Proteomics Analysis of Lymphatic Metastasis-Related Proteins Using Highly Metastatic Human Melanoma Cells Originated by Sequential in Vivo Implantation

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Metastasis of cancer cells to lymph nodes (LN) is a common modality of metastasis in clinical settings, but the mechanisms involved in lymphatic metastasis remain unclear compared to hematogenous metastasis to bones and the brain. To elucidate the molecular mechanisms responsible for melanoma LN metastasis, we first generated LN metastasis-prone melanoma cells (C8161F2) by the sequential in vivo transplantation of parental melanoma cells (C8161F0). Although the in vitro/in vivo proliferative potential of these melanoma cells were similar, the metastatic potential of the C8161F2 for LNs was significantly enhanced. We then conducted a proteomics analysis to identify the proteins and pathways that contribute to LN metastasis. We identified six proteins (three: up-regulated and three: down-regulated) whose expressions were statistically significantly different by more than 2-fold in the two cell groups. Some of these genes are responsible for the activation of the transforming growth factor-β (TGF-β)-related pathway, a well-known inducer of epithelial–mesenchymal transition (EMT). In addition, a gene ontology analysis revealed that the enhanced cell–cell adhesion appears to be involved in lymphatic metastasis. In conclusion, we established highly lymphatic metastatic melanoma cells, which would be valuable for studies of the molecular mechanisms responsible for lymphatic metastasis.

Key words melanoma; lymphatic metastasis; proteomics analysis; epithelial-to-mesenchymal transition

INTRODUCTION

Metastasis is reported to account for about 90% of deaths caused by cancer, and, as a result, the development of new treatments for metastasis is an urgent issue.¹ In particular, since lymphatic metastasis is known to be more frequent than hematogenous metastasis, suppressing lymph node (LN) metastasis is considered to be an important therapeutic target. However, many of the chemotherapeutic treatments that have been developed so far mainly involve the distribution of drugs via the bloodstream, i.e., by intravenous administration. Therefore, LN metastasis is currently resistant to chemotherapy due to the poor accumulation of drugs in the LN. It should also be noted that only limited LN metastasis-targeting agents have been developed. One reason for this is that most of existing metastasis models include a lung metastasis model that is delivered by intravenous administration,¹ a brain metastasis model delivered by intracerebral administration,² and a bone metastasis model delivered by left ventricular administration.³ That is, most models of hematogenous metastasis are simply models and only a few imitate lymphatic metastasis. For example, there is a known model in which cancer cells are transplanted to the sole of the forefoot and are thereafter metastasized to the LNs of the forearm several weeks later, but the number of lymphatic metastasis is overwhelmingly few due to technical difficulties compared to the hematogenous metastasis model. Thus, determining the factors (proteins, epigenome, etc.) that are determinant for LN metastasis is difficult due to the lack of a LN metastasis model. For the above reasons, establishing a cell strain that easily causes lymphatic metastasis has now become an urgent issue.

The aim of this study was to establish a melanoma cell strain that is capable of highly metastasizing in LNs, and to identify the genes that are involved in LN metastasis by comparison with parental melanoma cells (low lymphatic metastasis) and established melanoma cells (high lymphatic metastasis). We first developed cancer cells that were prone to LN metastasis, even with conventional subcutaneous transplantation. As a model of such a cancer, human melanoma cells C8161, which have a high frequency of LN metastasis,³ were used as a model cell. A new method for creating this characteristic cell line in terms of lymphatic metastasis; where a small number of cancer cells that have metastasized to LNs after sequential in vivo implantation of melanoma cells expressing the green fluorescent protein (GFP) and luciferase (luc) were isolated. The operation in which human leukocyte antigen (HLA) and GFP double-positive cells are isolated by a fluorescent-activated cell sorter (FACS) was repeated. The daughter strain of C8161 with a high ability to metastasize in LN (C8161F2) prepared by repeating this procedure twice showed a significantly improved LN metastasis as compared with the original parental strain (C8161F0). In addition, to extract genes that contribute to LN metastasis, these cells were analyzed by total protein proteomics. From this analysis, we found that the expression of six proteins had changed significantly by more than 2-fold (increased: 3 proteins, decreased: 3 proteins). Some of the identified genes were related to transforming growth factor-β (TGF-β). In summary, we provide
new insights into lymphatic metastasis of melanomas.

