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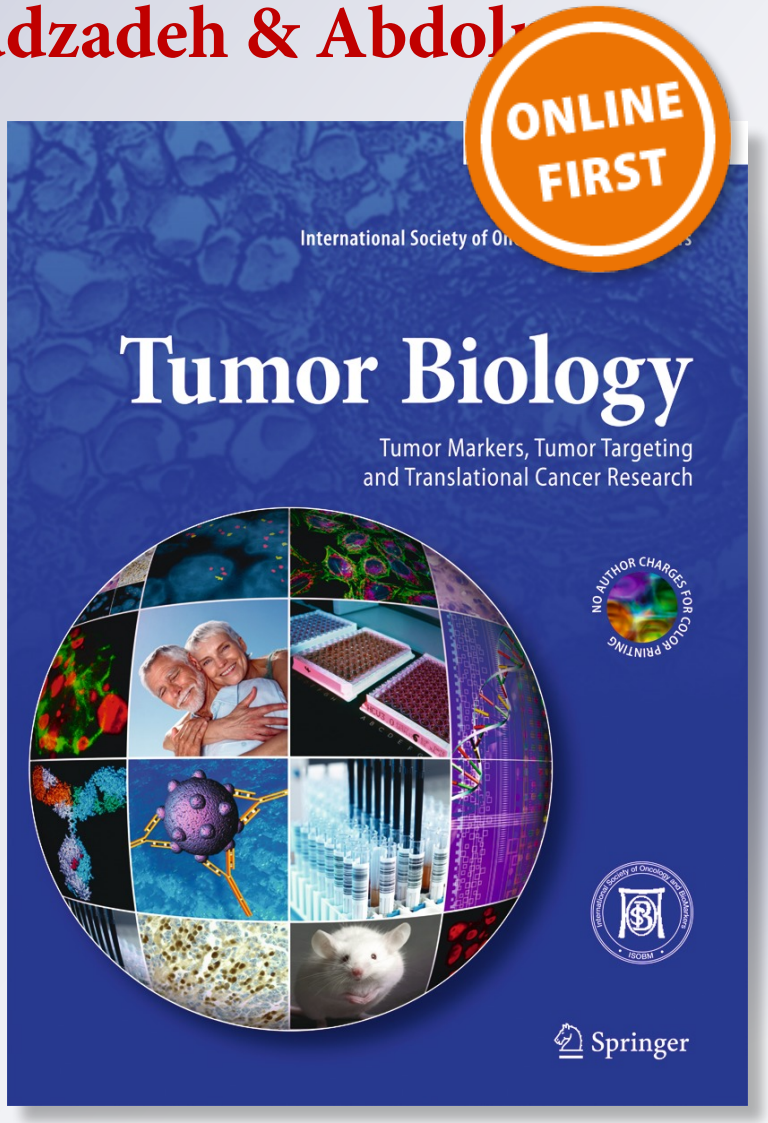

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ORIGINAL ARTICLE

Role of *Helicobacter pylori* on cancer of human adipose-derived mesenchymal stem cells and metastasis of tumor cells—an in vitro study

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Abstract Recent studies have shown that *Helicobacter pylori* has a special role in tropism of mesenchymal stem cells (MSCs) towards gastric tissues. This study aimed to find the effects of *H. pylori* on human adipose-derived mesenchymal stem cells (hA-MSCs) transforming toward cancer cells and also metastasis of tumor cells by synergic effects of *H. pylori* and gastric epithelial cells (AGS) on MSCs. The expressions of p53, bcl-2, MMP-2, and MMP-9 were examined in hA-MSCs by qRT-PCR technique. Our results demonstrated that *H. pylori* tries to improve the hA-MSCs carcinogenic activities by overexpression of bcl2 gene as an anti-apoptosis agent against the p53 gene expression as main apoptosis agent. In addition, it showed that *H. pylori* effects in metastatic activities of hA-MSCs by upregulation of related genes in this process. Perhaps, when hA-MSCs are attracted toward *H. pylori* chronic or ulcer infected tissues for their tissue healing function, they will be trapped under special gastric microenvironment. We demonstrated the direct and synergic effects of *H. pylori* in hA-MSCs through alteration of related genes involved in carcinogenesis processes. Hence, understanding of *H. pylori*-induced molecular pathogenesis could be a powerful strategy not only in identifying the origin and initiation of gastric cancer but also in the treatment of related disease and modification of stem cell therapy methods in the future.

