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Role of L1CAM in the Regulation of the Canonical Wnt Pathway and Class I MAGE Genes

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Molecule L1CAM is specific for nerve cells and tumors of various localizations. The expression of L1CAM is significantly higher in melanoma in comparison with benign nevi and correlates with the progress of melanoma and transition from radial to vertical growth. Monoclonal antibodies to L1CAM effectively and specifically attenuate melanoma growth, though stimulates the epithelial–mesenchymal transition. shRNA-mediated knock-down of L1CAM showed the involvement of L1CAM in regulation of activity of the canonical Wnt pathway and expression of genes of class I melanoma-associated antigens (MAGE).

Key Words: *shRNA; L1CAM; melanoma; MAGE; Wnt*

Cell adhesion molecule L1 (L1CAM) is a transmembrane glycoprotein of the immunoglobulin family composed of a highly conservative cytoplasmic fragment, a transmembrane region, and an extracellular component consisting of 6 immunoglobulin domains and 5 fibronectin type III repeats. The L1CAM is the first discovered member of L1 family of nerve cell adhesion molecules. In addition to L1CAM, this family includes proteins CHL1, NrCAM, and neurofascin [6].

It was previously assumed that L1CAM is specific for nerve cells, but further studies detected a relationship between this molecule and tumors of various locations. The expression of L1CAM is significantly higher in melanoma than in benign nevi and correlates with melanoma progress and transition from radial to vertical growth. Monoclonal antibodies to L1CAM effectively and specifically attenuate melanoma growth [5], but this treatment stimulates the epithelial–mesenchymal transition manifesting in enhanced vimentin expression and reduced E-cadherin level.

L1CAM can interact with plasma membrane molecules of the same (cis-reaction) or adjacent cells (trans-intereaction). L1CAM molecules can bind to each other (homophilic interaction) or to other molecules, including integrins and growth hormone receptors (heterophilic interaction). Three signal cascades related with L1CAM activity are known. The first of them is associated with L1CAM interactions with integrins or some growth factor receptors, which leads to activation of MAPK signal pathways related to ERK1/2 kinases. The second cascade is associated with intramembrane proteolysis of L1CAM and release of a conservative domain capable of penetrating into the nucleus and regulating the target gene expression. The third cascade maintains activation of the NF-κB pathway via interaction of L1CAM transmembrane form and integrins and ezrin [8].

Here we study the role of L1CAM in the regulation of signal cascades associated with tumor growth as exemplified by melanoma.

MATERIALS AND METHODS

MeWo cell line derived from melanoma metastasis to the lymph node served as experimental model.

L1CAM knock-down was achieved by transfection of MeWo cells with a vector pLVX (Clontech) carrying DNA encoding shRNA, siRNA precursor hairpin, and complementary part of *L1CAM* gene.

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Cloning was carried out as described previously [14]. The resultant plasmid clones were sequenced. Knockdown efficiency was evaluated by flow cytofluorometry. MeWo cell line with expression of L1 neuronal adhesion molecule protein not surpassing the basal level (3%) was used.

Cell samples were lysed in 500 µl QIAzol Lysis Reagent (Qiagen) as described previously [9]. RNA was isolated using miRNeasy Mini Kit, after which RNA concentration was measured spectrophotometrically on a NanoDrop 1000 spectrophotometer (Thermo Scientific). The quality of RNA was evaluated using Experion RNA StdSens Analysis Kit on Experion platform (Bio-Rad). Specimens with RQI (RNA Quality Indicator) ≥7 were used in further analysis.

Analysis of gene expression was carried out using GeneChip Human Transcriptome Array 2.0 (Affymertrix) according to the instruction. After pre-processing of the data of microarray studies, the expression was measured in the samples of the microarray kits. Preprocessing and evaluation of the expression by the RMA method were carried out using Affymetrix Expression Console (version 1.4.1.46).

Statistical analysis was carried out using Affymetrix Transcription Analysis Console (version 3.0.0.466). The expression logarithms were processed by ANOVA. Benjamini–Hochberg test was used for correction of *p*, with consideration for multiplicity of hypotheses verification.