MATERIALS AND METHODS

**Materials** D-MEM/Ham's F-12 with l-glutamine and phenol red, sodium azide, sodium deoxycholate, dithiothreitol (DTT), ammonium bicarbonate (AmBic), iodoacetamide, lysyl endopeptidase, mass spectrometry grade (Lys-C), ethyl acetate, and trifluoroacetic acid (TFA), were purchased from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Fetal bovine serum (FBS) were purchased from ThermoFisher Scientific (Waltham, MA, U.S.A.) or Sigma-Aldrich (St. Louis, MO, U.S.A.). A 100 units/mL of penicillin and 100 μg/mL of streptomycin mixed solution, 2.5 g/L of Trypsin and 1 mmol/L of ethylenediaminetetraacetic acid (EDTA) solution with phenol red, Dulbecco's phosphate buffered saline without Ca2+ and Mg2+ (D-PBS), bovine serum albumin and sodium lauroyl sarcosinate, were obtained from Nacalai Tesque (Kyoto, Japan). D-PBS powder was purchased from Nissui Pharmaceutical (Tokyo, Japan). Sequencing grade modified trypsin was purchased from the Promega Corporation (Madison, WI, U.S.A.). Purified anti-mouse CD16/32 antibody Rat immunoglobulin G (IgG) κ was purchased from Japan SLC (Shizuoka, Japan). D-PBS powder was purchased from Nissui Pharmaceutical (Tokyo, Japan). Sequencing grade modified trypsin was purchased from the Promega Corporation (Madison, WI, U.S.A.). Purified anti-mouse CD16/32 antibody Rat immunoglobulin G (IgG) κ was purchased from Japan SLC (Shizuoka, Japan). Purified anti-human HLA-A, B, C (###1406), and PE Mouse IgG2a Isotype Ctrl (FC) (#402013) were obtained from Biolegend (San Diego, CA, U.S.A.). Mice were purchased from Japan SLC (Shizuoka, Japan).

**Cell Cultures** GFP and luc were transduced into the human melanoma cell line C8161 as a reporter gene by RediFect Red-FLuc-GFP (PerkinElmer, Inc., Waltham, MA, U.S.A.). The successfully transduced C8161 cells were isolated by sorting based on GFP expression with a Cell Sorter SH800 (Sony, Tokyo, Japan). C8161 and cells obtained from lymph nodes were cultured in D-MEM/Ham's F12 containing both 10% (v/v) FBS and 1% (v/v) penicillin−streptomycin mixed solution. All cells were cultured at 37 °C in 5% CO2.

For in vivo cell culturing, melanoma cells were isolated from LN in tumor-bearing mice. After removing the super -natant, the cell pellet was washed twice with D-PBS, and approximately 1 million cells were suspended in 70 µL of D-PBS. Immunodeficient mice BALB/c Scl-nu/nu (male, 4-week-old) (n = 10) were inoculated with approximately 1.0 × 106 cells suspended in D-PBS on the left flank. At 5 weeks after the inoculation of the melanomas, the mice were intraperitoneally injected with 3 mg d-luciferin potassium salt (FUJIFILM Wako Pure Chemical Corporation) dissolved in 200 µL of D-PBS. The right inguinal LNs were then excised from mice, and luminescence was measured using an IVIS (PerkinElmer, Inc.).