Keywords Mesenchymal stem cells · Gastric cancer · Metastasis · *H. pylori*

Gita Eslami and Abdolreza Ardeshirylajimi contributed equally to this work.

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Introductions

Helicobacter pylori (*H. pylori*) was first discovered in 1983 by Warren and Marshal [1]. The International Agency for Research on Cancer (IARC) classify *H. pylori* infection as a class I carcinogen in 1994 [1, 2]. *H. pylori* is a Gram-negative bacterium that selectively colonizes in gastric epithelium. *H. pylori* has special virulence factors such as pathogenicity island (PAI), a cluster of approximately 30 genes, which are associated with severe gastric diseases, gastric adenocarcinoma, and gastric mucosa-associated lymphoid tissue (MALT) lymphoma [1, 3]. Today, gastric adenocarcinoma is the second leading cause of cancer-related death in the world [4]. The relationships between *H. pylori* infections and cancer have been investigated extensively; so far, cagA protein has been recognized as bacterial oncoprotein which implicate gastric carcinogenesis [3, 5, 6]. Microbial chronic infection and host immune response make a special microenvironment which is

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the very place that the progression of mucosal atrophy toward the gastric atrophy, metaplasia, dysplasia, and finally adenocarcinoma occurs [3, 7–13].

Mesenchymal stem cells (MSCs) were first separated from human and mouse bone marrow monolayer cell cultures in the 1950s [14]. MSCs are adult non-hematopoietic pluripotent stem cells [15]; these spindle-shaped and adherent cells proliferate rapidly to expand populations of clones [14]. They have been discovered to possess tissue replacement and wound healing properties, mainly due to their differentiation ability to many cell types [1, 14–18]. As recent studies demonstrated, stem cells have migrated towards the microenvironments which are infected by *H. pylori* [1, 6]; the main factors for attracting MSCs are chemotaxi cytokines that are particularly released in the chronic infection specially *H. pylori* infections [1, 19]. Stem cells have more abilities than other cells to temporarily put off and control the cells' normal growth and start extra proliferation for their innate duty in ulcer and wound healing [20, 21]. These special abilities can put them on problems too, when they are affected by wide range of different signals under specific microenvironments [22]. Recently, studies have shown that *H. pylori* induced MSCs migration through different ways towards the chronic infection microenvironments [1, 23]. Although cell migrations and metastasis have the same meaning of relocation, they are completely different at their biological phases because the metastasis of tumor cells is completely different from the migration of normal cells, immune cells, or MSCs. Several clinical and experimental studies have demonstrated that raising the amount of matrix metalloproteinase (MMP) production is associated with tumor growth, invasion, and metastasis of tumor cells which cut down the lifetime of the patients [24]. MMP-2 and MMP-9 play substantial roles within other types of MMPs in tumor cell progress and metastasis by degradation of type IV collagen, a major component of the extracellular matrix (ECM) [25]. It is the most important scaffold for the basement membrane (BM) proteins which helps maintain the BM cell junction and entirety [26]. MMP-2 and MMP-9 were correlated with progression and metastasis of cancer cells by degradation of the BM, which surrounds the cancer nests and vascular structures comprehensively [27, 28]. This study aimed to demonstrate the effective role of *H. pylori* in transforming human adipose-derived mesenchymal stem cells (hA-MSCs) to cancer cells and inducing some cancerous properties such as metastasis.

