Early detection of tumor cells in bone marrow and peripheral blood in a fast-progressing gastric cancer model

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Abstract. *Helicobacter pylori* (*H. pylori*) infection is a major risk factor for the development of gastric cancer. The authors previously demonstrated that in mice deficient in myeloid differentiation primary response 88 (*Myd88*$^{-/-}$), infection with *Helicobacter felis* (*H. felis*) a close relative of *H. pylori*, subsequently rapidly progressed to neoplasia. The present study examined circulating tumor cells (CTCs) by measuring the expression of cytokeratins, epithelial-to-mesenchymal transition (EMT)-related markers and cancer stem cell (CSC) markers in bone marrow and peripheral blood from *Myd88*$^{-/-}$ and wild-type (WT) mice. Cytokeratins CK8/18 were detected as early as 4 months post-infection in *Myd88*$^{-/-}$ mice. By contrast, cytokeratins were not detected in WT mice even after 7 months post-infection. The expression of Mucin-1 (MUC1) was observed in both bone marrow and peripheral blood at different time points, suggesting its role in gastric cancer metastasis. Snail, Twist and ZEB were expressed at different levels in bone marrow and peripheral blood. The expression of these EMT-related markers suggests the manifestation of cancer metastasis in the early stages of disease development. LGR5, CD44 and CD133 were the most prominent CSC markers detected. The detection of CSC and EMT markers along with cytokeratins does reinforce their use as biomarkers for gastric cancer metastasis. This early detection of markers suggests that CTCs leave primary site even before cancer is well established. Thus, cytokeratins, EMT, and CSCs could be used as biomarkers to detect aggressive forms of gastric cancers. This information may prove to be of significance in stratifying patients for treatment prior to the onset of severe disease-related characteristics.

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Abbreviations: H. pylori, Helicobacter pylori; WHO, World Health Organization; BMDCs, bone marrow-derived cells; *Myd88*$^{-/-}$, myeloid differentiation primary response 88-deficient; *H. felis, Helicobacter felis*; CTCs, circulating tumor cells; DTCs, disseminating tumor cells; EMT, epithelial-to-mesenchymal transition; CK8/18/19, cytokeratin 8/18/19; WT, wild-type; c-Kit, cluster of differentiation 117 (CD117); MUC1, mucin 1; ICC, immunocytochemistry; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; BHI, brain heart infusion; FBS, fetal bovine serum; PBS, phosphate-buffered saline; CD133, cluster of differentiation 133 (prominin 1); GAPDH, glyceraldehyde 3-phosphate dehydrogenase; MSCs, mesenchymal stem cells

Key words: bone marrow, circulating tumor cells, gastric cancer, *Helicobacter*, MyD88, peripheral blood

Introduction

Gastric cancer is the third leading cause of cancer-related mortality worldwide (1). *Helicobacter pylori* (*H. pylori*) infection is the most potent risk factor responsible for the development of gastric cancer, leading to the recognition of this bacterium by the World Health Organization (WHO) as class 1 carcinogen (2-4). *H. pylori* infections affect up to 80% of the population in certain parts of the globe (4). Mortality due to gastric cancer, similar to other common types of cancer, is the result of metastasis (5). Moreover, there are currently no available effective predictors for identifying recurrence and metastasis in gastric cancer. Consequently, the determination of factors that indicate the existence of metastasis is critical for therapeutic interventions with the goal of improving disease outcome. Cancer metastasis involves tumor cells referred to as circulating tumor cells (CTCs), leaving the original cancerous site by migrating to distant sites; these can be found in peripheral blood and bone marrow (6-9). In the bone marrow, these CTCs are referred to as disseminated cancer cells (DTCs). CTCs have been used as biomarkers of metastasis in a number of cancer types (8,9) and their presence is associated with a poor prognosis (5,10-13). In bone marrow, evidence of cancer cells at the time of surgical intervention has been associated with metastasis (8,14). While research into associating CTCs and DTCs with cancer metastasis has been extensive for breast and lung cancer (5), research into this topic for gastric cancer has been limited. Indeed, the CellSearch System (Veridex, LLC) was approved by the US Food and Drug Administration for the detection of CTCs in patients with breast, prostate and colorectal cancer (9,15-17); however, its use for the detection of CTCs in gastric cancer continues to be controversial (18). This has led to a lack of enthusiasm in studies detecting CTCs in gastric cancer patients, and consequently, in their routine usage in gastric cancer management.