**Proteomics Analysis** C8161F0 and C8161F2 were cultured up to confluence. The dissociated cells were then suspended in 4 mL of icd-D-PBS and then centrifuged for 3 min at 500 × g at 4 °C. These washing operations were repeated twice. Cells in 300 µL of phase transfer surfactant (PTS) containing 12 mM sodium deoxycholate, 12 mM sodium lauroyl sarcosinate, and 100 mM tris(hydroxymethyl) aminomethane–HCl (pH 9.0), were homogenized for 40 min by sonication with a Bioruptor II (TYPE24) (power: high, 30 s on/off; BM Equipment, Tokyo, Japan) on ice. The amount of the protein was measured by means of a Pierce bicinchoninic acid (BCA) Protein Assay kit (ThermoFisher Scientific). The samples were digested and purified by the PTS method.6-8 The peptide solution was dried by a vacuum centrifuge concentrator (CC-101 (TOMY SEIKO, Tokyo, Japan), UT-1000 (Tokyo Rikakikai, Tokyo, Japan), NVP-1000 (Tokyo Rikakikai)). The dried samples were dissolved in 50 µL of Buffer A containing 5% (v/v) acetonitrile and 0.1% (v/v) TFA and were desalted with GL-Tip SDB (GL Science, Tokyo, Japan) column tips. The desalted peptide solutions were dried again by a vacuum centrifuge concentrator. The dried peptides were dissolved in 50 mL of 0.1% (v/v) TFA, and 1 µg of samples of peptides were analyzed by LC-MS/MS. The peptide data were detected and identified by information-dependent acquisition and sequential window acquisition of all theoretical fragment-ion (SWATH) method. The proteomics data were analyzed by the ProteinPilot Software version 4.5 and PeakView Software version 2.1 (AB Sciex, Framingham, MA, U.S.A.) and are shown in a volcano plot. The proteins that were significantly (p < 0.05) increased in F2 compared to F0 were used for a gene ontology (GO) analysis using DAVID Bioinformatics Resources version 6.8.9 The clinical creditability of these genes was evaluated by human RNA-sequence data for a skin cutaneous melanoma (primary and metastasis) using UALCAN.10

**Statistical Analysis** Two-tail t-test Mann–Whitney U-test (only for Fig. 2B because of the data with no-Gaussian distribution) and were performed for pairwise comparison.

**RESULTS**

Establishment and Characterization of LN-Metastatic Melanoma Cells We established an LN-metastatic human HLA or an isotype control per sample at 4 °C. After adding 1.0 mL of FACS buffer, the cells were isolated by centrifugation for 5 min at 500 × g at 4 °C, and the supernatant was then removed. These washing operations were repeated twice. Finally, the cells were suspended in 500 μL of FACS buffer and passed through a nylon mesh, after which, the samples were analyzed by a NovoCyte (Agilent Technologies, CA, U.S.A.).

Evaluation of the Ability for Lymphatic Metastasis To evaluate the lymphotropic metastasis of parental melanoma C8161F0 or C8161F2 cells, luc activity in the LN was measured in vivo at 4–5 weeks after the inoculation. Immune-deficient mice BALB/c Scl-nu/nu (male, 4-week-old) (n = 10) were inoculated with approximately 1.0 × 106 of cells suspended in D-PBS on the left flank. At 5 weeks after the inoculation of the melanomas, the mice were intraperitoneally injected with 3 mg d-luciferin potassium salt (FUJIFILM Wako Pure Chemical Corporation) dissolved in 200 µL of D-PBS. The right inguinal LNs were then excised from mice, and luminescence was measured using an IVIS (PerkinElmer, Inc.).
melanoma by sequential in vivo sub-cultivating C8161 cells in athymic nude mice. The parental C8161 cells which expressed luc and GFP (C8161F0) were then inoculated into nude mice (Fig. 1A). At five weeks after the inoculation, inguinal LN as draining LN (dLN) was excised and dispersed in RPMI-1640, and the dispersed cells were then cultured (C8161F1). In several articles, the in vivo implantation was repeated, at least, twice to significantly enhance the metastatic capability, and hence compared the last daughter cell strain with parent one.11–13 Likewise, C8161F1 cells were serially implanted into nude mice, and subsequently the same isolation was repeated (C8161F2). To ascertain whether the obtained cells were human melanoma-derived cells, we examined the expression of the human histocompatibility complex, the HLA-A,B,C (Fig. 1B). All cells (Parental C8161, and daughter C8161F1 and C8161F2) expressed HLA-A,B,C, suggesting that melanoma cells were successfully isolated from dLN and cultured, and that the samples were not contaminated with murine immune cells.

