Mulberry Leaf Extract Inhibits Invasive Potential and Downregulates Hypoxia-Inducible Factor-1α (HIF-1α) in SK-N-BE(2)C Neuroblastoma Cells

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A neuroblastoma is an extracranial solid tumor diagnosed in childhood. Since tumor metastasis is the main cause of death for most neuroblastoma patients, an understanding of the mechanisms that modulate cancer cell invasion is a key to developing more effective chemotherapeutic agents. In the current study, we examined to determine whether mulberry leaf (ML) extract effectively inhibits the invasion potential of neuroblastoma cells in vitro. ML extract was found to suppress cell invasiveness as well as the activity and expression of matrix metalloproteinase-2 (MMP-2) under both normoxia and hypoxia in neuroblastoma. ML extract downregulated the expression of hypoxia-inducible factor-1α (HIF-1α), a well-known regulator of tumor metastasis, and its downstream targets, vascular endothelial growth factor (VEGF) and glucose transporter-1 (GLUT-1). Taken together, these results suggest that ML extract has chemotherapeutic effects on neuroblastoma cells by regulating invasion potential, thereby controlling the metastasis of neuroblastomas.

Key words: mulberry leaf; neuroblastoma; metastasis; hypoxia-inducible factor-1α

Neuroblastoma (NB) is the second most common solid pediatric tumor diagnosed, and it accounts for approximately 15% of deaths due to cancer in children.1 It is a childhood malignancy that derives from the sympathetic nervous system (SNS), and typically arises in the adrenal gland, abdomen, chest, or neck.1 NBs often form relatively localized, benign, and well-differentiated tumors. For stage I and II tumors, these often regress spontaneously, or are successfully treated by surgery alone. However, cases of regionally invasive (stage III) or metastatic (stage IV) NBs have poor clinical outcomes.2 Metastasis by NBs usually involves local lymph nodes, liver, bone marrow, and bones, and nearly 70% of NB patients have a metastasis event detected at the time of their primary tumor diagnosis.3,4 Patients with metastatic disease are considered high-risk, and their overall survival rate is less than 40%.5

Metastasis is the movement of cells from the primary tumor mass to distant locations, and constant progression leads to the death of most cancer patients.4 Over 90% of cancer patients do not survive due to the development of metastases, rather than the primary tumor itself.6 Cell motility and invasiveness are two critical processes for the invasion and metastasis of malignant cancer cells. During these processes, degradation of the extracellular matrix (ECM) is mediated by the secretion of several types of matrix metalloproteinases (MMPs), and these zinc-binding endoproteases can directly degrade most components of the ECM.7

In addition, one of the driving forces of metastasis is hypoxia, a condition in which the level of oxygen present in a tissue is decreased to 1%. Hypoxia is typically associated with solid tumors that show decreased blood flow, which is associated with decreased patient survival and advanced tumor stages for many types of cancer.8 Furthermore, hypoxic conditions can decrease the effectiveness of radiotherapy and chemotherapy.9 Hypoxia-inducible factor-1 (HIF-1) is a principle modulator of the tumor cell response to hypoxia, a heterodimer of an oxygen-dependent α subunit (HIF-1α), and an oxygen-independent β-subunit (HIF-1β). While HIF-1α levels are normally kept low by proteasomal degradation, they are stabilized under conditions of hypoxia.10 Upon stabilization, HIF-1α proteins translocate to the nucleus and dimerize with HIF-1β to transactivate target genes, including vascular endothelial growth factor (VEGF) and glucose transporter 1 (GLUT 1).11 Thus, regulation of hypoxia represents an important consideration in therapeutic strategies developed to target metastatic cancers.