Materials and methods

hA-MSC characteristic evaluation

The human adipose-derived mesenchymal stem cells (Fig. 1a), which are transduced, with a vector containing GFP and puromycin were obtained from the Department of Stem Cell Biology (Stem Cell Technology Research Center, Tehran, Iran) [29], and these hA-MSCs with puromycin resistance label can help us to separate stem cells from other cells in co-culture groups for applying them in next experiments (Fig. 1b). hA-MSCs were cultured to confirm MSCs' identity and their differentiation potency evaluation by treating under the osteogenic and adipogenic differentiation media [29]. Briefly, the cells were trypsinized, neutralized, centrifuged, resuspended, and then plated in four-well plates at 5×10^4 cells per well in Dulbecco's modified Eagle's medium ((DMEM). Then, they were treated under osteogenic and adipogenic differentiation medium. The medium was replaced every 2 days. After 3 weeks, the plates were washed with 1× PBS, fixed with 4 % paraformaldehyde and stained with Alizarin Red and Oil Red kites, and then examined with microscope. The cells were labeled with anti-human conjugated antibodies of CD105, CD90, CD34, and CD45 to confirm MSC immunophenotyping (eBioscience, USA). All the human antibodies (hAbs) were analyzed by Attune Acoustic Focusing flow cytometer system (Applied Biosystems, USA).

