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Original Research

Nuclear entrapment of p33^{ING1b} by inhibition of exportin-1: A trigger of apoptosis in head and neck squamous cell cancer

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Abstract: The effect of deregulation of nuclear export mediated by exportin-1, with consequent cellular mislocalization of p33^{ING1b}, a member of the tumor suppressor gene family, has not been previously investigated in head and neck squamous cell cancer (HNSCC). We evaluated the effect of reversing cytoplasmic p33^{ING1b} localization through inhibition of exportin-1 by leptomycin B (LMB) and the effect of nuclear entrapment of p33^{ING1b} on molecular alterations in primary and metastatic HNSCC lines. The expression and location of exportin-1 and p33^{ING1b} were analyzed by a quantitative real-time reverse transcription polymerase chain reaction PCR (qRT-PCR), a Western blot, and immunostaining. Cell proliferation and migration assays were conducted to determine the effect of exportin-1 inhibition on the cell lines. Exportin-1 was overexpressed in metastatic HNSCC, whereas p33^{ING1b} was poorly expressed. Exportin-1 inhibition induced nuclear entrapment and upregulation of p33^{ING1b}, extensive apoptosis, and growth arrest. It also suppressed cell migration. Cytoplasmic p33^{ING1b}-mediated regulation of cell growth and nuclear entrapment of p33^{ING1b} via inhibition of exportin-1 may be a key mechanism for inducing HNSCC apoptosis.

Key words: Head and neck cancer; Exportin-1; p33^{ING1b}; Nuclear export; Leptomycin B.

Introduction

Head and neck squamous cell cancer (HNSCC) accounts for approximately 650,000 new cases every year and the sixth most common cancer type worldwide, with a 5-year survival rate of just 40–50% in the last two decades (1). Surgery is the first-line therapy for HNSCC, but it results in a serious decrease in the quality of life in patients. Modern drug therapies also have serious adverse effects due to their toxicity or lead to the emergence of drug resistance (2). Despite technological advancements in treatment, HNSCC is characterized by high rates of relapse and metastasis and continues to be associated with high mortality rates (3). Thus, there is an urgent need to explore the molecular characteristics of HNSCC and develop novel therapeutic agents targeting specific cellular pathways to overcome potential drug resistance mechanisms.

The p33^{ING1b} protein, a member of the tumor suppressor gene family, is involved in proliferation, apoptosis, tumor growth, cellular aging, and modulation of cell cycle control points (4). Many studies have elucidated the altered expression patterns or products of p33^{ING1b} in cancerous tissues. However, the relevance of p33^{ING1b} in tumorigenesis cannot be understood using only genetic mutations or differential expression (5). Previous research reported that cytoplasmic inclusions and shifts in the nuclear localization of the p33^{ING1b} protein were closely correlated with metastasis in cancer tissues, including HNSCC (6). Research also suggested that cellular compartment shifting was an additional mechanism used by tumor cells to increase their proliferative potential. Aberrant cytoplasmic p33^{ING1b} expression was associated with loss of cellular growth suppression and resistance to apoptosis, whereas nuclear retention of p33^{ING1b} was linked to the inhibition of cell proliferation and enhanced apoptosis (7,8).

Exportin-1, also known as chromosome region maintenance or Crm1, is a member of the karyopherin family of receptor proteins and mediates the nuclear export of critical proteins, such as tumor suppressor proteins and transcription factors (9,10). Exportin-1 has a broad substrate range, distinguishing cargo proteins that have a leucine-rich nuclear export signal (NES), thus facilitating subcellular localization of target macromolecules (11). Exportin-1 plays a central role in carcinogenesis, suggesting that it may be an attractive cancer drug target. Recent studies reported that the expression level of exportin-1 was increased in many solid tumors and that inhibition of exportin-1 resulted in cancer cell death (12-20).