Mulberry (Morus alba L., family Moraceae) leaf (ML), is the primary food for silkworms, and is widely consumed by humans in China, Korea, and southeast Asian countries.12 Based on the experience of Oriental medicine, ML has many medical benefits, including anti-diabetic, anti-atherosclerotic, anti-oxidant, anti-inflammatory, and anti-cancer properties.12,13 In addition, ML extract was recently found to inhibit NB cell stemness and to induce cell differentiation.14 Although previous studies have examined the effects of ML extract on various cancers, the effects of ML extract on NB cell invasion and metastasis have not been examined, and the exact mechanisms remain uncertain. Hence, in this study a NB cell line, BE(2)C, was...
analyzed to identify anti-cancer effects induced by treatment with ML extract. In particular, the metastasis potential of BE(2)C cells was assayed.

Since cancer metastasis and invasion correlate with degradation of ECM and hypoxia, in the present study, the effect of ML extract on the invasive potential, level of MMP, and HIF-1α signaling of BE(2)C cells were also examined.

Materials and Methods

Preparation of ML extract and treatment. ML extract was kindly provided by Dr. Yunsook Lim (University of Kyunghee, College of Human Ecology, Seoul, South Korea), and was stored at −20°C. The preparation of ML extract was described previously. Briefly, harvested MLs were cleaned, dried, and pulverized. Dry powdered leaves (1 kg) were then extracted with 70% ethanol by occasional sonication overnight at room temperature. After filtration through a membrane, the extract was concentrated with a vacuum rotary evaporator at 40°C and lyophilized. It was stored at −20°C. A stock ML extract solution was prepared in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO) at a final concentration of 100 mg/mL. Further dilution of the solution into the cell medium was performed as needed. Control samples received the same concentrations of DMSO at a final concentration in the medium ranging from 0.01% to 0.04%.

Cell culture and induction of hypoxia. The SK-N-BE(2)C (BE(2)C) human NB cell line was obtained from the American Type Culture Collection (Manassas, VA), and was maintained in a 1:1 mixture of Minimum Essential Medium (MEM, Welgene, Daegu, Korea) and Ham’s F-12 medium (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS) (v/v) (Hyclone, Logan, UT) and was maintained in a 1:1 mixture of human NB cell line was obtained from the American Type Culture Collection (II) chloride hexahydrate (CoCl2, Sigma, St. Louis, MO) at a final concentration of 100 mg/mL. Carlsbad, CA). Cells were cultured at 37°C and 95% air.

Cell motility was evaluated by wound-healing assays. Wound healing assays. Cell motility was evaluated by wound healing assays. The SK-N-BE(2)C [BE(2)C] monolayer was treated with various concentrations of ML extract, and the cells were removed by a gentle washing with the medium. The wounded areas were seeded in 6-well plates and cultured to near confluence. Scratches were made using 24-well BD BioCoat Matrigel invasion chambers containing an 8-μm pore size PET membrane (Becton Dickenson, Bedford, MA) following the manufacturer’s instructions. Briefly, cells were treated with or without ML extract and prepared as cell suspensions in serum-free culture medium at a density of 3 × 105 cells/mL. Cell suspensions (500 μL) were then loaded into the upper chamber of each well. The lower chambers were filled with media containing 5% FBS as chemoattractant. After the invasion chambers were incubated for 48 h at 37°C under 5% CO2, the cells on the upper surface of the chamber (non-invasive cells) were removed using a cotton swab. Membranes with cells attached to the lower membrane surface (invasive cells) were fixed with methanol and stained with crystal violet (Sigma-Aldrich, St. Louis, MO). Five vision fields were selected randomly and the number of invaded cells for each was counted. All experiments were performed in triplicate.

Gelatin zymography assays. BE(2)C cells were cultured in serum-free medium with various concentrations of ML extract. After 48 h, the medium was collected and mixed with an equal amount of sample buffer, 4% sodium dodecyl sulfate (SDS), 20% glycerol, 0.01% bromophenol blue and 0.125 mM Tris–Cl (pH 6.8), without reducing agent and boiling. They were then loaded onto 8% polyacrylamide gels containing 0.1% gelatin. They were electrophoresed at 4°C, and then the gels were washed twice with renaturing buffer (2.5% Triton X-100) to remove SDS. The gels were incubated at 37°C overnight in developing buffer, 50 mM Tris–Cl (pH 7.5), 10 mM CaCl2, 15 mM NaCl, 0.2 mM ZnCl2. The gels were stained with 0.5% Coomassie Blue R-250 (Amresco, Solon, OH) at room temperature for 1 h, and then rinsed with de-stain solution (40:50:10 MeOH:H2O:Gacial-acetic acid). MMP activity was visualized as clear bands against the blue background of the stained gels.