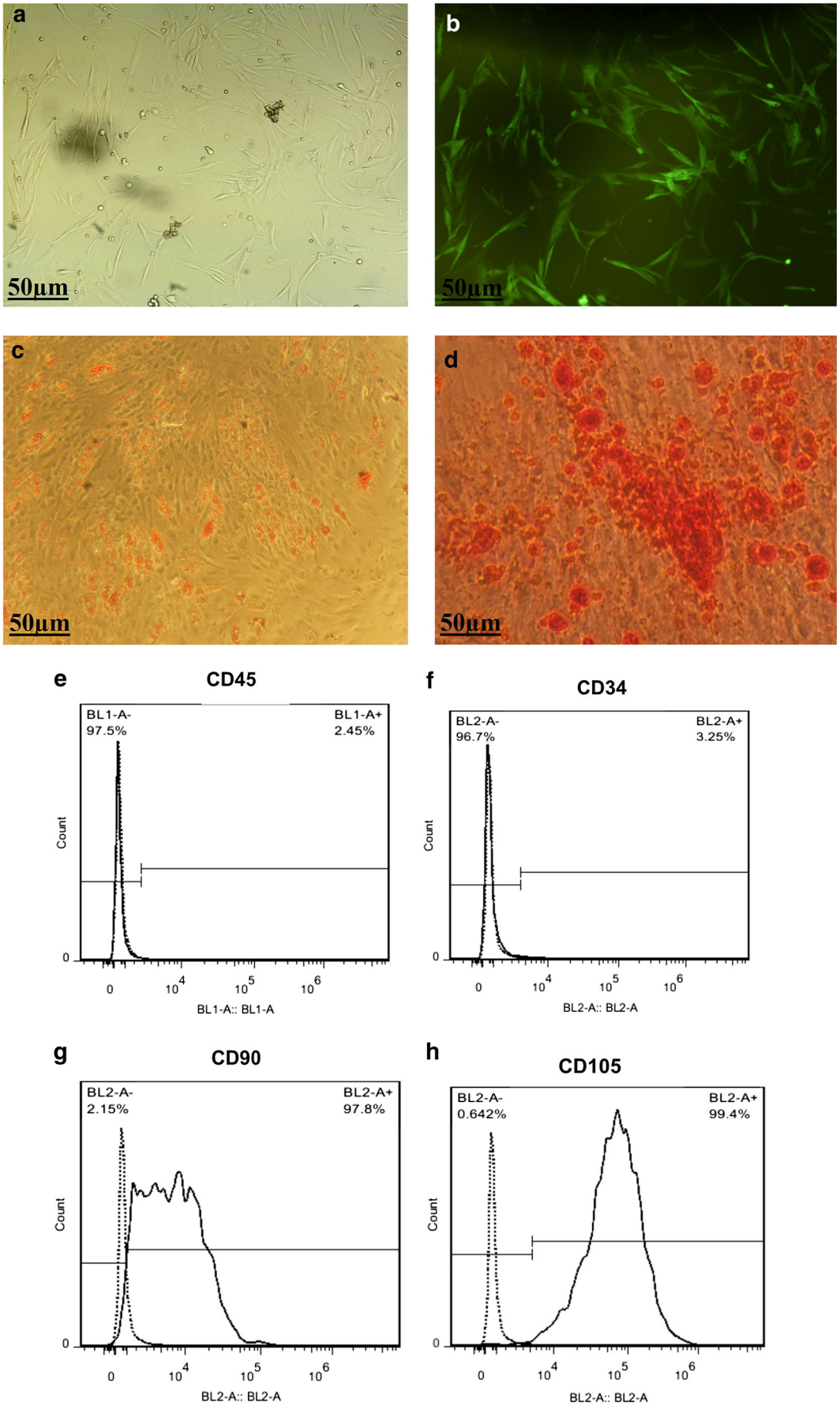
Cell culture

Gastric epithelial cells (AGS) were obtained from the National Cell Bank of Iran (Pasture Institute of Iran, Tehran, Iran). The AGS cells and hA-MSCs were plated into T-75 culture flasks (SPL Co.) and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco Life Technologies, USA) and incubated in humidity atmosphere and 5 % CO_2 at 37 °C.

Bacterial culture

The wild type of *H. pylori* strain was obtained from the Gastroenterology and Liver Diseases Research Center of Shahid Beheshti University of Medical Sciences, Tehran, Iran. *H. pylori* genotyping was based on its important virulence factors, such as cagA ($s_1/_2$) and vacA ($m_1/_2$). *H. pylori* strain was cultured on supplemented Brucella agar with 7 % sheep blood and antibiotics (vancomycin 2.0 mg, polymyxin 0.05 mg, trimethoprim 1.0 mg, and amphotericin B 2.5 mg/l). Incubation was performed in microaerophilic conditions at

Fig. 1 Human adipose-derived mesenchymal stem cell characteristic conforming. The hA-MSCs were uniform and showed fibroblastoid morphology (**a**). Florescence microscopic image of GFP - hA-MSCs (**b**). Microscopic image showed the typical morphology of adipocytes; adipogenic differentiation was determined by Oil Red (**c**). Osteogenic differentiation was evidenced by Alizarin Red staining (**d**). Immunofluorescent staining and flow cytometry analysis of cell surface markers showed that hA-MSCs were positive for CD105 and CD90 (**e**, **f**), but negative for CD34 and CD45 (**g**, **h**)



37 °C for 5–7 days in multi-gas incubator. Colonies were characterized by urease, catalase, and oxidase reactions and PCR as the molecular identification method. For co-infection experiments, *H. pylori* strain was grown at 37 °C for 24 h, resuspended in PBS, and adjusted for 1 McFarland (corresponding to 3×10^8 CFU/ml) in PBS before infection. For co-culture experiments, *H. pylori* strain was grown at 37 °C for 24 h, then used for infection with multiplicity of infection *(*MOI) of 100 (100:1).

Co-culture and co-infection

In six-well plates, 5×10^4 hA-MSCs and AGS were separately plated as control and independent groups without any co-culture or co-infection, and then, hA-MSCs were co-infected by *H. pylori* with MOI of 100 (100:1) and co-cultured by AGS cells with MOI of 10 (10:1) in different wells as binary groups and ternary groups; we co-cultured seeded hA-MSCs by AGS cells with MOI of 10 (10:1), then co-infected them by *H. pylori* with MOI of 100 (100:1). All groups were treated by cell culture method that we described previously in cell culture.

