Research Article

Lactobacilli Differentially Modulate mTOR and Wnt/β-Catenin Pathways in Different Cancer Cell Lines

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Abstract

Background: *Lactobacilli* are a group of beneficial bacteria whose anti cancer effects have been evaluated in different cancer cell lines as well as animal models and human subjects. Such anti cancer effects can be exerted via different mechanisms such as modulation of immune response as well as inhibition of pathogens colonization. In addition, *lactobacilli* have direct cytotoxic effects against cancer cells which may be exerted through modulation of expression cancer related pathways.

Objectives: The aim of this study is to find the mechanism of anti cancer effects of two *lactobacilli* strains, *Lactobacillus. crispatus* (LC) and *Lactobacillus. rhamnosus* (LR).

Materials and Methods: We analyzed expression of some mTOR and Wnt/β-catenin pathways genes in three cancer cell lines (HeLa, MDA-MB-231 and HT-29) following treatment with LC and LR culture supernatants.

Results: Of note, the expression of *CCND1* as a marker of cell proliferation, survival, and angiogenesis, has been decreased following LR treatment in all cell lines. In addition, the expression of *SFRP2*, an antagonist of Wnt pathway, has been increased in HT-29 following LR treatment and in HeLa cells following LR and LC treatments. Furthermore, we have demonstrated the downregulation of *S6K1* expression, a marker of poor prognosis, following LR treatment in HT-29 and following LR and LC treatments in MDA-MB-231 cell line.

Conclusions: Consequently, *lactobacilli* can modulate expression of mTOR and Wnt/β-catenin pathways genes in cancer cell lines in a strain specific as well as cell type specific manner.

Keywords: Cancer, mTOR, Wnt/β-Catenin, *Lactobacillus crispatus*, *Lactobacillus rhamnosus*

1. Background

Lactobacilli are a group of probiotic bacteria which colonize the human gastrointestinal tract as well as female urogenital tract (1). Several studies have shown tumor-suppressing properties for certain lactobacillus strains (2, 3). Such anti tumor effects have been postulated to be exerted via different mechanisms such as inhibition of pathogens colonization (4), induction of immune system (5, 6), direct cytotoxic effects on cancer cells (2, 3), antimutagenic effects (7) as well as modulation of carcinogens metabolism and prevention of DNA from oxidative damage (8).

MDA-MB-231 is an aggressive and highly metastatic cell line originated from a high grade tumor (9, 10). HeLa is a cervical cancer cell line in which integration of the human papilloma virus type 18 (HPV-18) genome is proposed as a initiator event in the tumorigenesis (11). Previously we have demonstrated that treatment with *lactobacilli* culture supernatants decreases the expression of HPV E6 oncogene so it may be of therapeutic value (12). HT-29 is a colorectal adenocarcinoma cell line in which the apoptotic effects of different *lactobacilli* strains have been evaluated (13).

The phosphoinositide 3 kinase (PI3K)/Akt/mammalian (or mechanistic) target of rapamycin (mTOR) pathway is an essential pathway leading to cell growth and tumor proliferation. This pathway is involved in resistance to endocrine therapy, HER2-directed therapy and cytotoxic therapy in breast cancer (14). In addition, this pathway has been shown to be frequently deregulated in cervical cancers (15) as well as colorectal cancers (16). Activation of mTOR complex 1 leads to phosphorylation of some factors and results in selective overexpression of cyclin D1, Bcl-2, Bcl-xL and vascular endothelial growth factor (VEGF) as well as the nucleocytoplasmic transport of selected mRNA

such as cyclin D1. Consequently, it increases cell prolifer- ation, survival, and angiogenesis. *S6K1* is a critical regula- tor of cell growth, which phosphorylates ribosomal pro- tein S6 and other important targets. Both *eIF4E* and *S6K1* are involved in cellular transformation, and their overexpres- sion has associated with poor cancer prognosis. mTOR and RICTOR are implicated in AKT phosphorylation and activa- tion and have role in AKT interaction with the apoptosis regulator BAD. mTOR complex 2 has been involved in the posphorylation of PRKCA (17) which is in turn implicated in various cellular processes such as cell adhesion, cell trans- formation and cell cycle checkpoint. PRKCA has been asso- ciated with metastatic potential of breast cancer through the activation of matrix metaloproteinases and has been regarded as poor prognostic marker as well as a therapeu- tic target in cancer patients (18, 19).

