CADM1/TSLC1 inhibits melanoma cell line A375 invasion through the suppression of matrix metalloproteinases

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Received October 23, 2013; Accepted May 14, 2014

DOI: 10.3892/mmr.2014.2556

Abstract. Increasing evidence has demonstrated that cell adhesion molecule 1/tumor suppressor in lung cancer 1 (CADM1/TSLC1) is crucially implicated in various biological processes, including proliferation, apoptosis, and invasion during tumorigenesis and development. However, the mechanism underlying its suppression of tumor growth and metastasis in melanoma remains elusive. The aim of the present study was to examine if CADM1/TSLC1 was able to induce growth suppression in melanoma. The plasmid pcDNA3.1-CADM1/TSLC1 was transfected into A375 cells (a human melanoma cell line). The expression of CADM1/TSLC1 in the transfected cells was determined by RT-PCR and western blotting analysis. Cell growth was measured by an 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide assay and cell apoptosis was determined by flow cytometry, while a transwell assay was utilized to measure the ability of invasion. The expression of MMP-2 and MMP-9 in the transfected cells was determined by western blotting analysis. RT-PCR and western blotting revealed that in pcDNA3.1-CADM1/TSLC1 the protein expression of CADM1/TSLC1 protein was higher than in the pcDNA3.1 and A375 cells. The expression of MMP-2 and MMP-9 was lower in the pcDNA3.1-CADM1/TSLC1 than that in the pcDNA3.1 and A375 cells. The growth of CADM1/TSLC1-transfected cells was significantly suppressed *in vitro* and the ability of invasion was also reduced**,** CADM1/TSLC1 was able to induce cell apoptosis. Furthermore, CADM1/TSLC1 was an anti-invasive gene, the overexpression of which inhibited the invasion of A375 cells. This inhibition may be due to the suppression of the MMP-2 and MMP-9 expression, which is relative to tumor metastasis and progression.

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Key words: cell adhesion molecule 1, tumor suppressor in lung cancer-1, matrix metalloproteinase-9, matrix metalloproteinase-2, proliferation, invasion, apoptosis

Introduction

Cell adhesion molecule 1/tumor suppressor in lung cancer-1 (CADM1/TSLC1) is a tumor suppressor gene that was first identified by Murakami *et al* in lung carcinoma (1). Murakami *et al* observed that loss of heterozygosity (LOH) on chromosome 11q23 occurred in patients with non-small cell lung carcinoma and found it had growth suppression effects on the tumor cells. Therefore, the gene was named tumor suppressor in lung carcinoma-1 (TSLC1).

Loss or reduction of CADM1/TSLC1 expression was frequently observed and demonstrated to be involved in the progression and metastasis of a growing number of different tumor types, including lung cancer (2), gastric cancer (3), T-cell leukemia (4), ovarian carcinoma (5), pancreatic cancer (6) and breast cancer (7). In our previous study (8), it was demonstrated that silencing of CADM1/TSLC1 in melanoma is consistent with promoter methylation, and that the incidence of the loss of expression and methylation of CADM1/TSLC1 significantly increased as the tumor stage advanced. The present study focuses on the functional role of CADM1/TSLC1 in the tumorigenesis of melanoma. To examine the possible growth-suppressive activity of CADM1/TSLC in melanoma, a stable CADM1/TSLC1-expressing cell line A375-CADM1/TSLC1 (A1) and empty vector-transfected control cell line A375-pcDNA3.1 (A0) were established by transfection with pcDNA-CADM1/TSLC1 or empty pcDNA3.1 vectors, respectively. An 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay, flow cytometry and transwell chambers were utilized to detect proliferation, invasion and cell apoptosis, respectively.

Metastasis is a complex process during which tumor cells become invasive, migrating away from the primary tumor and

passing through natural extracellular matrix (ECM)-based barriers impeding access to vascular or lymphatic vessels.

Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are two important members in the metalloproteinase superfamily which are structurally related proteolytic enzymes that facilitate the degradation of ECM and the basement membrane. It has been demonstrated that MMPs are closely correlated with tumor invasion, since their upregulation markedly facilitated cancer cell migration through the ECM (9).

To further examine the possible anti-invasive mechanism of CADM1/TSLC1, the expression of MMP-2 and MMP-9 in A375 cells with different treatment were analyzed by western blotting.