Evaluation of Lymphatic Metastasis of Newly Established C8161F2 Cells We next evaluated the extent of lymphatic metastasis of C8161F2. Luminescence of C8161F0 and C8161F2 in dLN was detected in 10 nude mice at 5 weeks after the inoculation. Luc luminescence in the C8161F2-bearing mice were dramatically higher than that in the C8161F0-bearing mice (Fig. 2A). A quantitative analysis suggested that the increased luminescence was statistically relevant (Fig. 2B). To exclude the possibility that the luc activity of C8161F2 was accidentally elevated in comparison to C8161F0, the in vitro luc activity of these cells were measured. The luc activity of C8161F2 was slightly lower than that of C8161F0 (Supplementary Fig. 1). Collectively, the in vivo luc activity confirms that the LN-metastatic properties of C8161F2 were provoked by the sequential in vivo implantation.

In vitro cell proliferation rates were investigated to exclude the possibility that the increase in lymphatic metastasis was not simply due to a difference in cell proliferation or tumor tissue growth. When the number of cells was measured at 24 and 48h after seeding in the culture plate, no change was observed in the speed of cell proliferation (Fig. 3A). In addition, the rate of in vivo tumor tissue growth was also evaluated. Tumor volumes were compared at 2 and 3 weeks after the administration of C8161F0 and C8161F2 cells to the immunodeficient mice, respectively. No difference was observed in the growth rate, even in vivo (Fig. 3B). These results suggest that the difference in LN metastasis is not simply due to difference in cell growth rate, but that there are differences in other steps in metastasis to the LNs.

Comparison of Parental Melanoma Cells and Daughter Melanoma Cells by a Proteomic Analysis To determine the factors that determine LN metastasis, a whole protein proteomic analysis was performed on C8161F0 cells and C8161F2 cells. The analysis showed a total 2073 proteins being expressed (Fig. 4A). Of these, the proteins whose difference between C8161F0 and C8161F2 were statistically significant \((p < 0.05)\) were 386 proteins (up-regulated in F2: 181 proteins, down-regulated in F2: 205 proteins). Moreover, the following proteins were identified as proteins with a significantly \((p < 0.05)\) and over 2-fold variation in expression, elevated: MAGUK p55 subfamily member 6 (MPP6), CCN family member 2 (CCN2), Interleukin-13 receptor subunit alpha-2 (IL13RA2). Decreased: Plastin-2 (LCP1), Centromere/kinetochore protein zw10 homolog (ZW10), plasminogen activator inhibitor-1 (PAI-1) (Fig. 4A). We performed a GO analysis with a focus on proteins that were statistically significantly increased (Fig. 4B). The top three pathways were related to cell–cell association: Cadherin binding involved in cell–cell adhesion, cell–cell adherent junction, cell–cell adhesion \((p < 10^{-10})\).

**Fig. 1. Preparation of Lymphotropic Metastatic Melanoma Cells**

(A) Schematic diagram for the preparation of lymphotropic metastatic melanoma cells. Immunodeficient mice were inoculated with human melanoma cells expression luc and GFP (F0) on the right flank. Tumor draining LN (dLN) was then excised and dispersed. Collected cells were propagated in vitro, and subsequently re-inoculated into immunodeficient mice. The procedure was repeated twice. We obtained lymphotropic metastatic melanoma cells (called F2). (B) Evaluation of HLA-A,B,C expression on the obtained melanoma cells isolated from dLN. Black and red lines indicate isotype control antibody-treated and PE-conjugated HLA-A,B,C antibody-treated cells, respectively. (Color figure can be accessed in the online version.)

**Fig. 2. Evaluation of Susceptibility for Lymphatic Metastasis**

(A) Five weeks after C8161F0 or F2 cells were inoculated into individual 10 nude mice on the right flank, inguinal LNs were then resected and the luc luminescence emitted by cancer cells was observed by IVIS. Images were shown in a luminescence descending manner. (B) Images were quantitatively analyzed. Circles denote each luminescence and bars indicates the mean of the samples. Statistical analysis was performed by two-tail Mann–Whitney U-test because luminescence of C8161F2 group don’t show Gaussian distribution. **: \(p < 0.01\) (\(n = 10\)). (Color figure can be accessed in the online version.)
DISCUSSION