Western blot assays. Western blot assays were performed as previously described. Briefly, cells were treated with various concentrations of ML extract and then lysed in lysis buffer, 50 mM Tris–Cl (pH 7.5), 15 mM NaCl, 1% Nonidet p-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM PMSF, 1 mM NaVO4, and 1 mM NaF (Sigma-Aldrich). Total protein concentrations were measured using BCA reagent assay kits (BioRad, Hercules, CA), and protein samples (100 μg each) were separated by SDS–PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA). The membranes were blocked with 5% non-fat milk in Tris-buffered saline containing Tween-20 (TBST) and incubated with the following primary antibodies against HIF-1α (Novus, Littleton, CO), α-tubulin (Sigma) or MMP-2 (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. After three washes with TBS, the membranes were incubated with an appropriate secondary horseradish peroxidase IgG for 1 h, followed by detection with chemiluminescence reagents (Animal Genetics, Suwon, Korea). Detection of α-tubulin was used as loading control.

Real-time quantitative PCR. Total cellular RNA was extracted from the cells using TRizol Reagent (Invitrogen, Carlsbad, CA). cDNA was synthesized using a RevertAid™ First Strand cDNA Synthesis Kit (Fermentas, Vilnius, Lithuania) and 1 μg of total cellular RNA samples. Real-time PCR was performed using a Step-One-Plus instrument (Applied Biosystems, Foster City, CA). Samples were combined with Power SYBR Green PCR Master Mix (Applied Biosystems) and subjected to the following conditions: initiation step at 95°C for 5 min, denaturation at 95°C for 15 s, annealing at 60°C for 30 s, and extension at 72°C for 10 s. All data were normalized to the expression of β-actin, and were analyzed by the CT method. The PCR primer pairs used included VEGF: 5'-GCA CCC ATG GCA GTA CT-3' (antisense), GLUT-1: 5'-GAT TGG TCG CTT CTC TCT GTG G-3' (sense) and 5'-TCA AAG GAC TTG ATC-3' (antisense), and β-actin: 5'-ATT GCC AAT GAG CCG TTC-3' (sense) and 5'-GGA TGC CAC AGG ACT ACA T-3' (antisense). All experiments were performed in triplicate.

Statistical analysis. Each experiment was performed at least 5 times independently in order to generate statistically relevant data. All statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). Results were expressed as mean ± standard error of the mean (SEM). Data were analyzed by Student’s t-test and one-way analysis of variance (ANOVA), followed by Tukey’s post-hoc test. Differences with a p-value of less than 0.05 were considered statistically significant.
Results

ML extract inhibited the migration of BE(2)C cells under both normoxia and hypoxia

To examine the effect of ML extract on cell toxicity, cell viability was examined. Compared to control, cell viability was not significantly affected following treatment with ML extract under normoxia or hypoxia. These results suggest that treatment of BE(2)C cells with ML extract ranging in concentration from 0 to 40 μg/mL for 48 h under normoxia (Fig. 1A) or hypoxia (Fig. 1B) is not cytotoxic.

Hypoxic tumor cells are more resistant to radiation therapy and chemotherapy due to their lower proliferation rate and their capacity to metastasize to remote organs.20,21) To determine whether BE(2)C cells are affected by ML extract under hypoxic conditions, cells were pretreated with ML extract for 24 h, and then treated an agent that induces hypoxia, DFO (100 μM) or CoCl2 (100 μM), for 24 h. Migration assay was performed under both normoxia and hypoxia (Fig. 2). SK-N-BE(2)C cells were observed to migrate more rapidly under hypoxia than normoxia. ML extract was also found to inhibit cell migration under both normoxia and hypoxia.