Materials and methods

Cell line and culture. The human melanoma cell line A375 was provided by Tumor Institute of Harbin Medical University (Harbin, Heilongjiang, China). The cells were maintained in the RPMI-1640 (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China), supplemented with 10% fetal bovine serum (Gibco, Eggenstein, Germany), 100 U/ml of penicillin and 100 ug/ml of streptomycin at 37˚C in a 5% CO_2 atmosphere.

Construction of plasmid expressing human CAMD1/TSLC1. Full length fragment of TSLC1 was amplified from RNA of hepatic cell L-02 by RT-PCR. The sequences of human CAMD1/TSLC1 primers were as follows: the forward primer, GACATGGCGAGTGTAGTGCT; the reverse primer, TGGGTCTGCAGGTTTCCAGT. PCR was performed using LA Taq System (TaKaRa Biotechnology (Dalian) Co., Ltd, Dalian, China) in 25 cycles of 94˚C for 50 sec, 58˚C for 30 sec and 72˚C for 90 sec. PCR products were ligated into the pcDNA3.1 vector (Invitrogen Life Technologies, Carlsbad, CA, USA) to obtain the pcDNA-CADM1/TSLC1 vector.

Establishment of stable cell lines. The A375 cells were maintained in high glucose RPMI-1640 supplemented with 10% PBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The A375 cells were transfected with pcDNA-CAMD1/TSLC1 and empty pcDNA3.1 vector, respectively, using Lipofectamine 2000 (Invitrogen Life Technologies) according to the manufacturer's instructions. The transfected cells were selected and maintained in full medium containing 600 μg/ml G418 (Invitrogen Life Technologies). The cells stably expressing CAMD1/TSLC1 were then isolated and the expression of CAMD1/TSLC1 was confirmed by PCR and western blotting.

Western blotting analysis. Western blotting was conducted according to standard methods as described previously (8). Briefly, the cells were washed with PBS, then treated with a lysis buffer and protease inhibitor mixture on ice for 25 min and centrifuged. The protein samples were separated with 12% SDS-PAGE and subsequently transferred onto PVDF membranes (Millipore, Billerica, MA, USA). The membranes were blocked with 5% skimmed milk solution in PBS for 60 min at room temperature, and incubated with rabbit anti-CADM1/TSLC1, MMP-2 and MMP-9 (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), followed by biotinylated goat anti-rabbit IgG (Sigma, St. Louis, MO, USA) each for 2 h at 37˚C. The membranes were stained with an enhanced chemiluminescence solution (PerkinElmer, Boston, MA, USA). The images were acquired using an Image Quant350 digital image system (GE Healthcare, Uppsala, Sweden).

Cell proliferation analysis. To examine the cell proliferation, the cell growth of A375, A0 and A1 cells were analyzed by an MTT assay. Briefly, $1x10^5$ cells were seeded 96-well plates and the cell growth was measured per 24 h until day 4. A volume of 30 μl of MTT (Sigma Chemical Co., St. Louis, MO, USA) solution (5 mg/ml) was then added and the cells were further incubated at 37˚C for 4 h. After 100 μl of DMSO was added, the photodensity value at 540 nm was determined by an Opsys MR microplate reader (Thermo Labsystems, Beverly, MA, USA). Measurements of cell growth by the MTT assay were expressed as a percentage of the inhibition according to the following formula: Inhibition rate (%) = [(A value of vector-alone transfectant - A value of CADM1 transfectant)/A value of vector-alone transfectant] x 100%.

Cell migration assay. The effect of CADM1/TSLC1 on tumor cell invasion was investigated *in vitro* by an cell migration assay that was performed using 96-well plate transwell chambers (BD Biosciences, Franklin Lakes, NJ, USA) according to the manufacturer's instructions. In brief, $2x10^5$ of A375, A0 and A1 cells in 500 μl serum-free medium were seeded into the upper part of each chamber of 96-well Matrigel chambers, respectively. A total of 24 h later, the cells were fixed and stained, and the number of cells on the lower surface of the filters was counted under the microscope (Olympus BX51; Olympus Corporation, Tokyo, Japan).

Cell apoptosis assay. For the apoptosis assay, $1x10^5$ A375, A0 and A1 cells were trypsinized at 48 h, washed with cold PBS, and resuspended in PBS. Then, 10 μl of Annexin V-FITC (BD Biosciences) and 5 ul of propidium iodide (PI) were added. After the cells were vortexed and incubated for 15 min at room temperature, 400 μl of binding buffer was added to the mixture. Flow cytometry was conducted on a FAC Scan instrument (BD Biosciences).

