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Changes in *SNAI1* and *VIM* gene expression in Caco2 cells after co-cultivation with bacteria from colorectal cancer biopsies

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The development of distant metastases is the final stage in the solid cancer progression and it is responsible for the majority of cancer-related deaths. Epithelial-mesenchymal transition (EMT) is involved in cancer progression and metastasis. In the present study, we used different types of intracellular bacteria isolated from colorectal cancer biopsies to examine their effect on the expression of *SNAI1* and *VIM* genes in Caco2 cell line. SNAI1 gene expression was significantly decreased after co-cultivation of Caco2 cells with *Pseudomonas aeruginosa*, *Proteus vulgaris*, *Proteus mirabilis*, *Enterococcus faecalis*, *Klebsiella pneumoniae* and *Bacillus cereus*, respectively ($p<0.05$). We observed more than 2-fold increase in *VIM* gene expression within Caco2 cells after co-cultivation with *Proteus vulgaris*. On the other hand, *VIM* gene expression decreased by half after co-cultivation with *Pseudomonas aeruginosa* and *Proteus mirabilis* ($p<0.05$). Our data suggest that bacteria presented in colorectal carcinoma tissues may cause changes in gene expression of EMT-associated genes. Further research is needed to find out whether bacteria are capable to support EMT and cancer progression.

Key words: bacteria, metastasis, colorectal cancer, SNAI1, VIM, vimentin, epithelial-mesenchymal transition (EMT)

Colorectal cancer (CRC) is a very common and lethal disease, which is influenced by both environmental and genetic factors. An overwhelming body of evidence has determined the relationship between certain bacteria and particular cancers [1]. The invasion and metastasis of colon cancer cells weakens the effect of the treatment, leading to a high mortality rate. Metastasis is closely related to the biological transformation process where cells gradually lose an epithelial phenotype and obtain a mesenchymal phenotype [2]. This is known as epithelial-mesenchymal transition (EMT). The EMT is a response by the whole organism to generate multiple layers and complex organs during normal development, and it is also a response to pathological conditions such as tumorigenesis, hypoxia, or chronic inflammation [3, 4]. It is a fundamental process of development and disease progression [5]. The transition from epithelial to mesenchymal states allows a cell to break away from its originating tissue and travel throughout the body. EMT is induced during activation of numerous signaling mechanisms and is regulated by several pathways. Some of the EMT-signaling pathways are upregulated by microbial pathogens, which suggests that pathogens may also be EMT inducers [6].

One of the most distinguishing features of the EMT phenotype establishment is the upregulated expression of mesenchymal markers [7]. One of these mesenchymal markers is Vimentin gene (*VIM*), which encodes highly conserved protein (53 kDa) that belongs to the type III intermediate filament family [8]. Vimentin is a multifunctional protein, responsible for maintaining cell shape, cytoplasm integrity, stabilizing cytoskeletal interactions, and it is also involved in the immune response [9]. Several Gram-negative pathogens such as the Enterobacteriaceae have been found to interact with vimentin both inside and on the eukaryotic cell surface [10, 11]. Hofman and Vouret-Craviari [6] speculate that most microbes that persist in the body have the potential to indirectly favor an EMT behavior.

VIM expression is altered by EMT-initiating transcription factors – *SNAI1* (Snail1) and *SNAI2* (Snail2). These transcriptions factors belong to a large superfamily known as SNAI and participate in cell differentiation and survival [12]. In the members of this family, the C-terminal domain is highly conserved in contrast to the divergent N-terminal region [13]. The C-terminal DNA-binding domain region of Snail1 and Snail2 differs in the zinc finger number: four (ZF1 to ZF4) in Snail1 and five (ZF 1 to ZF5) in Snail2. This difference might account for the differential interactions and/or binding affinities to target genes [14]. Snail2 expression is increased in patients with melanoma, lung, colon and

ovarian cancer [15]. Snail1 and Snail2 are linked to tumor progression and invasiveness by their ability to alter *VIM* expression [16, 17].

In the present study, we mainly focus on altered expression of two genes associated with EMT in human adenocarcinoma cell line Caco2 after co-cultivation with bacteria isolated from colorectal biopsies [18]. We used different types of intracellular bacteria to examine their effect on the *SNAI1* and *VIM* expression, and evaluated the possible role of these bacteria on cancer progression and metastasis.