In this study, we established an original cell strain that is capable of frequently metastasizing LN, and then investigated the genes that are involved in LN metastasis through a whole protein proteomics analysis. The cell line that spontaneously metastasized into LN shortly after the subcutaneous administration into the flank allowed us to readily develop an agent targeting lymphatic metastasis which was different from other previously reported models, in which melanoma cells were implanted into the forefoot pad. The metastatic potential of C8161F2 for LNs was significantly enhanced (Fig. 2). Although the in vitro/in vivo proliferative potential of these melanoma cells was slightly increased against C8161F0 (Fig. 3, not statistically significant difference), an approximately 4.5-fold increase in the metastatic ability of C8161F2 (Fig. 2). Additionally, the metastatic frequency was drastically elevated (C8161F0: 5/10, C8161F2: 10/10). This promoted metastatic ability would not be attributed to the negligibly increased proliferation potential of C8161F2.

We next investigated the molecular mechanism responsible for the accelerated lymphatic metastasis. Previous attempts to improve the metastatic potential of cancer cells by in vivo transplantation have been reported, in which BL6F10 cells were produced by the intravenous transplantation of BL6 cells, followed by the isolation and culturing of BL6 cells from the lung.13) These in vivo preparations have been reported to alter a variety of cell phenotypes. In the previous report on establishing a brain metastasis-prone breast cancer via in vivo transplantation, the cells associated with brain endothelial cells more strongly than parental cells via an elevation of ST6 N-acetylgalactosaminide alpha-2,6-sialyltransferase 5 (ST6GALNAC5).10) ST6GALNAC5 catalyzes the transfer of sialic acid units to cell surface proteins and subsequently modulates cell–cell contact. It was also reported that, in establishing cancer cells that metastasize to the bone, CD44 and CD49f were altered, which enhance the adhesion of such cells to other cells and/or extracellular matrices.11) From these reports, we conclude that the key factor is the elevation in adhesion capacity to the target organ. We speculated that similar changes occurred in the C8161F2 cells, namely, genes that enhance adhesion to the lymph node from the lymphatic flow. The results of the GO analysis of genes that were increased in proteomics indicated that molecules related to cell adhesion were, in fact, enhanced (Fig. 4B), suggesting that the subpopulation of cells that can strongly adhere to cells is enriched during the period that they were carried by the lymphatic flow in lymph nodes. This assumption is consistent with the result showing that the proliferation rate of C8161F2 was equal to that of C8161F0 (Fig. 3).

Judging from the proteins that were significantly altered (p<0.05, over 2-fold), migration and invasion ability might be enhanced in the C8161F2 cells. CCN2 (2.23-fold up-regulated in C8161F2: Fig. 4) is also referred to as connective tissue growth factor (CTGF) and is a major attractant for mitosis in connective tissue by vascular endothelial cells.16) CTGF is also a cofactor for transforming growth factor beta (TGF-β) to enhance the function of TGF-β.17) IL13RA2 (2.56-fold up-regulated in C8161F2: Fig. 4) is a gene coding for the interleukin 13 receptor alpha 2 subunit. Physiologically, IL13 is produced from activated T-helper 2 (Th2) cells, which stimulates immunoglobulin E isotype switching via the up-regulation of CD23 and major histocom-
patibility complex (MHC) Class II.\textsuperscript{18} When IL13RA2 binds to its ligand IL-13, the TGF-β promoter is activated and subsequently causes an increased level of expression of TGF-β.\textsuperscript{19}

TGF-β has a multifaceted effect on cancer progression and metastasis. For example, in ovarian cancer, it has been reported that TGF-β induces cancer metastasis through promoting epithelial-mesenchymal transition (EMT).\textsuperscript{19} EMT is the process by which epithelial cancers (colon cancer, breast cancer, etc.) acquire mesenchymal cell-like properties.\textsuperscript{20} The basic properties of epithelial cells are that they adhere to surrounding epithelial cells, have cell polarity, and are present in a neat and orderly manner. On the other hand, when cancer cells acquire mesenchymal cell-like properties via EMT, they no longer need to adhere to surrounding cells for survival and acquire a highly invasive and migratory potential. As a result, cancer cells are more likely to metastasize, which is a well-known mechanism.\textsuperscript{21}