Statistical analyses. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc., Chicago, IL, USA). Summary results were described as the mean ± standard error of the mean, and analysis of variance was used for the comparison of multiple means among the three groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

CAMD1/TSLC1 expression in A375 cells with different treatment. To evaluate the biological role of CAMD1/TSLC1 in melanoma, we established stable CAMD1/TSLC1-expressing cell line A375-CAMD1/TSLC1 (A1) and empty vector-transfected control cell line A375-pcDNA3.1 (A0) by transfection with pcDNA-CAMD1/TSLC1 or empty pcDNA3.1 vectors, respectively. The results of RT-PCR and western blotting demonstrated that the level of CAMD1/TSLC1 mRNA (Fig. 1) and protein expression (Fig. 2) were significantly increased in

Table I. Analysis of cell apoptosis by Annexin V/PI staining.

Cell line	Apoptotic cell (%)	Late phase apoptosis (%)	Living cell (%)	Early phase apoptosis (%)	Number of apoptotic cells (both phases)
A1	0.59±0.01	18.11±0.3[a]	68.32±0.6	16.59±0.1[a]	37.6±0.1[a]
A0	0.23±0.05	2.27±0.1	96.00±0.3	1.53±0.3	3.98±0.4
A375	0.08±0.03	3.36±0.2	93.58±0.1	2.58±0.5	5.62±0.9

[a]P<0.01 as compared with A0 and A357 cells. A0, A375-pcDNA3.1; A1, A375-CADMI/TSLC1; CADM1/TSLC1, cell adhesion molecule 1/tumor suppressor in lung cancer-1; PI, propidium iodide.

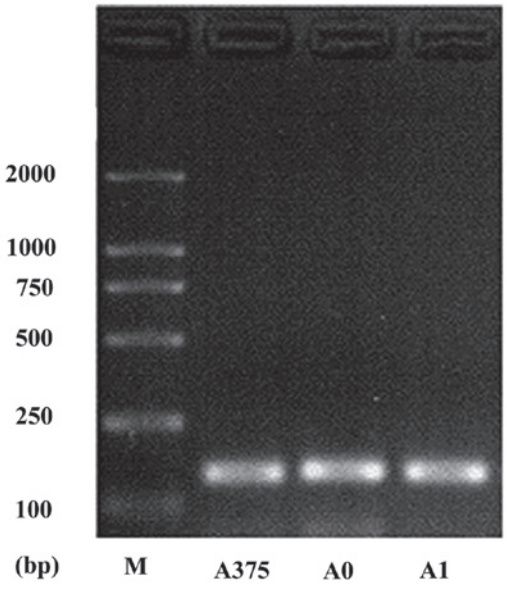

Figure 1. CADM1/TSLC1 mRNA expression. CADM1/TSLC1 mRNA expression levels were examined by RT-PCR. The expression level of the CADM1/TSLC1 gene was normalized to that of the housekeeping gene, β-actin. The relative levels of CADM1/TSLC1 mRNA in the three groups were counted using the formula $2^{-\Delta\Delta Ct}$. The values are expressed as the mean ± standard error of the mean. Compared with A375 and A0, A1 demonstrated a higher level of CADM1/TSLC1 mRNA expression (P<0.05). A0, A375-pcDNA3.1; A1, A375-CADMI/TSLC1; CADM1/TSLC1, cell adhesion molecule 1/tumor suppressor in lung cancer-1.