One of the most common methods for the detection of CTCs in solid tumors is with the use of surface markers, such as cytokeratins and mucin-1 (MUC1). Cytokeratins in general have been extensively studied in epithelial cancers, such as breast cancer (19) and specifically, cytokeratins such as CK8, CK18 and CK19 (20). These markers are of particular interest due to their abundant expression in epithelial cells and relatively low or no expression in mesenchymal cells (10,21). Recently, other markers, such as epithelial-to-mesenchymal transition (EMT)-related markers and cancer stem cells (CSCs) have been shown to be major components of CTCs due to their association with cancer progression (22-27).

EMT, which depicts changes in epithelial cells towards a malignant phenotype (28) is considered a crucial step in cancer progression (29). This process disrupts crucial activities, such as cell-cell adhesion, cell polarity (30) and extra cellular matrix degradation (31). There are a number of inducers of EMT, most notably factors, such as cytokines, innate and adaptive immune responses, and growth factors secreted by tumor microenvironment among others (32,33). This EMT process is tightly regulated by transcription factors, such as Snail, Twist and Zinc finger E-box-binding homeobox (ZEB). Snail and Twist have previously been shown to be overexpressed in *H. pylori*-infected patients (34). While patients with early stages of cancer do not exhibit EMT phenotypes, gastric cancer cell motility and metastasis are observed in the advanced stages of gastric cancer, which is implicated in the EMT process (35). While the clinical significance of EMT in other types of cancer has been confirmed (28,36,37), in gastric cancer, although the expression of EMT-related proteins has been studied, their significance remains questionable (38,39). CSCs, which are also suggested to be components of CTCs, are considered to contribute to a number of aggressive cancer characteristics, such as metastasis, tumor invasion, chemotherapeutic resistance and relapse (40).

Knowledge of micrometastatic cells, including when they arise and their detection, is critical since their dissociation from the primary tumor microenvironment and transportation to distant sites and finally, colonization is what ultimately leads to death. These cells are therefore important for the detection of metastasis or disease recurrence. The detection of CTCs is therefore crucial in identifying patients that are likely to relapse or develop metastases and can subsequently be targeted for the suppression of metastasis. Previous studies by the authors have demonstrated that the absence of myeloid differentiation primary response gene 88 (MyD88$^{-/-}$) leads to the development of an aggressive form of *Helicobacter*-induced gastric cancer, resulting in gastric cancer mouse models termed as slow [wild-type (WT)] and fast (*Myd88*$^{-/-}$) 'progressors' (41,42). *Myd88*$^{-/-}$ mice were shown to exhibit a rapid progression to precancerous and cancerous lesions in the stomach in response to infection with *Helicobacter felis* (*H. felis*) when compared to WT mice (41,42). In the present study, these gastric cancer models were used to evaluate the kinetics of CTCs and DTCs over a time span of 6 months. CTCs and DTCs were detected using surface markers, cytokeretins and mucins; EMTs and CSC markers, in the bone marrow and peripheral blood by employing immunocytochemistry (ICC), and/or reverse transcription-quantitative polymerase chain reaction (RT-qPCR). The data from the present study indicate that the early detection of metastasis in aggressive gastric cancers may be useful for gastric cancer management by providing information regarding proper prognosis and treatment intervention guidelines.

Materials and methods

Animals. Mice at 6-10 weeks old (weighing 20±5 g) were used in the present study. A total number of 40 male mice, WT (n=20) and *Myd88*$^{-/-}$ mice (n=16) with a C57BL/6J background were purchased from the Jackson Laboratory. In addition, some *Myd88*$^{-/-}$ mice (n=4) were bred inhouse. All mice were housed together prior to *H. felis* infection and for the duration of the study in a biosafety level II (BSL-2) facility with controlled temperature (23±2˚C) and relative humidity (45-60%) and had full access to food and water. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of California, San Diego, CA, USA. All procedures were performed using accepted veterinary standards.

Bacterial strains and growth conditions. *H. felis* strain CS1 (ATCC 49179) was used for the mouse infections. This strain was originally purchased from the American Type Culture Collection (ATCC). *H. felis* were maintained in both solid and liquid medium. The solid medium was composed of Columbia agar (BD Biosciences) supplemented with laked blood (5%, Hardy Diagnostics) and Amphotericin B (1%; Mediatech, Inc.). The liquid medium was composed of brain heart infusion (BHI; BD Biosciences) supplemented with 10%, heat inactivated fetal bovine serum (FBS). Bacteria were grown at 37˚C under microaerophilic conditions (5% O_2, 10% CO_2, 85% N_2) as described in previous studies by the authors (41,43). Bacteria maintained in solid media were passaged every 2-3 days. Prior to the infection of the mice, *H. felis* were grown in liquid medium for 48 h. Spiral bacteria were counted using a Petroff-Hausser chamber.