Deregulation of the canonical Wnt/β-catenin signaling pathway has been implicated in several cancers including breast, cervical and colorectal cancers (20). *SFRP2* encodes a soluble modulator of Wnt signaling. Methylation of this gene is a potential marker for the presence of colorectal cancer (21), cervical cancer (22) as well as breast cancer (23). Similar to other secreted Frizzled related proteins, *SFRP2* acts as antagonist of Wnt pathway by squelching Wnt lig- ands (24). *DKK3* codes for a Dickkopf (Dkk) protein which effectively inhibits Wnt signaling by preventing Wnt inter- action with LRPs (24). *DVL3* codes one of Disheveled (Dvl) proteins which becomes phosphorylated upon Wnt stim- ulation thereby stabilizes β-catenin (24). *TWIST* genes code for an essential factor for epithelial-mesenchymal transi- tion (EMT). The key TWIST isoform which couples aber- rant signals from EMT to senescence has been shown to be *TWIST2*. This isoform has been suggested as an impor- tant candidate biomarker for cervical cancer prognosis (25). TBLR1 has a critical role in nuclear β-catenin function. In addition, the depletion of TBL1-TBLR1 has considerably hindered Wnt-beta-catenin-induced gene expression and oncogenic growth in vitro and in vivo (26). Finally, *CCND1* codes for cyclin D1, an oncogene and an important positive regulator of the G1/S phase which is a downstream target of β-catenin (27). Figure 1 shows the position of selected pro- teins in the mTOR and Wnt pathways as well as the interac- tion of these pathways.

2. Objectives

In this study, we aimed at determination of cellular pathways involved in the cytotoxic effects of two *lacto- bacilli* strains namely*, L. rhamnosus* and *L. crispatus* against MDA-MB-231, HeLa and HT-29 cancer cell lines.

3. Materials and Methods

3.1. Selection of Genes From mTOR and Wnt/β-Catenin Path- ways

Based on previous expression studies, 5 genes were se- lected from mTOR pathway to evaluate expression of dif- ferent parts of this pathway: *RICTOR*, *S6K1*, *EIF4E*, *PRKCA* and *MTOR*. Furthermore, *SFRP2*, *TBLR1*, *DVL3*, *CCND1*, *DKK3* and *TWIST2* genes were chosen from Wnt/β-catenin pathway to evaluate their expression following treatment with *lacto- bacilli* supernatants. Selection of genes from this pathway was based on previous reports regarding epigenetic dereg- ulation in various cancer types. Fold changes in the expres- sion of these genes have been analyzed after certain treat- ments.

3.2. Cell culture

This study has been approved by the ethical commit- tee of Shahid Beheshti University of Medical Sciences. Hu- man cervical cancers (HeLa), breast cancer (MDA-MB-231), colorectal cancer (HT-29) as well as human lung fibroblst (MRC5) cell lines cell lines were purchased from the Pas- teur Institute, National Cell Bank of Iran and cultured ac- cording to previous studies (28).

3.3. Preparation of Supernatants From Lactobacillus Cultures

Microaerophilic conditions were applied for culture of *L. crispatus* strain SJ-3C-US and *L. rhamnosus* strain GG in de Man Rogosa Sharpe (MRS) broth (Merck; pH 6.5) as de- scribed in previous publications (28). To check the prob- able effect of lactobacilli-produced lactic acid on cell cul- tures, the pH of MRS controls have been adjusted by lactic acid based on the corresponding *lactobacilli* supernatant pH. In brief, the experiments comprised *L. crispatus* super- natant, pH 4.3 (LCS); *L. rhamnosus* supernatant, pH 4.05 (LRS); MRS, pH 6.5 and MRS adjusted with lactate (MRL) pH 4.05 or 4.3.