Materials and methods

Cell culture. The human adenocarcinoma cell line Caco2 was maintained and cultured in high-glucose (4.5 mg/ml) DMEM (Biochrom AG, Berlin, Germany) supplemented with 10% fetal bovine serum (FS) (Biochrom AG), an antibiotic/antimycotic mix (10 000 IU/ml penicillin; 5 µg/ml streptomycin; 2.5 µg/ml amphotericin) and 2 mM glutamine. The cells were maintained in a humidified atmosphere at 37 °C and 5% CO_2. All the chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA) if not stated otherwise.

Bacteria isolates. Bacteria obtained in our previous study were used for co-cultivation with Caco2 cell lines. Intestinal bacteria of CRC patients were isolated from colorectal biopsies using gentamycin protection assay [18] and maintained and cultured in LB medium. In this experiment, we used *Klebsiella pneumoniae, Proteus vulgaris, Proteus mirabilis, Pseudomonas aeruginosa, Enterococcus faecalis* and *Bacillus cereus*.

Co-cultivation. CaCo2 cells were seeded at a density of 5×10^6 cell/Petri dish and incubated at 37 °C until a confluent monolayer formed, followed by PBS washing. Each monolayer was infected with approximately 5×10^7 bacteria in a culture medium without antibiotics. After 3 hours of incubation at 37 °C, the monolayer was washed with PBS. To eliminate all extracellular bacteria, the CaCo2 cells were treated with DMEM which contained gentamicin (100 µl/ml). After 1-hour incubation, the cells were washed 2 times with PBS and cultured for 48 hours at 37 °C in 5% CO_2.

RNA isolation and cDNA synthesis. Total RNA was extracted from 5×10^5 cultured cells using the NucleoSpin RNA II kit (Macherey-Nagel, Dueren, Germany). RNA was depleted from genomic DNA by DNase treatment (RapidOut DNA Removal Kit; Thermo Fisher Scientific, Waltham, MA, USA). The light absorbance at 260 and 280 nm was measured by NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific). The RNA samples with optimum A260/A280 ratio were selected to synthesize complementary DNA. 1 µg of total RNA was reverse transcribed using SensiFAST cDNA Synthesis Kit (Bioline, London, UK). Reaction volume was 20 µl including: total RNA (1 µg), 5× TransAmp Buffer (4 µl), Reverse Transcriptase (1 µl) and DNase/RNase free-water (up to 20 µl). Then the samples were incubated for 10 min at 25 °C, 15 min at 42 °C and 5 min at 85 °C.

Primers. Snail1, Snail2 and Vimentin genes were selected as a target and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was chosen as a reference gene. Oligonucleotide sequences are listed in Table 1.

Quantitative Real-Time PCR analysis. Qualitative RT-PCR was performed in triplicates in 1×GoTaq® qPCR Master Mix, 0.25 pmol/µl concentration of primers and 0.5 µl template cDNA per one reaction. The protocol for RT-qPCR started with the activation step at 95 °C for 2 min, followed by 40 cycles which consisted of three steps: 15 sec at 95 °C for denaturation, 1 min at 60 °C for primer annealing and 5 sec at 75 °C for extension. PCR was performed in Bio-Rad 96FX cycler (Bio-Rad Laboratories, Hercules, CA, USA) and the results were analyzed by Bio-Rad CFX Manager software version 1.6. The gene for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a reference gene. The sequences of primers for selected genes are listed in Table 1.

The amplification efficiency for the target and reference genes was validated using a 2-fold dilution series of the cDNA template. A standard curve was drawn by plotting the logarithmic input cDNA concentration versus mean Threshold Cycle (CT) and the slope was determined. PCR efficiency was calculated using the formula: $E = 10^{-1/\text{slope}} - 1$. The relative quantification (RQ) of the gene expression was calculated using formula: $RQ=2^{-\Delta\Delta Ct}$ and $\Delta\Delta Cq = \Delta Cq\ (TAR) - \Delta Cq\ (REF)$.

Statistics. Relative Expression Software Tool (REST, version 2009) was used for statistical evaluation.

Table 1. List of primers.