Mouse infections. WT and *Myd88*$^{-/-}$ mice were infected with *H. felis* grown in BHI. A total of 10^9 organisms in 300 μl were administered to each mouse (14 mice from each mouse strain) by oral gavage every other day for a total of 3 inoculations as described in previous studies by the authors (41,43). Control mice (6 mice from each mouse strain) received 300 μl of BHI. *Myd88*$^{-/-}$ mice (2 or 3 mice) were euthanized each month for up to 6 months post-infection. WT mice (2 or 3 mice) were euthanized at 5, 6 and 7 months post-infection. Bone marrow and peripheral blood was aseptically removed and processed for experimental analysis.

Bone marrow isolation. Following euthanasia, femurs and tibias were aseptically removed from the mice taking care to remove any muscle on or near the bones as described in previous studies by the authors (44,45). Bone marrow cells were flushed using a 22-gauge needle and phosphate-buffered saline (PBS) by cutting the ends of the bones with sharp scissors. Cells were collected for downstream applications.

ICC. Samples collected from bone marrow and peripheral blood were deposited onto lysine coated slides using StatSpin CytoFuge (Beckman Coulter, Inc.). Briefly, cell samples were

loaded onto cell concentrators with lysine coated slides. The concentrators with sample were then placed into a cytofuge and spun at 55 x g for 4 min at room temperature (RT). Once cell samples were placed on slides, cells were fixed with 4% paraformaldehyde for 10 min at RT. The cells were then incubated with 1% bovine serum albumin (BSA) in 0.1% PBS supplemented with Tween-20 (PBST) for 30 min at RT. Cells were immunostained with antibodies specific for CK8/18 (ab215880), 1:2,000, Abcam and c-Kit, cluster of differentiation (CD)117 (c-Kit, sc-168, 1:200, Santa Cruz Biotechnology, Inc.). All primary antibodies were incubated in a humidified chamber at 4˚C overnight. After washing, the cells were incubated with goat anti-rabbit secondary antibody (1:1,000) with fluorochrome fluorochrome (Alexa Fluor 488 and 647; ab150077 and ab150083, respectively, Abcam) for 1 h at RT in the dark and the samples were then mounted with Fluoroshield mounting medium with DAPI (4',6-diamidino-2-phenylindole, Abcam). Slides were imaged using the Keyence BZX-700 Fluorescent Microscope (UCSD Microscopy Core).

Isolation of RNA and cDNA synthesis. RNA was isolated from the bone marrow and peripheral blood samples using the Direct-zol RNA mini kit (Zymo Research Corp) according to the manufacturer's instructions. Briefly, a total of 300 μl of TRI Reagent was added to bone marrow or blood plasma in a volume of 3:1. The samples were vortexed vigorously followed by RNA purification. The samples were passed through a collection column and washed with the accompanying buffers. The resulting RNA solution was passed through a filter cartridge and RNA eluted using nuclease-free water. RNA quality was determined using a Nanodrop system (Thermo Fisher Scientific, Inc.) by reading the absorbance levels at 260 nm. A total of 2 μg of RNA per sample was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription kit (cat. no. 4368814, Thermo Fisher Scientific, Inc.).

qPCR. qPCR was performed as described in previous studies by the authors (43,45,46). The expression of select genes, including CD44, SRY-box transcription factor (SOX)9, Prominin-1 (CD133), SOX2, octamer-binding transcription factor 4 (OCT4), NANOG, leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5), CK18, CK19, MUC1, Snail, Twist and ZEB. Briefly, 2 μl of cDNA were used per well in a 10 μl reaction mix for amplification using Step One Real Time PCR (Applied Biosystems; Thermo Fisher Scientific, Inc.). The amplification conditions consisted of an initial cycle of 95˚C for 5 min followed by 40 cycles of amplification with denaturation as follows: 95˚C for 15 sec, 60˚C for 20 sec, 72˚C for 40 sec. Gene expression levels were normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The data collected was analyzed using comparative cycle threshold calculations ($\Delta\Delta C_T$, Applied Biosystems; Thermo Fisher Scientific, Inc.) and plotted using GraphPad Prism software. The sequences of primers used are listed in Table SI.

Statistical analysis. Gene expression was analyzed using comparative cycle threshold calculations ($\Delta\Delta C_T$, Applied Biosystems; Thermo Fisher Scientific, Inc.) as described in previous studies by the authors (41). A fold change of >2 between infected and control mice was considered significant.