3.4. MTT Assay

Cell growth inhibition was computed by MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] assay kit (Sigma, St. Louis , MO) according to previous stud- ies (29). Overnight treatments with *lactobacilli* culture su- pernatants cells performed with different concentrations from 10% to 100% (v/v). Plates were incubated at 37°C under 5% (v/v) CO_2. Cell viability was computed using the follow- ing equation (Equation 1):

$$\text{Viability (percentage of control)}$$
$$= \frac{(A\text{bsorbance sample} - A\text{bsorbance blank})}{(A\text{bsorbance control} - A\text{bsorbance blank})} \times 100 \quad (1)$$
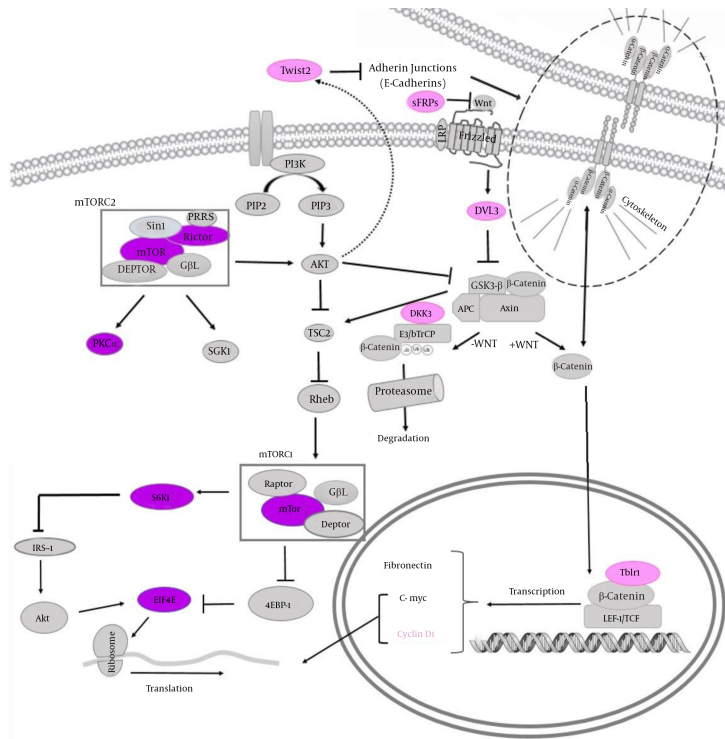
Figure 1. The Position of Selected Genes in mTOR (in Purple) and Wnt/ β-Catenin (in Pink) Pathways and Interactions of These Pathways

3.5. RNA Isolation, cDNA Synthesis and Quantitative RT-PCR (qRT-PCR)

The AccuZol™ total RNA extraction solution (Bioneer, Korea) was used to isolate total RNA from cultured cells. Nanodrop 2000 c spectrophotometer (Thermo Scientific) was used for determination of RNA concentration. Changes in mRNA expression of desired genes were analyzed by quantitative PCR (qPCR) after reverse transcription of 1 μg RNA from each sample with the PrimeScript RT reagent kit (Takara Bio, Ohtsu, Japan). mRNA quantification of genes was implemented in a rotor gene 3000 corbette detection system using SYBR Premix Ex Taq (Takara Bio, Ohtsu, Japan). Primer sequences are listed in Table 1. PCR condition was as follows: an initial denaturation at 95°C for 1 minute, and 40 cycles at 95°C for 15 seconds and 65°C for 1 minute. The final PCR reaction consisted 10 mL SYBR Green master mix, 2 mL cDNA, 0.5 mL each forward and reverse primer (10 pmol) and 7 mL nuclease-free water. Experiments were performed in duplicate for each data point. *B2ACTIN* mRNA was amplified as a normalizer, and fold changes in each target mRNA expression relative to *B2ACTIN* were calculated. Melting curve analysis was used to validate whether primers yielded a single PCR product.