Deregulation of exportin-1, with consequent cellular mislocalization of p33^{ING1b} has not previously been investigated in HNSCC. The p33^{ING1b} protein carries the nuclear localization signal (NLS). The way in which p33^{ING1b} in the nucleus is transferred to the cytoplasm is not known (8). Moreover, the effect of nuclear export of p33^{ING1b}, mediated by exportin-1 on molecular pathways in HNSCC has not been studied. Given the linkages of both tumor suppressor proteins and exportin-1 with subcellular localization and NES-dependent binding of exportin-1, we speculated that p33^{ING1b}-type tumor suppressor proteins might interact with various members of the karyopherin family, including exportin-1. This interaction could be associated with aberrant cytoplasmic p33^{ING1b} carrying a leucine-rich NES sequence.

Leptomycin B (LMB), a well-known exportin-1 inhibitor, binds covalently and irreversibly to cysteine 528 in the NES binding region of exportin-1, resulting in inhibition of nuclear export of cargo proteins (21). In the present study, we analyzed the subcellular distribution of the p33ING1b protein, evaluated the interaction of 33ING1b and exportin-1, and determined the effect of nuclear retention of p33ING1b on molecular alterations in HNSCC cells by exportin-1-inhibition using LMB. In addition, we analyzed the therapeutic effect of LMB in vitro in HNSCC cells and the mechanism underlying the antitumor activity of the LMB.

Materials and Methods

Cell culture

HNSCC lines. Primary (UT-SCC-74A [CVCL_7779]; UT-SCC-16A [CVCL_7812]) and metastatic lymph node tumor cells (UT-SCC-74B [CVCL_7780]; UT-SCC-16B [CVCL_7813]) that had been established from patients with tongue squamous cell carcinomas were kindly provided by Prof. Dr. Reidar Grenman (22).

The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/high glucose (Cat#SH30243.01; Hyclone, GE Healthcare), supplemented with penicillin (100 U/ml), streptomycin (100 µg/ml) (Cat#SV30010, Hyclone, GE Healthcare), 10% fetal bovine serum (Cat#SV30160.03; Hyclone GE Healthcare), 0.8% L-glutamine (Cat#SH30034.01; Hyclone, GE Healthcare), and 0.01% Plasmocin (InvivoGen ant-mpp) and then cultured at 37° C in a humidified atmosphere of 5% CO^2 and counted in a Neubauer chamber (23,24).

LMB treatment

To test the effect of pharmacological inhibition of exportin-1-dependent nuclear export, LMB (Cat#ab120501; Abcam, Cambridge, MA, USA) stored as a 10.2 µM stock in ethanol was used. The cells were treated with various concentrations of LMB.

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

RNA was isolated from the HNSCC lines using Trizol reagent (Invitrogen, Rockville, MD, USA) and transcribed into cDNA using a Transcriptor High Fidelity cDNA Synthesis Kit (Cat#05091284001, Roche Applied Science, Germany), according to the manufacturer's protocols. The qRT-PCR was performed using a SYBR Green qPCR kit (Cat#04887352001; Roche Applied Science, Germany) using the following primer pairs: exportin-1 (F 5′ GGGAAAACTGAAACCCACCT 3′ and R 5′ CTGAAATCAAGCAGCTGACG 3′), p33ING1b (F 5′ GCAGAGAAATGTCTCGCTGAT 3′ and R 5′ CTCACGATCTGGATCTTCTCG 3′), and β-actin (F 5′ TTCCTGGGCATGGAGTCCT 3′ and R 5′ AGGAGGAGCAATGATCTTGATC 3′). The relative expression levels of exportin-1, normalized to β-actin were calculated using the 2-ΔΔCT method (25, 26).

Western blot analysis

Protein was extracted from the HNSCC lines using radioimmunoprecipitation assay buffer (Cat#89900; Thermo Scientific, Vernon Hills, IL, USA). The protein concentrations were quantified using a bovine serum albumin (BCA) protein assay kit (Cat#23225; Pierce Biotechnology, Rockford, IL, USA), and the values were normalized using a standard BSA (Cat#9048-46-8; Sigma-Aldrich, St. Louis, MO, USA) curve.