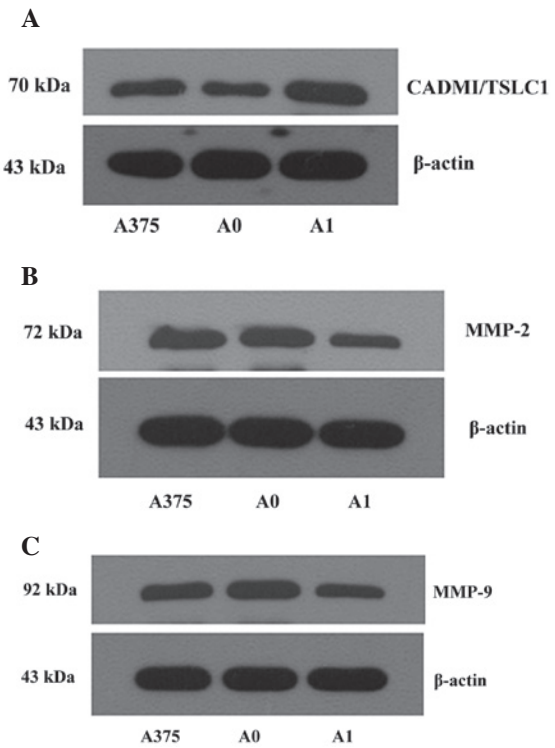

Figure 2. CADM1/TSLC1 and MMP-2, MMP-9 protein expression as determined by western blot analysis with an anti-CADM1/TSLC1 and MMP-2, MMP-9 antibody. The protein expression levels were semi-quantified by measuring the gray scale normalized to that of the housekeeping protein, β-actin. The relative protein expression levels were calculated by CADMI/TSLC1/β-actin ratios. The values are expressed as the mean ± standard error of the mean. (A) Compared with A375 and A0, A1 demonstrated a higher level of CADM1/TSLC1 protein expression (P<0.05); (B) compared with A375 and A0, A1 demonstrated a lower level of MMP-2 protein expression (P<0.05); (C) compared with A375 and A0, A1 demonstrated a lower level of MMP-9 protein expression (P<0.05). A0, A375-pcDNA3.1; A1, A375-CADMI/TSLC1; CADM1/TSLC1, cell adhesion molecule 1/tumor suppressor in lung cancer-1; MMP-2/9, matrix metalloproteinase-2/9.

A1 cells, compared with the A375 and A0 cells. However, there were no significant differences in the levels of CAMD1/TSLC1 mRNA and protein expression between the A375 cells and A0 cells (P>0.05).

Expression of CAMD1/TSLC1 inhibits cell proliferation. To study the effects of CAMD1/TSLC1 on cell growth, the cell viability of the transfected and non-transfected A375 cells was measured by an MTT assay. As demonstrated in Fig. 3, the growth of A1 clone was slower than that of A0 and A375 cells during the 96 h incubation period, but no significant differences between the A0 and A375 cells were observed (P>0.05).

Expression of CADM1/TSLC1 induces apoptosis. To determine the apoptotic cell death in A375 cells induced by CAMD1/TSLC1, A375, A0 and A1 cells were stained with Annexin V/PI. As demonstrated in Fig. 3 and Table I, the results of FACS analyses revealed that the proportion of positive cells were evidently increased in A1 cells (37.60±0.1%; P<0.01), compared with the A375 cells and A0 cells (3.98±0.4 and 5.62±0.9%, respectively). Furthermore, there was a significant increase in the number of apoptotic cells both in the early phase and late phase in A375-TSLC1 cells as well. However, there was no difference in apoptosis rate between the A375 and A0 cells (P>0.05), indicating that the overexpression of CAMD1/TSLC1 has an inductive effect on apoptosis of A375 cells.

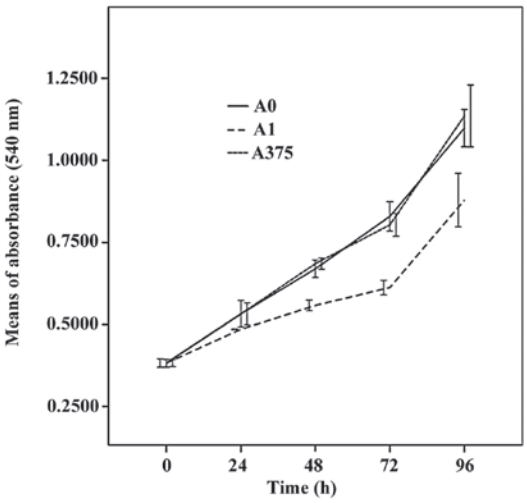

Figure 3. Effects of CADM1/TSLC1 on A375 cell proliferation as determined by an MTT assay. The value of absorbance (A540 nm) was measured for proliferation analysis in the microplate reader. The values are expressed as the mean ± standard error of the mean. Compared with A375 and A0, the growth of A1 clone was slower than that of A0 or A1 cells during the 96-h incubation period (P<0.05). A0, A375-pcDNA3.1; A1, A375-CADMI/TSLC1; CADM1/TSLC1, cell adhesion molecule 1/tumor suppressor in lung cancer-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide.