3.6. Statistical Analysis

Relative expression software tool (REST©) was applied for comparison of the total expression ratio of the genes between treated and control cells using a randomization test.

Mann-Whitney test was used for comparison of IC_{50} (concentration giving half-maximal inhibition) of cells treated with *lactobacilli* culture supernatants and pH- and lactate adjusted as well as pretreated controls in SPSS software (version 16.0). All data were expressed as a mean $\pm$ SE of three separate experiments. P < 0.05 was considered as statistically significant.

4. Results

4.1. The Effects of LCS and LRS on HeLa, MDA-MB-231 and HT-29 Cell Proliferation

LCS and LRS had no toxicity against MRC5 cells as we reported previously (30). The IC_{50} values of LRS against HeLa and MDA-MB-231 cells were 9 and 10% (v/v) and those of LCS were 11 and 13% (v/v) respectively. The cytotoxic effects of LCS and LRS against HeLa and MDA-MB-231 cells were higher than those of MRS and MRL (MRS with pH adjusted to that

Table 1. Sequence of Primers Used in This Study

Primer		Sequence	Product Size, bp
B2ACTIN			105
	Forward	AGATGAGTATGCCTGCCGTG	
	Reverse	GCGGCATCTTCAAACCTCCA	
SFRP2			162
	Forward	ACCGAGGAAGCTCCAAAGGT	
	Reverse	GCTCTTGGTCTCCAGGATGATT	
TBLR1			141
	Forward	GGGAGGAGAATGGAGCACAT	
	Reverse	CAGGGTTCCAGGCACAGATA	
DVL3			130
	Forward	TGGACGACGATTTCGGAGTG	
	Reverse	TTATCAGCACAGAAGGGGGC	
CCND1			180
	Forward	GAGGCGGAGGAGAACAAACA	
	Reverse	GAGGCGGTAGTAGGACAGGA	
DKK3			112
	Forward	CCTGGCAAACTTACCTCCC	
	Reverse	AGTCTGGTTGTTGGTTATCTTGT	
TWIST2			142
	Forward	GTGACATCGGACAGAAGA	
	Reverse	CAAACATAAGACCCAGAAGAAA	
RICTOR			195
	Forward	ACAACAGAGCAACGAGGTA	
	Reverse	TCTGGATTCTGAAGTGCTAGTT	
S6K1			167
	Forward	TGCTTAATCACCAAGGTCAT	
	Reverse	TCCCAAACTCCACCAATC	
EIF4E			159
	Forward	CCAGGGCCAAACGGACATA	
	Reverse	GGGATTAGGAGTAGGGGTGGT	
PRKCA			131
	Forward	TGCAAAGGACTGATGACCAAAC	
	Reverse	GGCTGGATCTCCCTGTTCTC	
MTOR			168
	Forward	TGGGGACTGCTTTGAGGTTG	
	Reverse	ACACTGTCCTTGTGCTCTCG	

of LCS and LRS) (P < 0.05) (Figures 2A and 2B). LRS but not LCS has cytotoxic effects against HT-29 cell (Figure 2C). The IC$_{50}$ of LRS against HT-29 cells was 14% (v/v). These results