Results

Detection of epithelial markers in bone marrow and peripheral blood in response to Helicobacter infection. The epithelial markers, CK8/18, were used to detect CTCs and DTCs in peripheral blood and bone marrow from mice in response to infection with *H. felis*, respectively. c-Kit (also known as CD117) was used as a standard surface marker expressed in hematopoietic cells and progenitor cells in bone marrow (47,48). In the present study, in the fast 'progressor' gastric cancer model, bone marrow was analyzed for epithelial markers monthly for up to 6 months post-infection. Epithelial cell markers were detected in the bone marrow as early as 3 months and their expression levels increased as the disease progressed, with maximum expression observed at 6 months post-infection (Fig. 1). These markers were not detected at 1 (Fig. 1) or 2 months (Fig. S1). CK8/18 were not detected in peripheral blood. However, the increased expression of CK18 and CK19 in both peripheral blood and bone marrow (Fig. 2) was observed as the disease progressed using RT-qPCR. Moreover, MUC1 expression was observed in peripheral blood at both at 4 and 6 months, whereas its expression in bone marrow was only observed at 6 months (Fig. 2B). On the other hand, no epithelial markers were detected in the slow 'progressor' gastric cancer model (*H. felis*-infected WT mice) at 5 and 6 months post-infection (Fig. S2). Therefore, all subsequent experiments were only performed in the fast 'progressor' gastric cancer model (*H. felis*-infected *Myd88*$^{-/-}$ mice).

Evidence of epithelial transition to a mesenchymal phenotype. The EMT transcription factors, Snail, Twist and ZEB, were analyzed to determine their expression during *H. felis*-induced disease progression. Increased expression levels of Snail were observed in bone marrow as compared to negligible or below threshold levels at both 4 months and 6 months in peripheral blood (Fig. 3). On the other hand, although Twist was expressed above threshold levels at both 4 and 6 months post-infection, the peak levels differed, peaking at 6 months in peripheral blood (Fig. 3A) and at 4 months in bone marrow (Fig. 3B). ZEB was expressed in peripheral blood; however, its expression was undetectable in bone marrow (Fig. 3).

Expression of cancer stem cells markers in bone marrow and peripheral blood. LGR5 is the most well-known gastric cancer stem cell marker and has been extensively studied to validate its importance in gastric cancer (49). In the present study, the expression of LGR5 increased gradually from 4 to 6 months in peripheral blood and bone marrow, with higher expression levels observed in bone marrow (Fig. 4). The expression of CD44, which was the first gastric cancer stem cell marker identified (50,51) peaked at 6 months in the bone marrow. On the other hand, CD133 was expressed at high levels in peripheral blood (Fig. 4A) but was undetectable in the bone marrow (Fig. 4B). Other cancer markers evaluated included OCT4, NANOG, SOX2 and SOX9; their expression levels were detected in both bone marrow and peripheral blood (Fig. 4).