RNA extraction and cDNA synthesis

Total hA-MSCs samples were performed by using an RNA extraction kit according to the manufacturer's protocol (TRIzol, Invitrogen). Briefly, before extraction, all hA-MSCs and co-cultured pellets were treated by puromycin-containing media with 2 μg/ml puromycin. Puromycin-containing media were changed every day for 2 days, and then, hA-MSCs were lysed, 200 μl of chloroform was added, and the samples were incubated for 5 min at room temperature. After centrifugation (12,000×*g* for 15 min at 4 °C), total RNAs were precipitated from the aqueous upper phase using 500 μl isopropanol and then resuspended in 50 μl RNase-free water and stored at −80 °C. The amount of extracted RNA was determined by measuring the absorbance at 260 nm. cDNA synthesis of 2 μg of total RNA was performed in 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM MgCl, 10 mM dithiothreitol, 300 μM dNTP, and 2.7 Mm random hexamers primers (Vivantis) in a final volume of 46 μl. The samples were heated at 65 °C for 10 min and rapidly chilled on ice, and 100 U of M-MuLV reverse transcriptase was added (Vivantis). The final mixture was incubated at 37 °C for 60 min and then heated at 95 °C for 5 min.

qRT-PCR

Real-time polymerase chain reaction (qRT-PCR) was performed in Rotor-Gene Q (Qiagen) using SYBR Green Master Mix (Takara Bio) detection and a two-step PCR protocol. The samples were run in triplicate, and thresholds were uniformly set across all genes with a particular reference standard to calculate

Table 1 Primers used in the qRT-PCR technique

Gene name	Primers 5′→3′
MMP-2	F: GCTCGTGCCTTCCAAGTC R: AGTCCGTCCTTACCGTCAA
MMP-9	F: CGGACCAAGGATACAGTTTGT R: CTCAGTGAAGCGGTACATAGG
P53	F: GGAGTATTTGGATGACAGAAAC R: GATTACCACTGGAGTCTTC
Bcl-2	F: ATGTGTGTGGA GAGCGTCA R: AGAGACAGCC AGGAGAAATC
Beta actin	F: GTCCTCTCCCAAGTCCACAC R: GGGAGACCAAAAGCCTTCAT

ΔCT=CT (gene)−CT (standard). β_2-Microglobulin was used as a housekeeping gene, and the expression level was evaluated for internal control; the primer pairs is shown in Table 1.

Statistical analysis

The relative expression of all genes was evaluated as the observed Ct values and measured using the relative expression software tool (REST, http://rest.genequantification.info) and RT2 profiler software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php) [18]. All experiments were performed at least three times, presented as mean±standard deviation (SD), and analyzed by Student's *t* test. The *P* values of <0.05 or less were considered statistically significant.

Results

hA-MSC characteristic evaluation

After 21 days of culturing hA-MSCs in adipogenic and osteogenic media, hA-MSCs were stained with Oil Red and Alizarin Red kites for osteogenic and adipogenic potency evaluation (Fig. 1c, d). Flow cytometry system was applied for immunophenotyping of hA-MSCs immunostained with antibodies against the indicated antigens CD105, CD90, CD45, and CD34. Flow cytometry analysis of the cell surface markers showed that hA-MSCs were positive for CD105 and CD90 and negative for CD34 and CD45 (Fig. 1e–h).