showed that the main cause of cancer cell death was not the acidity. This cytotoxicity against cancer cells can be attributed to a substance other than lactate in the supernatant of the lactobacilli. In addition, cytotoxicity effect of LRS was significantly higher than LCS in three cancer cell lines examined (P < 0.01).
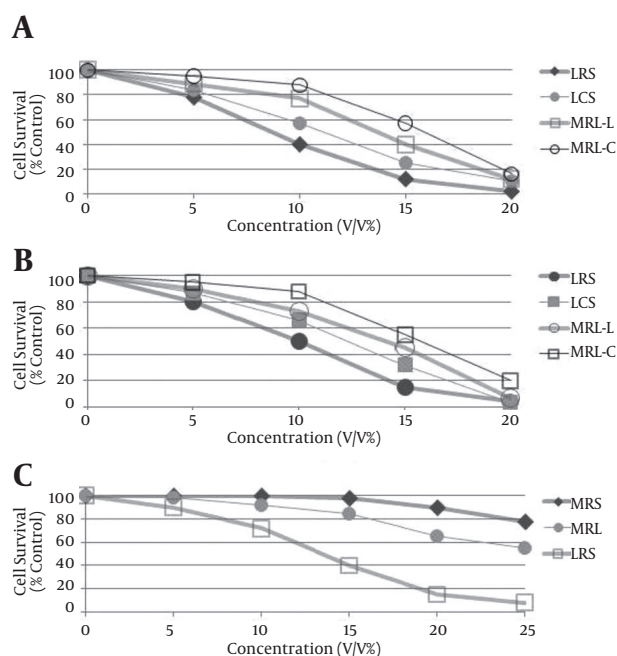

Figure 2. Cell Growth Inhibitory Effects of Different Concentrations of *Lactobacillus crispatus* Culture Supernatant (LCS), *Lactobacillus rhamnosus* Culture Supernatant (LRS), MRS and MRL on 2A, HeLa; 2B, MDA-MB-231; 2C, HT-29 Cells Respectively

4.2. The Effects of LCS and LRS on Expression of mTOR and Wnt/β-Catenin Pathways Genes

mRNA levels of mentioned genes in cancer cell lines were measured by qRT-PCR before and after treatment with LCS and LRS. After 4 hours treatment of cancer cells with certain percentages (v/v) of culture supernatants (based on the observed IC$_{50}$ for each LS against a cell line), the effects of LRS and MRS on genes expression was compared with MRS and MRL. All genes have been expressed in three cell line before treatment except for *SFRP2* whose expression has not been detected in HeLa cells before treatment but has been significantly upregulated following both LRS and LCS treatments. Figure 3 shows the effects of LCS and LRS on the expression of mTOR and Wnt/β-catenin pathways genes in three cell lines respectively.

Figure 3. The Effects of *Lactobacillus crispatus* Culture Supernatant (LCS), *Lactobacillus rhamnosus* Culture Supernatant (LRS), MRS and MRL on Expression of Selected Genes From mTOR and Wnt/ β-Catenin Pathways in HeLa, MDA-MB-231 and HT-29 Cells Respectively

5. Discussion

In this experiment, we have demonstrated downregulation of some mTOR related genes following LS treatment in MDA-MB-231, HeLa and HT-29 cell lines. Of note, the effects of LS on expression of genes have been strain specific as well as cell line specific. For instance, expression of *EIF4E* has been decreased in MDA-MB-231 cells following LRS treatment by the factors 87. However, LCS has resulted in a 100 fold reduction in *S6K1* expression in the same cell line. mTOR inhibitors have shown anti-tumor activity against various human cancers. Combinations of mTOR inhibitors with other treatment strategies such as cytotoxic chemotherapy as well as a variety of targeted molecular agents have shown promising results in many patients (15). Among mTOR inhibitors which are currently in clinical use are rapamycin and its analogs. These drugs have been shown to bind to a domain rather than the catalytic site and inhibit various mTOR functions. A potential drawback of these drugs is that they may activate an mTOR-dependent survival pathway resulting in treatment failure. However, small molecules that compete with ATP in the catalytic site have been shown to inhibit all of the kinase-dependent functions of mTOR without activating the survival pathway (31). Here we have shown that supernatants from two *lactobacilli* cultures significantly downregulates expression of some genes in mTOR pathway and can be regarded as a mechanism by which these *lactobacilli* exert their cytotoxic effects against cancer cells. As we have shown the cytotoxic effects of these *lactobacilli* on cancer cells, the possibility of activating the survival pathway by these *lactobacilli* is probably ruled out. In addition, considering the role of mTOR pathway in resistance to target spe-

cific therapies in breast cancer, downregulation of some mTOR genes in triple negative MDA-MB-231 cells by *lactobacilli* may be of therapeutic value.

