of BVES upon *H. pylori* infection with all strains. The lack of statistical significance in the decrease by co-culture with strain 60190 at four hours may become statistically significant with more replicates. Although strain 60190 showed a significant decrease compared to the uninfected control at 6 hours, it was concluded that the effects of *H. pylori* on BVES transcription are *cag* PAI-independent due to the lack of statistical significance between measured values of the wild-type strains and their corresponding mutants. This allows for the findings of this study to be applied to a greater population of *H. pylori* infections, not just those harboring the *cag* island. Strains lacking the *cag* PAI may still be subject to disease progression as a result of the presence of other virulent genes, such as *vacA* which suppresses T-cell response to *H. pylori* infection, thereby increasing infection longevity [6].





**Figure 3.** BVES and occludin were tagged with fluorescent antibodies. Nuclei were stained with DAPI. Immunofluorescence was used to determine localization of BVES compared to previously established tight junction protein occludin. Analysis from immunofluorescent microscopy demonstrated a reduction in BVES expression and translocation from the membrane to the cytoplasm in MGECs co-cultured for 48 hrs with *H. pylori* strains 60190 and 7.13. Images are representative of twenty fields of view.

Because BVES was decreased at the transcriptional level, it can be concluded that *H. pylori* infection alters transcriptional regulation. This conclusion can be related to previous findings that attribute marked decreases in BVES to transcriptional mechanisms [8]. In the current study, it was also observed that the rodent-adapted wild-type strain 7.13 exerted more profound effects on transcription levels compared to the lab-adapted wild-type strain 60190 suggesting that this difference reflects the use of a rodent-adapted strain in a rodent cell line. This suggests that different strains of *H. pylori* may dictate the degree to which BVES is decreased.

It was also evident that the decrease in levels of BVES caused by *H. pylori* infection was sustained from four hours to six hours. The increase from four hours to six hours, which is still decreased compared to the control, is interesting and warrants further investigation into the transcriptional regulation mechanisms.

The final analysis of the effects of *H. pylori* infection in this study was conducted using immunofluorescence microscopy. MGECs were infected with strains 60190 and 7.13 and tagged with immunofluorescence antibodies designed to recognize and bind to BVES and occludin. A previous study showed that the mesenchymal phenotype could be acquired after loss of BVES at the cell membrane [9]. Immunofluorescence microscopy results in this study show an overall reduction in BVES from control to *H. pylori* strain 60190-infected to *H. pylori* strain 7.13-infected cells. A translocation of BVES from the membrane to the cytoplasm succeeding infection is also observed. These results are consistent with previous findings and show that they can be extended from human corneal epithelial cells to gastric epithelial cells.

#### CONCLUSION & FUTURE WORK.

The search for a biomarker that can be used to indicate disease progression is one of the ultimate goals of defining mechanisms by which *H. pylori* induces gastric adenocarcinoma. Due to previous findings in colorectal carcinoma [8], BVES was identified as one such indicator and was chosen for further examination in gastric cancer. Previous research also warranted investigation of BVES in relation to a metastatic process, EMT.

This study provides justification for further investigation of BVES as a biomarker of gastric cancer as it was shown that levels of BVES are decreased in gastric cancer cell lines. The direct relationship between levels of BVES and confluence also supports the ability to employ BVES as a prospective biomarker in the future as its levels decrease as disease progresses to adenocarcinoma.

In attempts to eventually employ BVES as a biomarker of gastric adenocarcinoma, it would be advantageous to identify the mechanism by which *H. pylori* regulates transcription of BVES. A previous study on transcriptional regulation of BVES in colorectal cancer showed promoter hypermethylation to be one such mechanism [8]. Similarly, a study performed using gastric cancer showed promoter hypermethylation to be responsible for silencing of BVES [10]. An experiment designed to delineate the role of *H. pylori* in transcriptional regulation could measure transcriptional activity of infected cells prior to and following treatment with a demethylating agent, such as 5-Aza-2' deoxycytidine, previously shown to restore decreased BVES transcription in colorectal cancer cell lines [8]. The implications of promoter hypermethylation and consequent decrease in transcription of BVES could be observed by measuring tight junction integrity through transepithelial electrical resistance. Furthermore, quantitative bisulfite pyrosequencing could be conducted to determine percent methylation of the BVES promoter, as done previously in normal and colorectal carcinoma tissues [8].

To examine translational regulation of BVES by *H. pylori*, Western blots coupled with densitometry could be used to identify BVES and quantify protein levels before and after co-culture. Results from these experiments could yield insight into translational regulation by *H. pylori* and merit investigation of the mechanisms by which such translational regulations occur.

Future research may also include an extension to *in vivo* models. Tissue samples from rodent models of *H. pylori* infection and gastric cancer could be examined for localization of BVES using immunohistochemistry. Results from this study could warrant further examination of human gastric biopsies.

The overall goal of this work is to define mechanisms that culminate into the development of a method aimed at early detection of tumorigenesis initiated by decreased levels of BVES in clinical populations. The findings in this study have identified a decrease in BVES in gastric cancer cell lines as well as a decrease in both BVES transcription and translation with *H. pylori* infection. These results with confirmations using *in vivo* studies could be promising for the use of BVES as a marker of progression to more severe disease in *H. pylori*-infected individuals.

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#### SUPPORTING INFORMATION.

**Figure S1.** Epithelial-mesenchymal transition

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