Gene expression analysis

To analyze the effects of *H. pylori* on the gene expression pattern of hA-MSCs, the carcinogenesis-related genes were investigated by real-time PCR technique. The regulation of the tumor suppressor gene p53 expression was changed in all *H. pylori* involved groups and upregulated significantly in hA-MSCs+AGS+ *H. pylori* ternary culture groups when compared with hA-MSCs+AGS binary group and AGS

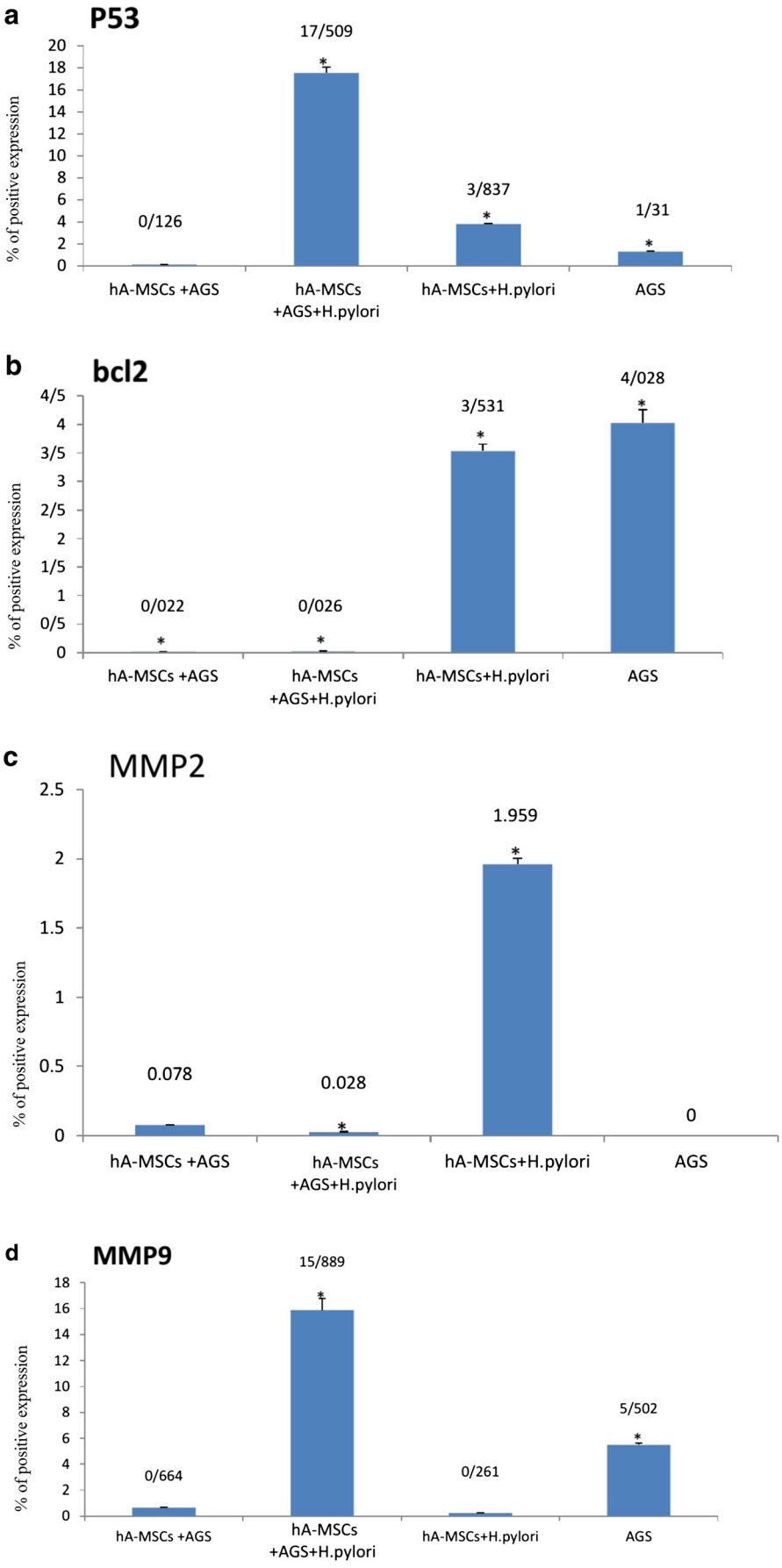


Fig. 2 Gene expression analysis. The gene expressions were investigated by real-time PCR method. The bcl2, p53, MMP-2, and MMP-9 relative expression (**a**–**d**)

control groups (Fig. 2a). In addition, *H. pylori* significantly upregulated the expression of bcl-2 gene in hA-MSCs+ *H. pylori* binary group when compared with other groups specially hA-MSCs+AGS+ *H. pylori* ternary and hA-MSCs+AGS binary groups (Fig. 2b).