The proteins were separated by electrophoresis in an SDS-PAGE gel and transferred to polyvinylidene difluoride HybondTM-ECLTM nitrocellulose membranes (Cat#RPN2020D; Amersham, Buckinghamshire, UK). The membranes were incubated at 4° C overnight with primary antibodies against exportin-1 (1:1000; Cat#ab24189; Abcam, Cambridge, MA, USA), p33ING1b (1:250; Cat#sc-7566-goat polyclonal (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and β-actin (1/20000; Cat#sc-47778; Santa Cruz Biotechnology, Santa Cruz, CA, USA). The membranes were subsequently incubated with horseradish peroxidase-linked secondary antibody anti-exportin-1 rabbit IgG (1:3000; Cat#ab9705; Abcam, Cambridge, MA, USA), anti-p33ING1b goat IgG (1:5000; Cat#P0449, Dako, Glostrup, Denmark), and anti-β-actin mouse IgG (1:2500; Cat#7076P2; Cell Signaling, Danvers, MA, USA) at 37° C for 1 h, with shaking. The bound proteins were then visualized using ECL substrate (Cat#1705060; Bio-Rad, USA) with the Chemidoc MP Imaging System (Bio-Rad, USA). The relative intensities were evaluated using Image J software (National Institutes of Health, Bethesda, MD, USA) and normalized to the signal intensity of β-actin.

Immunofluorescence analysis

The cells were first seeded onto 24-well plates (3×10^5 cells/well) on 13 mm coverslips (Cat#174950; Nunc Thermanox, Thermo Scientific, USA). At 48 h post-LMB treatment, the medium was removed, and the cells were fixed for 10 min with 4% formaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in phosphate-buffered saline (PBS) at room temperature. Following two washes with PBS and fixing, the cells were permeabilized in 0.5% Triton X-100 (Roche, Mannheim, Germany) in PBS for 10 min. After blocking with 1% BSA (Sigma-Aldrich, St. Louis, MO, USA) in PBS for 30 min, the cells were incubated with exportin-1 and p33ING1b primary antibodies (respectively, Cat#sc-5595; rabbit polyclonal, 1:1000 dilution and Cat#sc-7566; goat polyclonal 1:50 dilution, both Santa Cruz Biotechnology, Santa Cruz, CA, USA) in blocking buffer for 1 h. After washing twice in PBS, the cells were incubated with Alexa-Fluor 488 (Cat#Z25302; Life Technologies, USA) in a 1:200 dilution and 546 labeled secondary antibody (Cat#Z25004: Life Technologies, USA) in a 1:250 dilution for 30 min. After washing, the cells were counterstained with 10 µg/mL of diamido2-phenylindole dihydrochloride (DAPI) and mounted on coverslips with ProLong® Gold Antifade Reagent (Cat#P36934; Life Technologies, USA).

Apoptosis assays

The apoptotic status of the cells was investigated using a Caspase 3/7 Activity Assay Kit (Cat#12012952001; Roche Life Sciences, USA). Briefly, 1×10^5 cells/well were placed in 96-well plates and treated with LMB. Caspase 3/7 activity was measured 48 h after LMB treatment, and luminescence was monitored using a