ML extract inhibited the invasive potential of BE(2)C cells under both normoxia and hypoxia

Transwell invasion assays were performed further to determine whether treatment of BE(2)C cells with ML extract would inhibit their invasiveness. Cells were plated in the upper wells of migration chambers with or without ML extract (40 μg/mL). The lower chambers contained 5% FBS. After 24 h, the cells were treated with DFO (100 μM) for 24 h to induce hypoxia. The invasive potential of BE(2)C cells was significantly increased under hypoxia, but treatment with ML extract reduced the invasive potential of BE(2)C cells relative to the untreated control cells under both normoxia and hypoxia (Fig. 3A). When the numbers of invasive cells per microscopic field were counted, a 30% increase in the number of invasive BE(2)C cells was recorded for hypoxia versus normoxia (p < 0.001) (Fig. 3B). In contrast, treatment with ML extract resulted in 84% and 86% decreases in the numbers of invasive of BE(2)C cells under normoxia and hypoxia respectively as compared to the control cells (p < 0.001). These results indicate that ML extract can inhibit NB cell invasion as well as NB cell migration under both normoxia and hypoxia.

ML extract downregulated the expression of HIF-1α and suppressed MMP-2 under hypoxic conditions

Western blot assays performed, the protein levels of HIF-1α were observed to be upregulated during DFO- or CoCl2-induced hypoxia, yet were undetected in the
vehicle-only controls (Fig. 4A). This indicates that DFO or CoCl₂ mimicked the hypoxic condition that upregulated HIF-1α. Moreover, treatment of BE(2)C cells with 40 μg/mL, ML extract resulted in downregulation of HIF-1α during DFO- and CoCl₂-induced hypoxia.

MMPs promote tumor invasion and metastasis by degrading the ECM. To determine the effects of ML extract on the secretion of MMPs from BE(2)C cells and the expression of MMP-2, gelatin zymography assays and Western blot were performed. As shown in Fig. 4B, treatment with ML extract suppressed the activity of the MMP-2 enzyme under both hypoxic and normoxic conditions. Furthermore, expression of MMP-2 was downregulated following treatment with ML extract under hypoxic conditions (Fig. 4C). These data suggest that ML extract can inhibit hypoxia-induced metastasis of BE(2)C cells via inhibition of MMP-2 enzyme activity and expression.

**ML extract downregulated the mRNA expression of downstream target genes of HIF-1α**

VEGF and GLUT-1 are downstream target genes of HIF-1α that play important roles in tumor metastasis.²²,²³ To determine whether ML extract can downregulate hypoxia-regulated VEGF or GLUT-1 expression, real-time PCR analysis was performed. In the assays performed, mRNA expression of VEGF and GLUT-1 was found to be upregulated under hypoxic conditions (Fig. 5A and B). In contrast, ML extract did not affect the mRNA expression of VEGF or GLUT-1 under normoxia. However, the mRNA expression of both targets was down-regulated when cells were pretreated with 40 μg/mL ML extract prior to induction of hypoxia by DFO. Taken together, these data indicate that ML extract can inhibit hypoxia-regulated gene expression.
Discussion

NB arises from embryonic neural crest cells in the nervous system, and often spreads to other organs of the body before symptoms manifest.24 NB cells show three distinct cellular phenotypes: a substrate-adherent (S-type) cell, a neuroblastic (N-type) cell, and a morphologically intermediate (I-type) cell.25 The BE(2)C cells used in this study are I-type cells, and they exhibit increased tumorigenicity, malignancy, and metastatic potential compared to the N- and S-type cell lines.26,27

More than half of all NB patients suffer from metastasis. In addition, despite recent advances in cancer chemotherapy, patients with malignant NB usually exhibit resistance to conventional chemotherapy or radiation therapy. Current therapeutic agents can also cause serious side effects, including neurotoxicity and nephrotoxicity.28,29 To prevent unfavorable side effects and ultimately improve patient survival, more effective supplements with low toxicity are required.