Moreover, the relative expression of MMP-2 gene was significantly upregulated in hA-MSCs+*H. pylori* binary group when compared with hA-MSCs+AGS+ *H. pylori* ternary and hA-MSCs+AGS binary groups. Also, we did not see any significant expression on AGS group (Fig. 2c). The expression of MMP-9 gene was significantly upregulated in hA-MSCs+AGS+ *H. pylori* ternary group when compared with the other groups (Fig. 2d). The magnitude comparison of the co-regulation of mutagenic and metastatic gene expression subset-related transcriptions across the treated samples and the control group is shown in Fig. 3.

Hence, these results show that *H. pylori* affects expression of genes involved in tumor development in cells that attracted to the tumor environment specially, hA-MSCs.

Discussion

Regarding the previous experimental data, *H. pylori* is able to activate the migration properties of MSCs directly in in vivo and in vitro studies [1]. Therefore, it provides a special microenvironment with special situation in stomach. This study provides evidence that *H. pylori* as incorporator part of this microenvironments can change the regulation of carcinogenesis- and metastasis-relevant genes' expression on migrated cells like MSCs.

Our results demonstrated that first *H. pylori* induced the expression of apoptosis at the cells around them in gastric chronic microenvironment by changing the regulation of p53 gene. When MSCs are attracted toward the *H. pylori* chronic infection tissue, *H. pylori* induces the cellular apoptosis by enhancing the expression of p53 gene. Actually, apoptosis reaction is the first biological expected action of cells when meeting mutagen agents, and here, enhancing of p53 may be interpreted as the first reaction of hA-MSCs in front of *H. pylori* as a mutagenic infection risk factor [30, 31]. However, in the response to expression of p53 gene, overexpression of bcl2 gene occurs directly or indirectly due to *H. pylori* infection.

Therefore, this interruption on the apoptosis direction may provide more risky situations for transforming of MSCs to metaplasia and dysplasia phases because MSCs have more susceptibility than other cells to move for cell atrophy [32–34]. Also, our findings showed that two kinds of main MMP (MMP-2 and MMP-9) expression were induced by *H. pylori* in different co-culture situations. In binary groups, *H. pylori* enhanced significantly the MMP-2 expression when co-cultured with hA-MSCs; this upregulation of MMP-2 proteins can be the most significant sign of the direct effects of *H. pylori* on metastasis activities too. Most recent studies emphasize on the invasive and metastatic roles of MMPs on tumor cells [26–28, 30], but here, we have shown the enhancing of MMP-2 gene expression on other cells like hA-MSCs is important too, because they were migrated already to *H. pylori* chronic infection tissues where there may be already cancer tumors (Fig. 2c).

To investigate more on main MMPs which involved genes in metastasis, we investigated the expression of MMP-9 gene. The results showed that the MMP-9 expression was enhanced in hA-MSCs on ternary groups where it was co-cultured with AGS and *H. pylori*. Perhaps, in this group, the AGS and *H. pylori* had been cooperating together in overexpressing MMP-9 gene in hA-MSCs. In fact, here, it shows the synergistic effects of *H. pylori*, *AGS* on hA-MSCs which consequently will affect the tumor cells' metastasis. Thus, upregulating MMP (MMP-2 and MMP-9) expression level on stem cells by *H. pylori* can increase the rate of degradation of type IV collagen as a major component of the extracellular matrix (ECM) [28] and definitely increase the damage and