The increased expression of IL13RA2 and CTGF enhance the function of TGF-β. Since melanoma cells are not epithelial cells, the concept of EMT could not be applied to melanoma cells, that is an “EMT-like phenotype.” In other words, melanoma cells might become prone to metastasized to LNs by acquiring the ability to infiltrate and migrate via the enhanced TGF-β signaling-mediated EMT-like mechanism. Actually, it has been reported that EMT-inducing transcription factors (EMT-TFs) promote malignancy by disrupting the differentiation-de-differentiation function of melanoma cells.\textsuperscript{22} Further studies should be required for an elucidation of the involvement of TGF-β in lymphatic metastasis.

PAI-1 (also known as serine protease inhibitor family E member 1; SERPINE) was significantly reduced (0.27-fold down-regulated in C8161F2: Fig. 4). Although PAI-1 is usually known as a protein that inhibits the activity of urokinase-type plasminogen activator (uPA), the binding of PAI-1 to the uPA/uPAR complex enhanced endocytosis, and the internalized PAI-1 was then degraded in the cell.\textsuperscript{23–25} Due to inhibitory effect of PAI-1 on the uPA/uPAR system, a decrease in the levels of PAI-1 would be expected to enhance the fibrinolytic system and promote metastasis through the degradation of extracellular matrix and other substances.

The direct involvement of other genes in LN metastasis is unclear. MPP6 is an abbreviation for MAGUK p55 subfamily member 6, which forms a part of the RNA exosome component in the cytoplasm.\textsuperscript{26} It has been reported that the exosome component is involved in RNA processing and quality control through its degradation.\textsuperscript{27} Since MPP6 has hardly been reported to be associated with cancer, it may be a new therapeutic target. In fact, when the results of transcriptome studies in melanoma patients were extracted from the database of The Cancer Genome Atlas (TCGA), the expression of MPP6 in metastatic lesions was found to be significantly higher than that in primary lesions (Supplementary Fig. 2). Likewise, the RNA expressions of other IL13RA2 and CTGF was predominantly high in metastatic lesions in clinical specimens.

ZW10 (0.39-fold down-regulated in C8161F2: Fig. 4) is also known as Centromere/kinetochore protein zw10 homolog. Physiologically, ZW10 plays an essential role in mitotic checkpoint, which prevents it from transmitting to the anaphase until the maturation of the chromosome attachment to the spindle apparatus through the formation of a ROD-Zw10-ZW10 (RZZ) complex.\textsuperscript{28,29} It has been reported that the suppression of ZW10 expression by small interfering RNA (siRNA) in colorectal cancer increases cell migration and proliferation.\textsuperscript{30}

LCP-1 (0.27-fold down-regulated in C8161F2: Fig. 4) is a hematopoietic series-specific protein that contributes to the stability of actin-containing structures by cross-linking actin filaments.\textsuperscript{31} It has specific functions in various immune cells, including stabilizing the immune synapses of T cells (Three-dimensional segregation of supramolecular activation clusters in T cells) and participating in T/B cell motility.\textsuperscript{32} The high level of expression of LCP-1 in malignant cells has been reported to be correlated with the intensity of the malignancy.\textsuperscript{32} It has also been reported that the ectopic expression of LCP-1 enhances the proliferation and invasive ability of colon cancer cells. The mechanism responsible for the contribution of MPP6, ZW10 and LCP-1 to LN metastasis remains to be clarified in the future.

In summary, our comparative proteomics analysis of low- and high-LN metastatic melanoma cell lines (C8161F0 vs. C8161F2) suggests that TGF-β signaling might be involved in melanoma LN metastasis. Moreover, GO analysis suggests that cell attachment ability might be involved with lymphatics metastasis. On the other hand, changes in expression were also observed in a group of proteins for which their function and metastasis are not reported to be related. Further study of these gene functions may lead to the development of innovative therapeutic agents that will permit lethal lymphatic metastasis to be avoided.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials The online version of this article contains supplementary materials.

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