CADM1/TSLC1 suppresses cell migration. To investigate the effect of CAMD1/TSLC1 on cell metastasis, the cell migration ability of A375, A0 and A01 cells was examined by transwell assay. As demonstrated in Fig. 4, the cell migration assay revealed that the proportion of cells that transferring through the matrigel was significantly suppressed by 86.7% in A1 cells compared with that in the A0 (43.4%) and A375 (25.8%) cells. However, no significant differences between the A0 and A375 cells were observed in the transwell assay.

CADM1/TSLC1 downregulates MMP-2 and MMP-9 expression. To further investigate the molecular mechanisms involved in the effect of CADM1/TSLC1 overexpression on tumor cell invasion, the expression of MMP-2, -9, were determined by western blotting in A375, A0 and A1 cells. As demonstrated in Fig. 2B and C, the expression of MMP-2 and MMP-9 were significantly downregulated in the A1 cells, compared with that in the A0 and A375 cells.

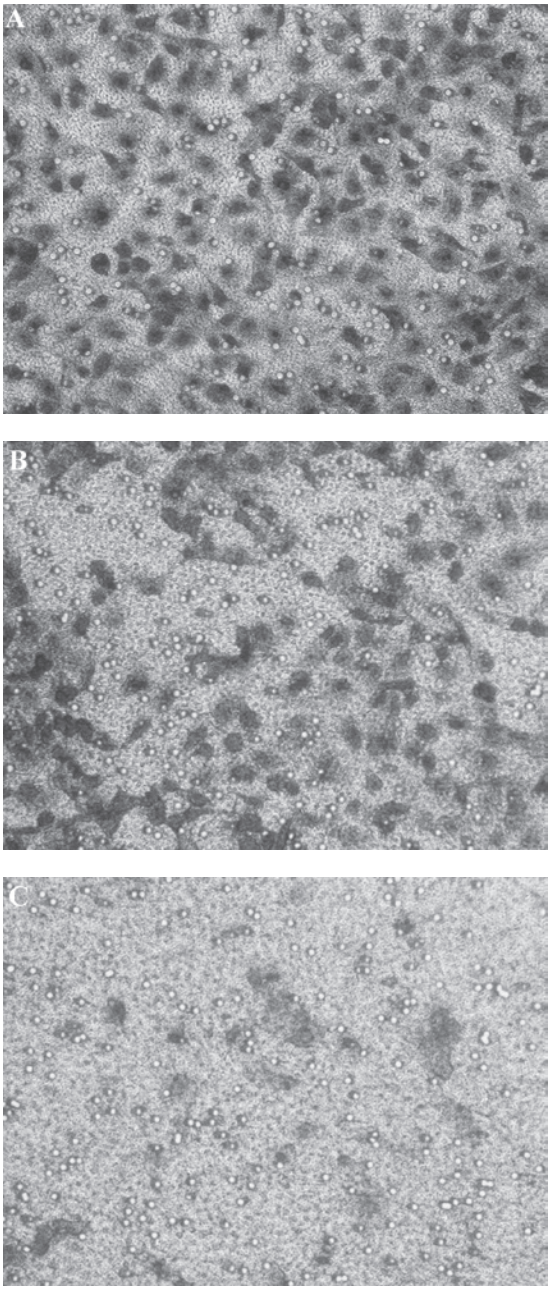

Figure 4. Effects of CADM1/TSLC1 on A375 cell invasion as determined by a transwell assay. All data were analyzed by SPSS 13.0 software, which was expressed as the mean ± standard error of the mean. The invasive capacity was significantly suppressed in A1 cells compared with that in A0 and A375 cells (P<0.05). A0, A375-pcDNA3.1; A1, A375-CADMI/TSLC1; CADM1/TSLC1, cell adhesion molecule 1/tumor suppressor in lung cancer-1.

Discussion

Melanoma is a malignant tumor of melanocytes that causes the majority of skin cancer-associated mortalities (10). The 5-year survival rate decreases from 95% for patients with a maximum tumor thickness of 1 mm lacking metastases, to <10% for patients with visceral metastasis. There is a conspicuous difference in survival between localized and metastatic disease (5-year survival of 98 and 15-62%, respectively) (11). The occurrence of metastasis is associated with high mortality rates due to the aggressiveness characteristics of the tumor and the lack of effective therapies to combat its spread. Therefore, identifying the molecular mechanism underlying tumor progression and metastasis in cancer is urgently required.