RESULTS

shRNA knockdown of L1CAM reduced *L1CAM* gene expression by 9.5 times (p=0.002). This was not associated with obvious signs of epithelial–mesenchymal transition at the transcription level [11]. The expression of SNAIL and Slug transcription factors increased (*SNAI1* expression increased 3.36 times, p=0.024, and *SNAI1* expression increased 1.7 times, p=0.007). On the other hand, the expression of E-cadherin, occludin, claudine-1, placofilin-3, vimentin, and smooth-muscle actin genes did not change.

Analysis of the expression of cell adhesion molecules showed changes in the expression of some genes associated with the aggressive phenotype of tumor cells (Table 1). These changes could indicate activation of mechanisms aimed at compensation for L1CAM suppression and activities of the corresponding signal cascades. Enhanced expression of integrins α4, α7, and β3 genes is worthy of note, as these integrins are responsible for interactions with leukocyte receptors, laminin, and RGR receptors, respectively [1].

shRNA-mediated knock-down of L1CAM in MeWo cells led to significant reduction in the expression of some melanoma antigen encoding (MAGE) genes (Table 2). Class I MAGE proteins encoded by gene located in X chromosome are subdivided into groups A, B, and C. Normally, the proteins are expressed only in the male gametes and placenta and are characteristic of malignant tumors [13]. Later homologous autosomal genes have been discovered encoding class II proteins, from MAGE-D through MAGE-L, are more widely expressed and were found in normal tissues. All MAGE proteins is characterized by the presence of a MAGE homologous domain (MHD) encoded by a single exon, showing as much as 98% homology within MAGE families [15]. The function of the majority of MAGE proteins remains unknown, but correlations between their expression and tumor development, aggressive clinical course, or resistance to chemotherapeutic agents have been demonstrated [15]. Class I MAGE proteins promote p53 degradation by activating E3 ubiquitin ligase that labels proteins for subsequent destruction by proteasomes; these proteins regulate activity of a universal nuclear skeleton protein KAP1 involved in chromatin folding, gene repression, and DNA reparation, and modulates activity of proapoptotic tumor suppressor ZNF382 [15]. High level of MAGE-C2 protein leads to ubiquitation of tumor suppressor BS69, which, in turn, suppresses canonical and noncanonical activation of NF-κB [7]. In our experiment, at least 40-fold reduced expression of MAGE-C2 indicated attenuation of the inhibitory effect on tumor growth suppression.

It was found for MAGE that activity of permanent gene demethylation can be less important than activity of promoter-associated process protecting the 5'-region

TABLE 1. Differentially Expressed Genes Encoding Adhesion Molecules under Conditions of L1CAM Suppression by RNA Interference

Gene	Change, linear scale	*p* (ANOVA)
CTNND2	-2.1	0.001
CADM1	+2	0.009
ITGA7	+2.1	0.013
COL18A1	+2.1	0.031
TNC	+2.2	0.011
COL5A2	+2.6	0.004
ALCAM	+2.8	0.011
ITGB3	+2.9	0.005
ITGA4	+3	0.002
FN1	+5.8	0.002
THBS1	+7.3	0.001
CDH19	+10.4	0.008