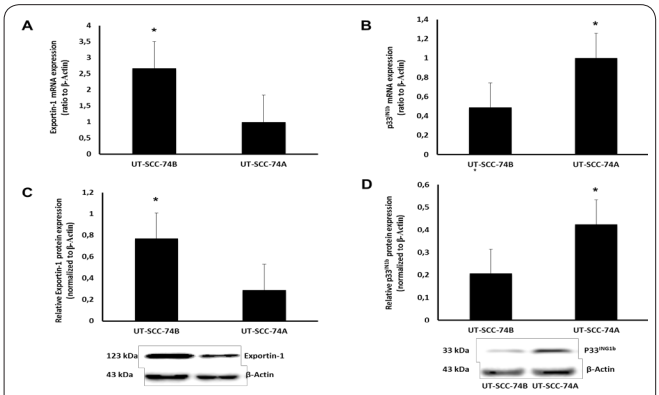

Figure 1. Expression of exportin-1 and p33^{ING1b} in metastatic HNSCC cells. Relative exportin-1 mRNA levels and p33^{ING1b} in HNSCC cell lines as determined by qRT-PCR analysis (A–B). The data were normalized to β-actin mRNA levels and was compared in the UT-SCC-74A primary HNSCC cell line and UT-SCC-74B metastatic lymph node tumor cells line. **A.** Relative exportin-1 mRNA expression levels were significantly upregulated in metastatic HNSCC ($p < 0.05$). **B.** The expression of the p33^{ING1b} in the primary HNSCC line was higher than that in the metastatic cell line ($p < 0.05$). Representative Western blot images showing the levels of exportin-1 and p33^{ING1b} proteins in the HNSCC lines and a histogram showing the relative signal intensity, as evaluated by Image J software. Protein levels were determined by Western blot analysis, as described in the Material and Methods. The signal intensity of protein bands in each sample was normalized to that of β-actin, and fold changes are presented in the histogram. (C–D). **C.** Exportin-1 protein expression was significantly increased in metastatic HNSCC cells ($p < 0.05$). D. The expression of p33^{ING1b} was markedly increased in primary HNSCC cells ($p < 0.05$). The results are presented as the mean ± SEM. *, represents significant suppression ($p < 0.05$). Exportin-1, also known as chromosome region maintenance 1 protein or Crm1. p33^{ING1b}, inhibitor of growth protein 1b.

VeritasTM Microplate Luminometer.

Cell proliferation

The cell proliferation status of the cells was analyzed using the xCELLigence Real Time Cell Analyzer System (RTCA DP) (Roche, Mannheim, Germany) and an XTT cell proliferation assay (2,3-is-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) (27).

For the xCelligence assay, 100 ml of DMEM containing 2% fetal bovine serum were added to the wells. After 1 h of equilibration with the medium, 100 ml of cell suspension (1×10^5 cells) were added to each well. Measurements were obtained at intervals of 15 min, and the results were analyzed using RTCA software. For the XTT assay, the cells were cultured separately in 96-well plates in fresh serum-free medium (1×10^5 cells/well) 24 h after inhibition (28). Briefly, the cells were incubated with 50 ml of XTT solution (Cat#11465015001; Roche, Mannheim, Germany) for 4 h at 37° C in a humidified atmosphere of 5% CO^2.

Migration assay

The cells were cultured separately in 6-well plates in fresh serum-free DMEM (3×10^5 cells/well). After 24 h, when the cells had reached about 80% confluence, the cell monolayer was scratched in the middle of the wells using a sterile 200-µl micropipette tip and photographed using a Leica inverted microscope (Cat#DM100; DFC 295, Germany). The images were captured at 10× magnification.

Statistical analysis

The data were statistically analyzed using the Mann–Whitney *U* test, and a *p*-value of < 0.05 was considered statistically significant.

Results

Exportin-1 expression was significantly upregulated in metastatic HNSCC lines, whereas p33^{ING1b} expression was downregulated

We analyzed exportin-1 and p33^{ING1b} gene expression data from primary and metastatic HNSCC lines by qRT-PCR analysis. Exportin-1 was significantly overexpressed in both metastatic HNSCC lines in comparison with primary cell lines ($p < 0.05$) (Fig. 1A), with a greater increase in UT-SCC-74A than UT-SCC-16A (data not shown). The primary HNSCC lines also exhibited higher p33^{ING1b} gene expression in comparison with that of the metastatic cell lines ($p < 0.05$) (Fig. 1B).