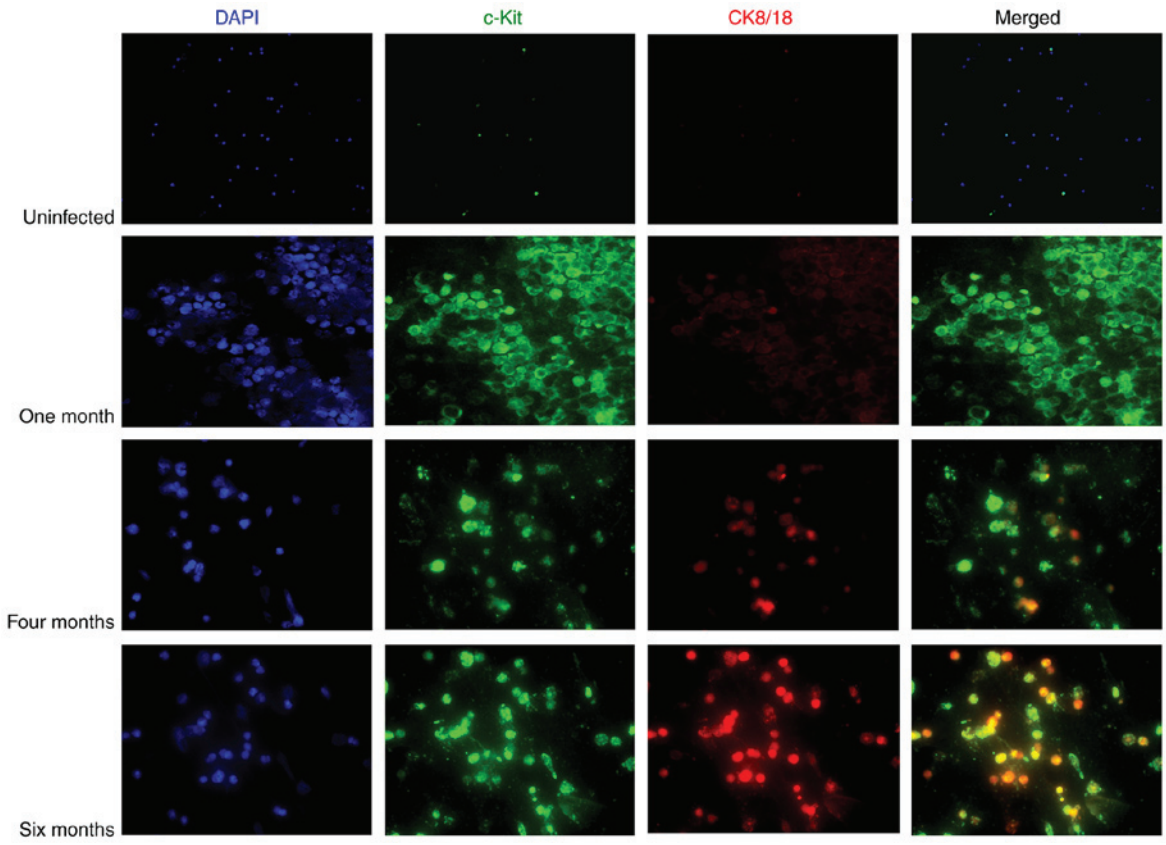

Figure 1. Infection with *Helicobacter felis* induces epithelial marker expression in the fast 'progressor' gastric cancer model (*H. felis*-infected *Myd88*$^{-/-}$ mice). Representative images of immunofluorescence staining of bone marrow for c-Kit (green) and CK8/18 (red) are shown with DAPI staining of nuclei in blue. *Myd88*$^{-/-}$ mice were infected with *H. felis*, and bone marrow was checked monthly from 1 to 6 months to determine epithelial marker expression. Images shown are: Uninfected (first panel), 1 months post-infection (second panel), 4 months post-infection (third panel), and 6 months post-infection (fourth panel). Images were taken at x40 magnification. *Myd88*$^{-/-}$, myeloid differentiation primary response 88- deficient; CK8/18, cytokeratin 8/18.

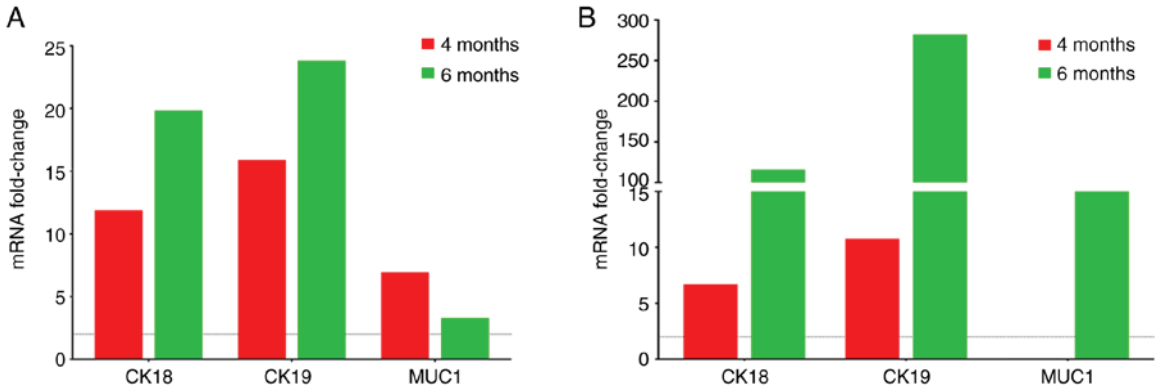

Figure 2. Epithelial markers in bone marrow and peripheral blood are overexpressed. Gene expression of CK18, CK19 and MUC1, in (A) peripheral blood and (B) bone marrow from *Myd88*$^{-/-}$ *Helicobacter felis*-infected mice. Data are reported as the fold induction of uninfected *Myd88*$^{-/-}$ mice. *Myd88*$^{-/-}$, myeloid differentiation primary response 88-deficient; CK, cytokeratin; MUC1, mucin 1.