Here, we provide the first evidence that treatment of BE(2)C cells with ML extract can effectively suppress the invasive potential of this NB cell line. Metastatic cells can possess cell motility, surface adhesion properties, and extracellular proteases activity. Moreover, interactions between cells and the ECM are hypothesized to be important for the invasion, migration, and metastasis of tumor cells.30 A key component of these interactions is proteolytic degradation of the ECM, needed for the invasion and migration of cells into the basement membrane. MMPs have been found to degrade the ECM and to facilitate the entry of cancer cells into the blood or lymphatic system for metastasis.31 In particular, MMP-2 can degrade components of the basement membrane and enhance the invasion of cancer cells.

Gelatin zymography is used mainly for the detection of MMP-2 and MMP-9. It is not the preferred substrate for other MMPs, including MMP-1, MMP-8, and MMP-13. Therefore, signals for other MMPs are very weak or undetectable.32 Moreover, very low levels of MMP-9 activity detected in SK-N-BE(2)C cells by the zymography are consistent with previous reports.33,34 Neuroblastoma cells express MMP-2, which degrades the basement membrane, and they express low or undetectable levels of MMP-9.35 While it has been reported that zymography is more sensitive than Western blot analysis,36,37 and thus can detect changes in MMP-2 activity (even at 20 μg/mL of ML extract), Western blot analysis cannot detect changes in expression at the same dose. Furthermore, MMPs are secreted in latent form as pro-MMPs, which require activation, and a tissue inhibitor, TIMP, creates a complex with a proenzyme (zymogene) in gelatinases, including MMP-2 and MMP-9, and regulates the activity of MMPs.34 Activation of proMMP-2 requires the participation of integral plasma membrane MMP (membrane type-MMP, MT1-MMP).35 Thus the different effects of 20 μg/mL of ML extract on MMP-2 activity and protein expression can be explained by the involvement other TIMPs and MT-1-MMP on MMP-2 activity. Future studies to investigate TIMP and MT1-MMP are warranted.

Here, we found for the first time that ML extract can reduce the secretion and expression of MMP-2 as well as the invasion of BE(2)C cells through a matrigel-coated membrane. These findings indicate that it can reduce the extent of NB cell invasion by suppressing MMP-2 activity and expression. There are several other natural agents containing flavonoids that have been found to mediate anti-metastatic and anti-invasive effects by inhibiting MMP-2 activity.36 For example, anthocyanins from mulberry fruits have been found to inhibit the invasion of metastatic A549 lung cancer cells, to decrease the expression of MMP-2, and to activate c-Jun and NF-κB.37 These results are consistent with those of the present study in that ML extract exhibited anti-invasive potential against a certain metastatic cancer-like NB.

Hypoxia induces a wide range of biological changes. These include decreases in cell proliferation,38,39 increased expression of drug resistance genes,40,41 selection of apoptosis-resistant clones,42 and enhanced tumor cell invasion and metastasis,43,44 but gene expression induced by HIF-1α allows cells to respond to hypoxic conditions by enhancing oxygen transportation or by adapting the cells to lower levels of oxygen in metastatic cancers.45 Hence, targeting of HIF-1α might represent...
an effective strategy for controlling metastasis events. A significant finding of the present study is that ML extract inhibited hypoxia-induced expression of HIF-1α in NB cells. Several studies have reported that hypoxia is associated with the regulation of gene expression and/or the activity of members of the MMP family, including MMP-2.\(^46,47\) In addition, the activity of MMP-2 was found to be enhanced under hypoxia as compared to normoxia. Correspondingly, the present study found that ML extract decreased both MMP-2 enzyme activity and expression, and further suppressed hypoxia-stimulated secretion of this enzyme very effectively. Thus, ML extract might mediate an anti-invasive effect on NB cells by suppressing hypoxia-regulated mechanisms. 