The results of the Western blot analysis of protein expression of exportin-1 and p33^{ING1b} in cultured cell lines are shown in Figure 1C–D. Exportin-1 was overexpressed and p33^{ING1b} was poorly expressed in the metastatic HNSCC cell lines as compared with that in the primary HNSCC cell lines ($p < 0.05$) (Fig. 1C, 1D). The results of immunostaining of exportin-1 and p33^{ING1b} levels in HNSCC lines confirmed the qRT-PCR and Western blot analysis findings (Fig. 2).

HNSCC exhibited predominant cytoplasmic p33^{ING1b} localization, which was reversed by inhibition of exportin-1

We observed marked cytoplasmic p33^{ING1b} staining in HNSCC cells. In addition, LMB-induced inhibition of exportin-1 initiated nuclear accumulation of p33^{ING1b} (Fig. 3).

Inhibition of exportin-1 induced nuclear entrapment and upregulation of p33^{ING1b}

To test the causal correlation of nuclear p33^{ING1b} and HNSCC apoptosis with exportin-1-inhibition, we treated HNSCC cells with LMB for 48 h. The results revealed overexpression of p33^{ING1b} in the HNSCC cells. In addition, LMB-induced inhibition of exportin-1 in-

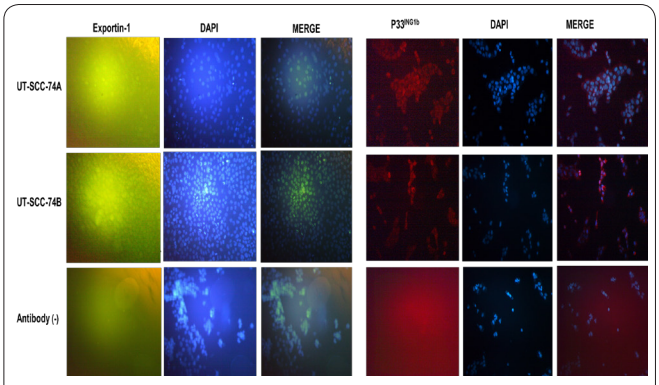

Figure 2. Immunohistochemical analysis of exportin-1 expression and p33^{ING1b} in HNSCC lines. Merged images following incubation with anti-exportin-1 antibody and anti-p33^{ING1b} antibody and DAPI. DAPI, diamido2-phenylindole dihydrochloride.

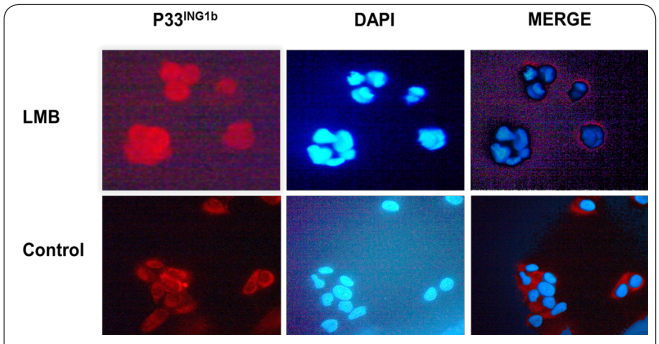

Figure 3. Effect of LMB treatment on the inhibition of exportin-1 and nuclear accumulation of p33ING1b. Nuclear staining of UT-SCC-74B cells plated on coverslips and treated with 10 nM LMB for 48 h. The cells were stained for p33ING1b (red) and treated with DAPI to stain the nucleus (blue). The cells were imaged by fluorescence microscopy.

duced nuclear entrapment of p33ING1b ($p \leq 0.01$) (Fig. 4A, 4B). To shed light on events prior to LMB-induced inhibition of exportin-1 and apoptosis and understand the potential role of p33ING1b, we studied caspase activity in a caspase 3 assay. The assay revealed increased apoptosis and increased caspase 3 activity in the LMB-treated cells as compared with the control ($p \leq 0.01$) (Fig. 4C).