Discussion

Examination and diagnostic tools for confirming the presence of gastric cancer are often invasive with endoscopy being the main test used to detect stomach cancer. At times, signs and symptoms are not very distinguishable for many patients and with no protocol in place in countries where incidence of gastric cancer is low, the chance of early stage detection is very minimal. For the majority of patients, gastric cancer is diagnosed in the locally-advanced or late stages, as either screening is not performed or the disease is detected only following the development of symptoms. Early detection will help increase patient survival by decreasing the chance for metastatic progression. Indeed, detection of CTCs in peripheral blood and bone marrow in gastric cancer patients has been suggested to be indicative of metastasis (52,53). However, the clinical significance of CTCs and DTCs as indicators of metastasis has not been appropriately utilized

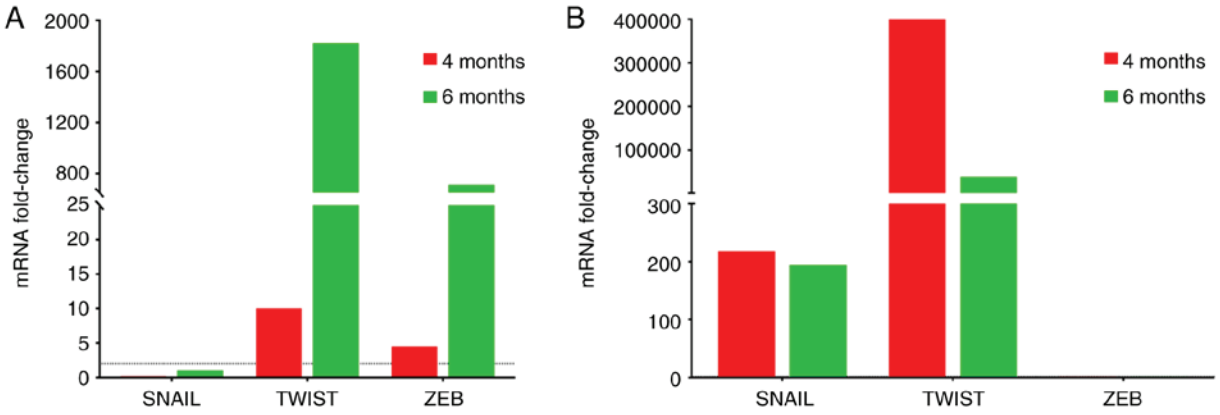

Figure 3. Expression of epithelial-mesenchymal transition (EMT)-related markers. Snail, Twist and ZEB levels were assessed in (A) peripheral blood and (B) bone marrow from *Helicobacter felis*-infected *Myd88*$^{-/-}$ mice at 4- and 6-months using RT-qPCR. Data are reported as the fold induction of uninfected *Myd88*$^{-/-}$ mice. *Myd88*$^{-/-}$, myeloid differentiation primary response 88- deficient; CK, cytokeratin; ZEB, zinc finger E-box-binding homeobox.