Oligo name	Sequence (5′→3′)	Length	Amplicon (bp)
GAPDH-F	GAAGGTGAAGGTCGGAGTC	19	226
GAPDH-R	GAAGATGGTGATGGGATTTC	20	
Snail1-F	CAACTGCAAATACTGCAACAAGG	23	245
Snail1-R	ACTTCTTGACATCTGAGTGGGTC	23	
Snail2-F	CTTTTTCTTGCCCTCACTGC	20	224
Snail2-R	GCTTCGGAGTGAAGAAATGC	20	
Vimentin-F	CAGGGCGCGTCCTCTGCC	18	228
Vimentin-R	TGGACATGGCTGCGGAGGGT	20	

Results

In our study, we used relative quantification to analyze the changes in gene expression in Caco2 cells affected with bacteria relative to an unaffected control. We used six different types of bacteria isolated from colorectal biopsies for co-cultivation: *P. aeruginosa, P. vulgaris, P. mirabilis, E. faecalis, K. pneumoniae* and *B. cereus*. Relative quantification describes the change in target gene expression relative to reference group such as an untreated control.

The slope of the standard curves for the *VIM, SNAI1* and *GAPDH* genes was –3.4, –3.27 and –3.28, respectively (Figure

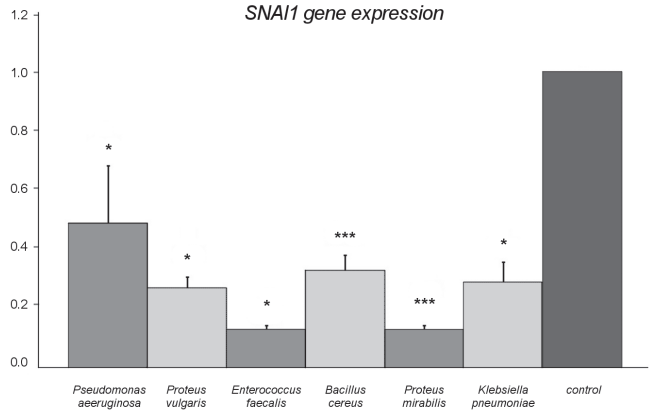

Figure 1. *SNAI1* expression in Caco2 cells affected with different types of bacteria isolated from colorectal biopsies. ***p<0.001, *p<0.05; p-values in comparison to control.

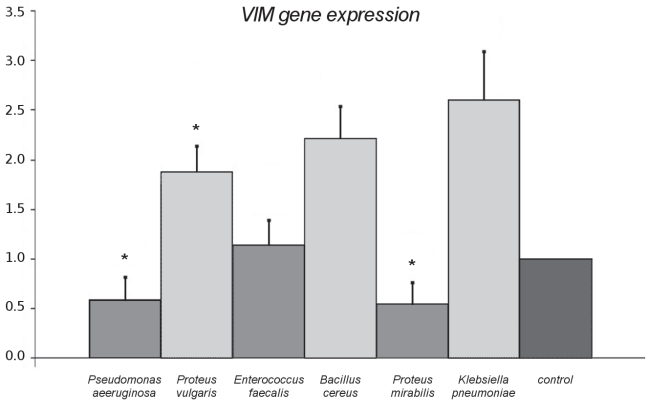

Figure 2. *VIM* expression in Caco2 cells affected with different types of bacteria isolated from colorectal biopsies. *p<0.05; p-values in comparison to control.

S1). The PCR efficiency (E) was 96.9% for *VIM*, 102.3% for *SNAI1* and 101.6% for *GAPDH*.

Quantitative analysis showed downregulation of *SNAI1* expression in all affected cells (Figure 1). *SNAI1* gene expression was significantly decreased ($p<0.05$). Notably, gene expression decreased significantly in Caco2 cells affected with *E. faecalis* and *P. mirabilis, SNAI1*, approximately 10-fold ($p<0.001$). Data analysis showed reduced *SNAI1* gene expression by more than a half in Caco2 cells after co-cultivation with *P. aeruginosa, P. vulgaris, K. pneumoniae* and *B. cereus*.

Vimentin has higher gene expression pattern in affected Caco2 cells than *SNAI1* (Figure 2). Quantitative results have shown that *VIM* gene expression is increased more than 2-fold after co-cultivation with *P. vulgaris, K. pneumoniae* and *B. cereus,* but this increase was significant only for *P. vulgaris* ($p=0.05$). *VIM* gene expression decreased by half in Caco2 cell affected with *P. aeruginosa* and *P. mirabilis* ($p<0.05$).