Inhibition of exportin-1 repressed cell proliferation in HNSCC cells

The effect of LMB-induced inhibition of exportin-1 on cell proliferation in primary HNSCC and metastatic cell lines was studied in xCELLigence and XTT assays. Cell proliferation was monitored 72 h after LMB treatment. Interestingly, inhibition of exportin-1 had a significant impact on mediation of cell proliferation repression in both primary and metastatic cancer cells ($p < 0.05$) (Fig. 5A, 5B). The XTT assay showed that an increase in LMB concentrations did not enhance cell growth suppression (Fig. 5C). As shown by the assay, LMB treatment of HNSCC lines at concentrations of

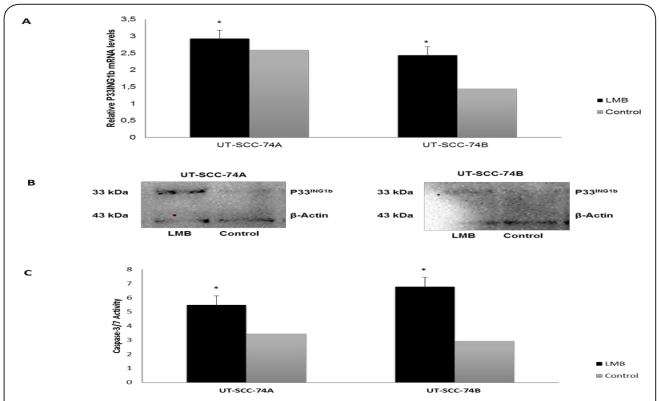

Figure 4. Influence of LMB-induced inhibition of exportin-1 on nuclear entrapment and upregulation of p33ING1b. **A.** Relative p33ING1b mRNA levels in primary and metastatic HNSCC lines, as determined by qRT-PCR analysis ($p \leq 0.01$ and $p \leq 0.01$, respectively). The data were normalized to β-actin mRNA levels and then was compared in UT-SCC-74A and UT-SCC-74B. The error bars show ± SD. **B.** Western blot images showing the levels of the p33ING1b protein in HNSCC lines 48 h after LMB treatment. The signal intensity of the protein bands was normalized to the signal intensity of β-actin. **C.** Cellular viability was reduced, and caspase 3/7 activity was enhanced in the primary and metastatic cell lines after LMB treatment ($p \leq 0.01$ and $p \leq 0.01$, respectively).

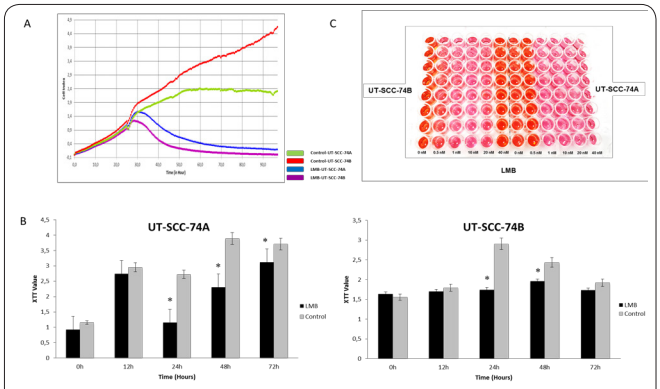

Figure 5. Effect of inhibition of exportin-1 on cell proliferation in primary and metastatic HNSCC lines. Impact of LMB-induced inhibition of exportin-1-dependent nuclear export on cell proliferation in primary and metastatic HNSCC cells (A–B). **A.** Cell proliferation was significantly decreased in the LMB-treated cells as compared with that in the control, as shown by the xCELLigence RTCA-DP system ($p < 0.05$). **B.** An XTT cell proliferation assay confirmed the results of the xCELLigence analysis ($p < 0.05$). **C.** Representative photograph of the XTT assay of head and neck cancer cells cultured in a 96-well 72 h after LMB treatment. The dark orange color represents the quantity of cell proliferation, and the pale orange color indicates toxicity. In the XTT assay, after incubation with XTT solution, LMB treatment of UT-SCC-74A and UT-SCC-74B at concentrations of 0, 0.5, and 40 nM induced a strong orange color, whereas treatment at concentrations of 1, 10 and 20 nM induced a light orange color.