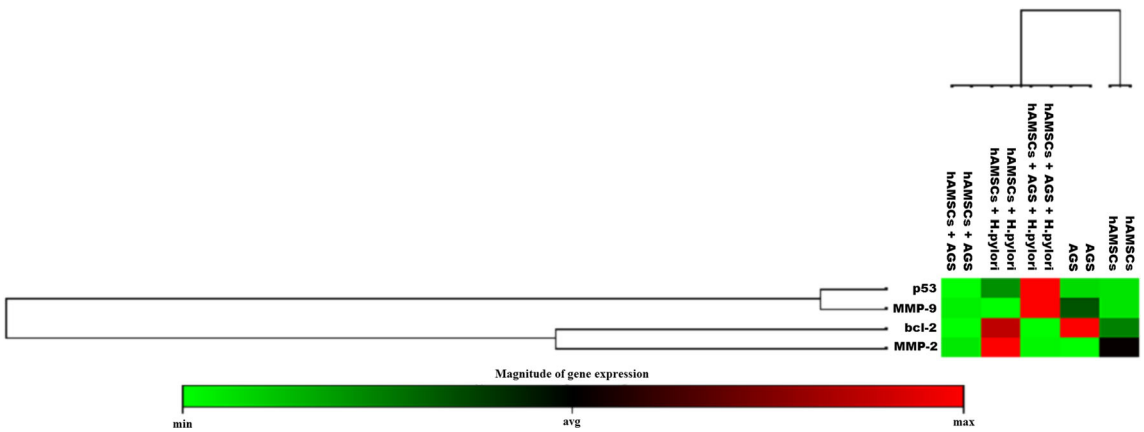


Fig. 3 Co-regulation of mutagenic and metastatic gene expression. Comparison of the co-regulation of mutagenic and metastatic gene expression subset-related transcriptions across the treated samples and the control group (qRT-PCR assays)

devastation of the BM, which surrounds the cancer nests and vascular structures, and consequently increase the invasion and metastasis of tumor cells too [25, 26].

Perhaps, when hA-MSCs are attracted towards the chronic *H. pylori*-infected tissue and gastric ulcer, for their innate duty for tissues and ulcers healing activities, they will be trapped under special gastric microenvironment that may include *H. pylori* infection and tumor cells. Previous reports have shown that contribution of *H. pylori* and other cell groups like immune cells in this microenvironment not only attracts hA-MSCs toward these sites but also may lead them to providing transformation of metaplasia, dysplasia, and cancer cells because there may be setting of conflicting different signals [22]. Therefore, if in these microenvironments we have already tumor cells, it gives more opportunity to *H. pylori* for increase of metastasis of tumor cells by inducing and upregulating MMP expression on hA-MSCs which are attracted to these microenvironments already.

Conclusion

Stem cell therapies are increasing every day around the world, and researchers try to improve stem cell usage methods in wide range of medical stem cell therapies such as tissue engineering and cancer therapies with different protocols as the same as direct injections of stem cells on patients body. Therefore, considering applying stem cells for different patients with different situations is important, and also, attention to abusing of stem cells by other microorganisms can help us to find more solutions for better results of stem cell therapies. Additionally, in gastric cancer therapies, these results are helpful for attention to the overexpression of MMPs in other cells like hA-MSCs which surrounded the gastric tumor cells by synergic effects of microorganisms like *H. pylori*.

Bottom of form

The findings we demonstrated here may have implications for clinical stem cell therapies and cancer-metastasis treatments in the future. It is better before starting any stem cell therapies or cancer and metastasis treatments we first look for the *H. pylori* or *H. pylori* chronic infections and treat it completely, because it plays a key role to achieve the best successful results in these treatment methods for patients. Actually, understanding of *H. pylori's* effective roles could be a powerful strategy not only in identifying the origin and initiation of gastric cancers but also in the treatment of related diseases such as gastric ulcers and cancerous process like metastasis. Further experiments are required to elucidate the related mechanisms and potential applications of this study.

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Compliance with ethical standards

Conflicts of interest None

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