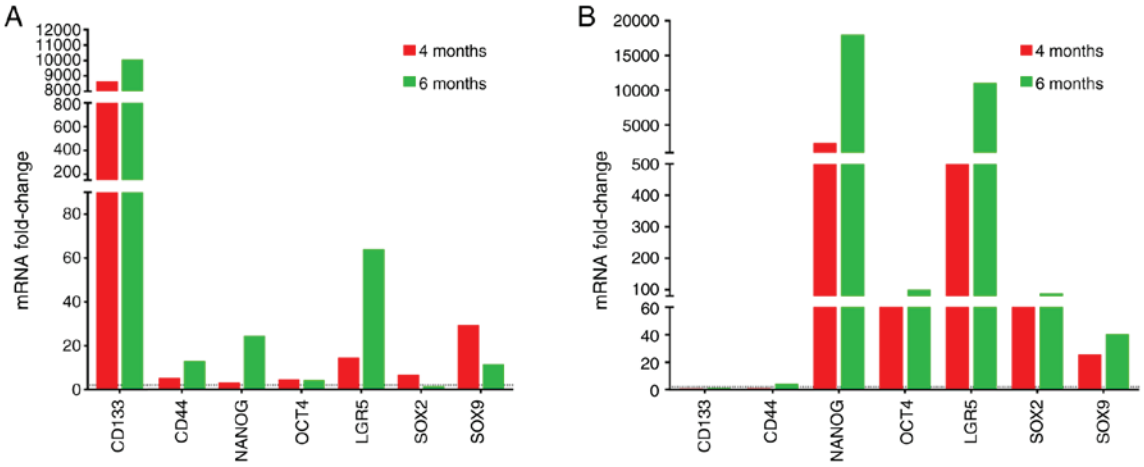

Figure 4. Quantification of cancer stem cell marker levels. CD133, CD44, NANOG, OCT4, LGR5, SOX2 and SOX9 levels were assessed in (A) peripheral blood and (B) bone marrow from *Helicobacter felis*-infected *Myd88*$^{-/-}$ mice at 4- and 6-months using RT-qPCR. Data are reported as the fold induction of uninfected *Myd88*$^{-/-}$ mice. *Myd88*$^{-/-}$, myeloid differentiation primary response 88- deficient; CD133, cluster of differentiation 133 (Prominin 1); OCT4, octamer-binding transcription factor 4; LGR5, leucine-rich repeat-containing G-protein coupled receptor 5, SOX, SRY-box transcription factor.

in gastric cancer compared to breast and lung cancer (5). In the present study, three subsets of biomarkers were utilized, namely cytokeretins, EMTs and CSCs, to detect CTCs and DTCs indicative of metastasis using a previously established fast 'progressor' gastric cancer model at an early stage (41). CTCs were detectable as early as 3 months compared to our slow 'progressor' model (WT type) where they were undetectable even at 6 months. This suggests that in an aggressive form of cancer the transformed cells, CTCs begin moving to secondary locations even before the cancer is well established at the primary site. The presence of epithelial gastric surface markers within bone marrow and peripheral blood indicate that not only have tumor-like cells left the microenvironment of the gastric mucosa, but have successfully begun infiltrating these areas leading to micro metastatic tumors throughout the body (19). CK8, CK18 and CK19 have previously been identified as markers whose expression is found in almost all epithelial-based carcinomas (48,54). Cytokeratins, such as 8 and 18 are found in >90% of gastric cancer tumors (55) rendering them reasonable targets to evaluate as positive markers of gastric metastasis. In addition, the present study detected MUC1 in both the peripheral blood and bone marrow. MUC1 is an oncoprotein found in a number of adenocarcinomas (59), which under normal conditions is known to protect the gastric epithelium (56-59). However, in the presence of *H. pylori*, MUC1 expression has been shown to be considerably decreased (57). MUC1 is one of the markers used for detecting CTCs and DTCs in epithelial solid cancers (5) and has been linked to cancers such as non-small cell lung cancer (60), as well as DTCs within the bone marrow of breast cancer patients (61). The role of MUC1 in carcinogenesis has not been well elucidated and in particular, its role in gastric cancer is contradictory (62-64); however, its overexpression has been associated with cancer metastases (65). Recent studies have suggested regulation of MUC1 by mir-206 inhibits proliferation and migration of gastric cancer cells (66). Thus, reinforcing the role of MUC1 as a gastric cancer metastases biomarker. Moreover, MUC1 promotes cell proliferation by Wnt signaling pathway and EMT activation through Snail in renal carcinoma (67).

EMT is described as the transition of cells from an epithelial to a mesenchymal state that is associated with

the suppression of E-cadherin resulting in an invasive cell phenotype (27-29,68,69). This change in expression is induced by EMT-transcription factors (EMT-TFs), which include Snail, Twist and ZEB. The increased expression of these EMT markers is associated with the transition of the epithelium into a malignant phenotype (28). As gastric cancer progresses, epithelial cells begin to lose these phenotypic markers and begin to acquire a mesenchymal phenotype (70), which is associated with the loss of cell-cell adhesion of epithelial cells, as well as changes in cell polarity, which eventually allows for the easy migration of cells (69). The concomitant expression of these EMT markers with epithelial markers in our gastric cancer model indicates that these EMT markers may be used as indicators of metastasis in gastric cancer. Recent findings suggest that acquisition of mesenchymal markers in tumors is a poor prognostic cancer factor (27,71,72). Hypoxic conditions in tumors are suggested to trigger mesenchymal stem cell (MSC) migration (73-76). The presence of these MSCs in the tumor stroma is associated with EMT stimulation. Once stimulated it is indicated that these MSCs may promote the invasion and spread of tumor cells in systemic circulation (77). As an example, studies carried out by Yang *et al*, 2004 (78), in breast cancer have suggested that high levels of the continued expression of Twist are essential for metastasis. The findings of the present study also demonstrated the continued expression of Twist in both peripheral blood and bone marrow, thus, suggesting a role of Twist in metastasis in an aggressive form of gastric cancer. Snail is a potent suppressor of E-cadherin and is closely associated with cancer metastasis and tumor progression via the Wnt pathway (79). Previous research on breast cancer has demonstrated that Snail is required for lymph node metastasis (28). High expression levels of Snail in the bone marrow may indicate DTCs in the bone marrow. ZEB, in addition to its function as an EMT inducer, also plays a role in hematopoiesis. ZEB has been associated with acquisition of cancer stem cell (CSC) properties (80). Thus, the expression of ZEB, also shown in the present study, indicates early metastasis in fast-progressing gastric cancer.