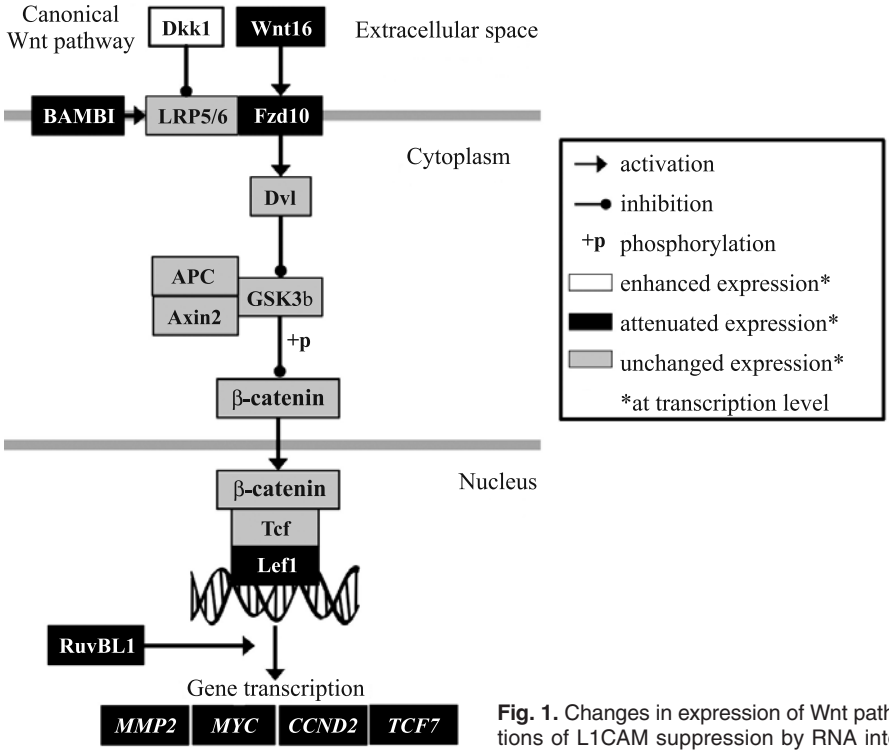

Fig. 1. Changes in expression of Wnt pathway genes under conditions of L1CAM suppression by RNA interference.

from methylation and thereby supporting MAGE activity in tumor cells. Mutations of in ETS transcription factor binding sites of MAGE-A1 promoter impaired defense from methylation, which attested to a relationship between MAGE family genes and these transcription factors [4]. In our experiment, only ETV4 of ETS transcription factor family decreased significantly (1.5 times, p=0.031). However, it is known that activities of some ETS family members directly depend on phosphorylation by MAPK cascade kinases [12]. In our case, suppression of L1CAM blocked a series of MAPK-activating interactions associated with this molecule, which suggested suppression of activities ETS family transcription factors without changing mRNA synthesis. In addition, according to published reports [4], methylation of MAGE family genes could be regulated by other transcription factors, in addition to ETS family factors.

We also analyzed expression of Wnt pathway genes under conditions of L1CAM suppression (Fig. 1). The Wnt pathway is the most important proliferation and differentiation regulation cascade. It is involved in carcinogenesis and progress of many tumors, including melanoma [10]. Activation of the canonical Wnt pathway by attachment of ligand molecules to the transmembrane receptor complexes leads to accumulation of nonphosphorylated β-catenin in the cytoplasm, which is then released into the nucleus and binds to Tcf and Lef transcription factor families in order to modify the transcription of target genes. Studies of the canonical Wnt pathway activity have detected low expression of *WNT16* gene, encoding one of the ligand molecules and of *FZD10* gene encoding Wnt pathway receptor not studied in melanoma. On the other hand, low expression of *BAMBI* Wnt pathway co-receptor gene is detected, this gene associated with melanoma with a high invasive and migration potential [2], and high expression of *DKK1* gene inhibiting the canonical pathway receptor complex, this gene suppressing the migration activity of melanoma cells [3]. These transcription changes indicate suppressed activation of the canonical Wnt pathway at the receptor level. In addition, changes in the nuclear protein genes, mediating the effects of Wnt pathway, are detected. Low expression of LEF-1 transcription factor and stimulator of transcription of Wnt pathway target gene RUVBL are detected. The picture of canonical Wnt pathway suppression is confirmed by low expression of target genes of this pathway: *MMP2*, *MYC*, *CCND2*, and *TCF7*. Analysis of Tcf factor family has

TABLE 2. Changes in MAGE Expression

Gene	Change of expression, linear scale	p (ANOVA)
MAGEC2	-39.9	<0.001
MAGEC1	-5.7	0.002
MAGEC3	-1.4	0.040

demonstrated high expression of the long noncoding RNA gene *TCF7L1-IT1*, located in the Tcf-3 transcription factor intron. This long noncoding RNA is for the first time detected in tumors of the skin.

Our results demonstrate the important role of L1CAM in regulation of the canonical Wnt pathway and in expression of class I MAGE genes. Presumably, L1CAM-associated signal cascades modify melanoma growth rate, invasive potential, and the intensity of the metastatic process.

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