The oxygen-sensitive HIF-1α subunit is expressed in human cancers, and regulates the expression of the genes involved in tumor angiogenesis and glucose metabolism, including VEGF and GLUT-1.\(^10\) For example, hypoxia-induced expression of VEGF stimulates angiogenesis, thereby maintaining oxygen homeostasis in tumors,\(^11\) and hypoxia-induced expression of GLUT-1 increases the production of ATP, supporting cancer cell survival.\(^11\) Hence, high levels of HIF-1α expression are positively associated with VEGF or GLUT-1 expression. Correspondingly, an anti-cancer mechanism involving inhibition of HIF-1α is identified when downregulation of VEGF and GLUT-1 is detected. In the present study, hypoxia-induced mRNA expression of VEGF and GLUT-1 was downregulated following treatment with ML extract. This suggests that ML extract has an inhibitory effect on the angiogenesis and glucose metabolism that occur under hypoxic conditions, but further studies are needed to confirm this.

Several studies have demonstrated that plant-derived materials and naturally occurring phytochemicals have anti-migration and anti-invasion effects on various cancers. In a report by Chu et al., it was proposed that Terminalia catappa L. leaf extract (0–100 μg/mL) mediated anti-metastatic effects on lung cancer cells by downregulating MMP-2, MMP-9, and urokinase plasminogen activators.\(^48\) Another study, by Im et al., found that the butanol fraction of Guava (Psidium cattleianum Sabine) leaf extract (1–100 μg/mL) had an anti-invasion effect by suppressing the expression and activity of MMP-2 and MMP-9 via ERK1/2 MAPK.\(^49\) Furthermore, a methanol extract of Elaeagnus glabra (0–100 μg/mL), a Korean medicinal plant, has been found to inhibit HT1080 fibrosarcomas by blocking MMP-2 and MMP-9.\(^50\) In the present study, ML extract was found to inhibit the migration and invasion of neuroblastoma cells at a lower dose (0–40 μg/mL) than the doses applied in other studies. Furthermore, the effects of ML extract on invasion via activation of MMP-2 or increases in MMP-2 expression were found to be accompanied by increases in HIF-1α expression, which might be a promising target for anti-metastatic therapy using plant-derived materials. 

Compounds from various plants have been reported to exhibit superior potential as safe and effect supplements for cancer prevention and treatment.\(^51\) For example, extensive research has demonstrated that plant extracts, including blueberry, green tea, and phyllanthus, have inhibitory effects on tumor metastasis by inhibiting the migration and invasion of cancer cells.\(^52,53\) It has also been demonstrated that MLs contain several polyphenols, including quercetin 3-(6-malonylglucoside) (900 mg per 100 g), rutin (573 mg per 100 g), isoquercitrin (194 mg per 100 g), and astragalin (31 mg per 100 g).\(^54,55\) Flavonoids are non-toxic compounds associated with a wide range of beneficial bioactivities, including anti-oxidant, anti-inflammatory, and anti-tumorogenic activities, as well as the capacity to regulate enzyme activities.\(^56\) In the present study, the anti-cancer potential exhibited by ML extract might have been due to the combination of several bioactive compounds that were present in the extract. Further studies are needed to confirm these effects, and to identify the activities of individual bioactive components of the ML extract on cancer cell invasion both in vitro and in vivo.

In conclusion, here we report the first evidence that ML extract can inhibit the invasiveness of human NB cells, and that it has inhibitory effect on (i) the expression of HIF-1α, (ii) the expression of VEGF and GLUT-1 (HIF-1α target genes), and (iii) the activity and expression of MMP-2. These findings have clinical significance as they help to elucidate the roles of ML extract in the regulation of NB metastasis, and provide mechanistic insight into the progression of malignant tumors, but further studies are necessary to develop ML extract as a safe anticancer agent either alone or in combination with other anti-metastatic drugs for the treatment of NB in humans.

**References**

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