Discussion

Gut microbiota has been proved to be involved in the initiation and progression of CRC in many ways [19]. The structural difference observed in the fecal microbiota community of CRC patients was related with the non-cancerous population, and some microbiota belonging to the genera of *Enterococcus, Escherichia/Shigella, Klebsiella*, *Streptococcus*, and *Peptostreptococcus* were richer in CRC patients [20]. Bacterial adhesion and invasion might perpetuate the inflammatory response, eg. *Streptococcus bovis* wall-extracted antigens promote carcinogenesis in CaCo2 cells by over-expression of cyclo-oxygenase 2 [21]. Enterotoxigenic *Bacteroides fragilis* has induced tumor growth in mice by upregulation of the inflammatory chemokines, interleukin 17 and interleukin 23 [22].

The role of bacteria in cancer progression via EMT signaling pathway has not been studied sufficiently so far. In our experiment, we have selected two EMT-associated genes (*SNAI1 and VIM*) and monitored changes in their expression induced by short co-cultivation of Caco2 cells with certain bacteria. CaCo2 cell line is derived from large intestine carcinoma (stage X, grade II) with decreased *VIM* expression [23]. The results show significantly reduced expression of the *SNAI1* gene in Caco2 cells after co-cultivation with all types of bacteria which we used. Similar results were obtained by monitoring the *SNAI2* gene expression (not published), but these results were not significant because the level of expression was very low. This can be a reaction of cells to the bacterial attack, since inhibition of Snail1 expression downregulation led to inhibition of the bacterial translocation across the epithelium [24].

Many studies and investigators reported that in majority of the cancers *VIM* gene expression is varied and revealed an association between their expression and cancer aggressiveness [25–28]. It seems that vimentin expression is not only a marker associated with invasive phenotype but required for carcinoma cell motility. Notably, 70% inhibition of vimentin expression was sufficient to impair migration and invasion [25].

One possible mechanism for bacteria to influence the carcinogenesis progression could be their involvement in the regulation of vimentin expression. We have detected both increased and decreased expression according to particular bacteria used for co-cultivation. *P. mirabilis* caused decreased expression of both *VIM* and *SNAI1*. This observation may indicate its protective effect in the tumorigenesis process, which has been suggested in several studies. Already in 1965, Toyoichiro et al. [29] reported that *P. mirabilis* RMS-203 (Murata strain) possessed oncolytic effects in murine tumors. Zhang et al. [30] in their study evaluated the suppressive effect of *P. mirabilis* on breast cancer growth and metastasis *in vitro* and *in vivo*. Results showed that *P. mirabilis* could adhere to cells and inhibit cell growth in a non-specific way *in vitro*, preferentially accumulate in tumor tissues and can markedly

suppress both tumor growth and pulmonary metastasis in a mouse breast cancer model. *P. aeruginosa* caused decreased expression of both *VIM* and *SNAI1*. Borthwick et al. [31] reported no effect on EMT marker expression compared to control in co-cultured cells with *P. aeruginosa*-activated THP-1 cells. *P. aeruginosa* did not drive EMT directly but could accentuate TGF-β1 driven EMT indirectly via activation of innate immune cells [31].

In our observations, *K. pneumoniae* upregulated expression of vimentin more than twice, but this increase was not significant. Leone et al. [32] described changes in production of the intracellular reactive oxygen species in cells exposed to *K. pneumoniae*. The ROS (reactive oxygen species) production is connected to *VIM* expression, i.e. *VIM* expression is upregulated by ROS [33]. This can be a possible mechanism by which *K. pneumoniae* upregulate *VIM* expression. Similarly, another intracellular bacterium leading to pulmonary infections *M. tuberculosis* induced upregulation of the vimentin expression in human monocytes [34]. Vimentin may be a key regulator of several tumorigenic pathways, as it forms a complex with 14-3-3 that may prevent dephosphorylation of proteins in the complex, thereby inhibiting antitumor activity within the cells. Vimentin has been shown to be required for maturation of invadopodia and to mediate cellular migration and formation of lamellipodia [35].

As we can see from our results, as well as from literature, the ability of some bacterial pathogens to activate EMT is a consequence of the local chronic inflammatory response persistence or depends on a direct interaction of the pathogens with the host epithelial cells. Interestingly, only a short co-cultivation was sufficient to invade cells and influence the level of gene expression. Further research is needed to better define the signaling pathways activated during the early phases of interactions between the bacterium and the host cells. Herein, we have shown that most pathogenic bacteria associated with CRC increased the *VIM* expression. Vimentin might be a relevant target for cancer therapeutic intervention and has immense potential in terms of novel clinical prognostic and diagnostic tools.

Supplementary information is available in the online version of the paper.

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