In order to translate the result of these kinds of studies into the clinical use, it is necessary to find the fraction of culture supernatant which is responsible for such effect. However, it is possible that different fractions have synergic effects. Future studies should focus on identification of *lactobacilli* fractions which confers cytotoxic effects against cancer cells as well as those modulate cancer-related pathways. Furthermore, in this study we just evaluated expression of these targets at mRNA level. As phosphorylation status of different proteins in mTOR pathway is important in regulation of this pathway, future studies should investigate the effect of lactobacilli-derived products on phosphorylation of these proteins.

In addition, we have demonstrated modulation of different parts of Wnt/ β-catenin pathway following *lactobacilli* treatment in different cell lines. Of note, *SFRP2* expression has not been detected in HeLa cells before *lactobacilli* treatment, but considerably has been upregulated following treatment. As revealed by a former study, restoration of the expression of *SFRP2* has resulted in decreased Wnt signaling in CaSki cervical cancer cells, decreased abnormal accumulation of free β-catenin in the nucleus, and inhibited cancer cell growth. In addition, *SFRP2* inhibited the expression of three transcription factors involved in the EMT program including *TWIST* (32). However, in our experiment, upregulation of *SFRP2* in HeLa cells has not been accompanied by downregulation of *TWIST2* expression. In addition, treatment of HT-29 cells with LRS has increased the expression of *SFRP2* while de-

creased the expression of *CCND1*. *CCND1* has been regarded as an unfavorable prognostic factor for colorectal cancer (33), so its downregulation following LS treatment may be of clinical value. As *SFRP* genes are regarded as targets of cancer specific hypermethylation in the colon (34, 35), upregulation of *SFRP2* expression in HT-29 cells following LRS treatment implies a role for *lactobacilli* in epigenetic regulation of gene expression, which should be evaluated in future studies.

Additionally, we have demonstrated downregulation of TBLR1, a prognostic marker in cervical cancer with a critical role in the invasion and metastasis (36) in HeLa cells following LRS treatment. However, as such effect has been seen following MRS treatment as well, it is not considered as significant. In addition, *CCND1* has been downregulated in HeLa cells after LS treatment. As *CCND1* is regarded as a marker of poor prognosis in early stage cervical cancer (37), its downregulation by LS may have a clinical significance.

A previous study has shown that the transformation of HPV expressing human keratinocytes needs activation of the Wnt pathway (38). Furthermore, E6 and E7 have been shown to be involved in β-catenin nuclear accumulation and activation of Wnt signaling in HPV-induced cancers (39). Downregulation of Wnt-β catenin pathway in cervical cancer following *lactobacilli* treatment in addition to our previous data regarding down regulation of HPV E6 oncogene by *lactobacilli* in these cells (3) implies that certain *lactobacilli* strains can defeat cervical cancer by various means.

In addition, we have demonstrated that treatment with LRS can result in downregulation of *CCND1* and *DVL3* expressions in MDA-MB-231 cells by the factors 22 and 68 respectively. Downregulation of these Wnt agonists by *lactobacilli* provide a possible explanation for beneficial effects of *lactobacilli* in the treatment of breast cancer patients. However, *DKK3* is regarded as a putative Wnt signaling inhibitor (40) whose expression has not been significantly changed following LS treatments.

5.1. Conclusions

Lactobacilli can modulate expression of mTOR and Wnt/ β-catenin pathways genes in cancer cell lines in a strain specific as well as cell type specific manner. Considering the role of *lactobacilli* in cancer prevention and treatment, understanding how the lactobacilli-derived products inhibit cancer-related signaling pathways may shed new insights on design and development of novel anti cancer strategies.

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