The present study also detected CSCs, including LGR5, CD44, CD133, OCT4, SOX2, SOX9 and NANOG in peripheral blood and bone marrow during disease progression. Cancer stem cells play a vital role in cancer metastasis (12,40). For all tumor-associated stem cell markers detected in the current fast-progressing gastric cancer model, the expression levels were always greater in the bone marrow than in the peripheral blood. This is in line with research on challenges associated with the detection of CTCs in blood due to very low numbers of tumor cells in blood (81). Indeed, the levels of CTCs are generally lower in peripheral blood compared to bone marrow (82). Notably, CD133 was highly expressed in peripheral blood and undetectable in bone marrow. The reason for this differential expression remains to be investigated. CD133 is a known cancer stem cell marker in cancers, such as colorectal cancer and liver cancer and for is its role in metastasis in these types of cancer (83). Of all these CSC markers, LGR5 and CD44 are well-known targets of the Wnt signaling pathway, and have been implicated in cancer invasion and metastasis through their involvement in tumor formation and proliferation (84-86). LGR5 induces the Wnt/β-catenin pathway, enhancing tumor formation and cancer cell proliferation (84). The gradual increase in the expression of LGR5 observed in the present study was similar to that observed for cervical cancer (84). LGR5 expression increased as the disease progressed. The expression of CD44 also gradually increased as the disease progressed although the level of expression was lower compared to LGR5. The findings of the present study demonstrating a close association in expression between EMT transcriptional factors and stem cells markers are in agreement with those of studies indicating a link between EMT and acquisition of stem cell properties (87,88). In addition, studies have indicated that EMT facilitates the generation of CSC traits for metastasis, but also for self-renewal properties required for initiating secondary tumors attributed to NANOG, OCT 4, SOX2 to name a few (89-91). These provide credence to the current observation that these markers can be used to detect gastric cancer metastasis and predict aggressive and fast-progressing gastric cancers. Future studies are required to confirm that these cells are indeed cancer cells by use of a xenograft mouse model. In considering the future translation of the current study to humans, the data suggest that the detection of tumor cell biomarkers could be performed when *H. pylori* infection is diagnosed in a patient, given that currently, gastric cancer is diagnosed when it has already progressed to the late stages of the disease. Granted that bone marrow biopsy is an invasive procedure, performing peripheral blood tumor cell biomarker detection screens alone would be a better prophylactic approach at first and depending on the results, further confirmation for gastric cancer could be performed by bone marrow aspiration procedures. Therefore, including peripheral blood tumor cell biomarker detection in regular health screens upon diagnosis of *H. pylori* infection is potentially a good preventive measure.

To date, at least to the best of our knowledge, there are no published data available showing the stage at which gastric cancer metastasizes. Using the present mouse models of gastric cancer (41), the expression of EMT, stem cell markers and cytokeratins was detected in the fast 'progressors' gastric cancer model by 4 months, but not in the slow 'progressors', suggesting that these factors are involved in the early events of tumorigenesis; thus, these factors may represent early indicators of disease dissemination and therefore, metastasis. The present study using mice suggests that dysplastic gastric epithelial cells begin seeding themselves in other tissues, including the bone marrow early during the disease progression to gastric cancer and before the emergence of gastric cancer *in situ*. In addition, the present study revealed an association between cytokeratins, EMTs and CSCs with an aggressive form of gastric cancer. Studies on tumor cell biomarker detection in human gastric cancer patients are required to confirm these findings. Nonetheless, the present study sets up a proof of concept that longitudinal monitoring of CTCs as an indicator of metastasis in gastric cancer is an achievable goal similar to the current management of breast cancer (19,20,27). Therefore, the findings of the present study may lead to the development of early detection strategies for CTCs in patients with an aggressive form of gastric cancer, so that appropriate treatment can be provided in a timely manner.

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Availability of data and materials

The datasets generated and analyzed during this study are available from the corresponding author upon reasonable request.

Authors' contributions

MO designed the study concept. ILP and CP acquired the data. PB, ILP and MO analyzed and interpreted the data. PB and IL drafted the manuscript. PB and MO edited the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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