CADM1, also known as TSLC1, as a novel tumor suppressor, has been extensively investigated in various tumors (12-16). Loss or reduction of TSLC1 expression has been frequently found and demonstrated to be involved in the occurrence and progression of multiple different human tumors (17-20). Our previous results demonstrated that the silencing of TSLC1 through methylation is an important event in the pathogenesis of melanoma. Loss of the expression of TSLC1 in the cytoplasm of melanoma is associated with later tumor stage and decreased patient survival. TSLC1 thus constitutes a clinically important prognostic marker and a potential target for the development of novel therapies.

Accumulative evidence has revealed that CAMD1/TSLC1 is a crucial regulator of cell proliferation, invasion and

apoptosis (21-23). In the present study, to verify the molecular mechanism of the tumor-suppressing effect of CAMD1/TSLC1 in melanoma, a A375 cell line stably expressing CAMD1/TSLC1, A1, was successfully established and was used to investigate the role of CAMD1/TSLC1. *In vitro*, cell growth suppression by CAMD1/TSLC1 expression was demonstrated in that the cell proliferation was evidently inhibited in the A1 cells compared with the A375 and A0 cells ($P<0.05$), whereas there was no significant difference in the proliferation between the A375 and A0 cells ($P>0.05$). It was concluded that the inhibition of CAMD1/TSLC1 on cell invasion was, at least in part, due to the inhibition of proliferation.

It is well established that apoptosis is an important physiological process responsible for maintaining the balance of homeostasis and that it has a central role in the progression and development of tumors. In the present study, Annexin V/PI staining demonstrated a significant increase in the number of total apoptotic cells of A1 other than in A375 and A0 cells. This indicated that the overexpression of CAMD1/TSLC1 has an inductive effect on the apoptosis of A375 cells.

Malignant tumor invasion is a dynamic, continuous process. Tumor cells migrate away from the primary site, first invading the extracellular matrix (ECM) and the basement membrane and the interstitial cells in some molecular adhesion, and then activating cell synthesis and the secretion of various degradation enzymes, that assist in the migration of tumor cells through the ECM into the blood vessels. Then under the regulation of certain factors, including running through the vessel wall leakage to the secondary site, they continue to proliferate and subsequently result in the formation of metastases with peeling, adhesion, degradation, mobility, proliferation and transfer throughout the course of malignant tumor invasion.

In the present study, to determine whether the invasion ability of the A375 cells, matrigel, which simulates the metastatic process of tumor cells to travel through the ECM and basement membrane components, was inhibited, the cell migration ability of A375, A0 and A01 cells was examined by a transwell assay. As demonstrated in Fig. 4, the transwell assay revealed that the invasive capacity was significantly suppressed in A1 cells compared with that in the A0 and A375 cells , but no significant differences between the A0 and A375 cells were observed, which indicated that the overexpression of CAMD1/TSLC1 had an inductive effect on the migration ability of melanoma.

MMPs are a family of structurally related proteolytic enzymes that facilitate the degradation of ECM and the basement membrane. MMP-2 and MMP-9 are two important members in the metalloproteinase superfamily, which are involved in a wide range of proteolytic events, including tumor growth, migration, metastasis and angiogenesis (24). In the present study, to further examine the probable anti-invasive mechanism of CAMD1/TSLC1, the expression of MMP-9 and MMP-2 in A375, A0 and A1 cells was examined. As is demonstrated in Fig. 2., the A1 cells revealed a marked expression of MMP-9 and MMP-2. Therefore, it was hypothesized that the invasion inhibitory effect of CAMD1/TSLC1 may be due to the suppression of MMP-9 and MMP-2 expression, which subsequently prompts tumor metastasis and invasion. The details of the mechanism involved required further validation.

In conclusion, the present study demonstrated that CADM1/TSLC1 had anti-invasive effects on A375 cells. This inhibition was correlated with the expressional downregulation of MMP-2and MMP-9, which are associated with tumor metastasis and progression.

Acknowledgements

This study was supported by the Foundation of Technique of Heilongjiang Province, China (no. C201317). The authors would like to thank Xiwen Sun for providing valuable suggestions and assistance and Yuyan Ma for his excellent technical assistance.

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