10 and 20 nM markedly diminished the proliferation of HNSCC cells ($p < 0.05$ for both; data not shown). However, HNSCC cells showed a modest decrease in proliferation after LMB treatment at a concentration of 40 nM as compared with that of LMB at concentrations of 10 and 20 nM.

Inhibition of exportin-1 suppressed HNSCC migration capability

We investigated the migration of primary and metastatic HNSCC cells in the presence and absence of LMB in a scratch assay. Images were taken 0 h, 12 h, 24 h, and 48 h post-LMB treatment. A greater number of cells migrated in the control than HNSCC lines. The HNSCC lines displayed significant migration loss at an LMB concentration as low as 0.5 nM as compared with the control ($p < 0.05$) (Fig. 6A, 6B).

Discussion

HNSCCs are one of the most common types of cancer and a frequent cause of cancer mortality (1). Exportin-1 and p33ING1b proteins have different functions in cell physiology and are known to play important roles in carcinogenesis (4,13). In the current study, we investigated the expression of exportin-1 and p33ING1b in HNSCC cell lines. Previous research reported that p33ING1b was downregulated in several types as cancer, especially in metastatic cell lines (5), consistent with the findings of the present study.

p33ING1b plays a key role in the cell cycle and proliferation (4). Previous research demonstrated that alterations in p33ING1b and exportin-1 appeared to affect cancer progression and that upregulation of exportin-1 and downregulation of p33ING1b expression seemed to in-

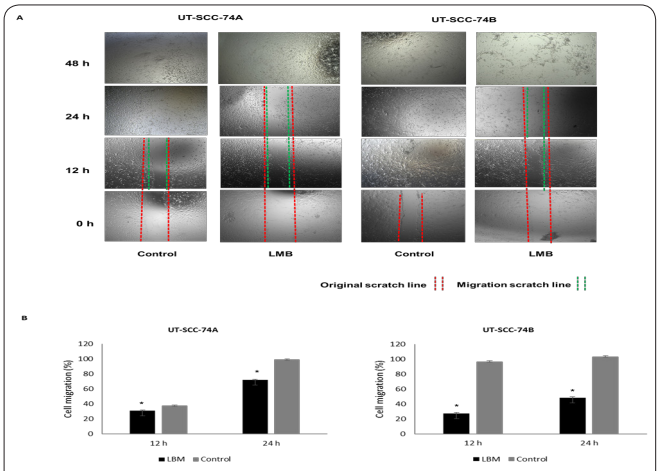

Figure 6. Influence of inhibition of exportin-1-dependent nuclear export on tumor cell migration. **A.** Cell motility in primary and metastatic HNSCC lines was examined by a scratch assay. The red dotted lines mark the edges at the start of the experiments, and the green dotted lines mark the edges at the end of the experiments. Cell migration was observed by microscopy and photographed (10× magnification) (scale bar, 250 μm). **B.** The cell number that migrated was counted, and the percentage of cell migration was plotted. *, represents significant suppression ($p < 0.05$) of cell migration in the LMB-treated cells as compared with the control.

crease malignancy (6,23). A number of previous studies showed that nuclear p33^{ING1b} was particularly important for the control of malignant growth and survival of HNSCC cells (5-8). Our data and those of previous reports demonstrated increased protein levels of p33^{ING1b} in the cytoplasm of HNSCC cells, suggesting that it could act as a tumor suppressor in cytoplasm (5,8). However, as p33^{ING1b} mutations are very rare, mislocalization of p33^{ING1b} in tumorigenesis is likely due to some mechanism that remains to be uncovered (5).

The exportin-1 protein is a nuclear exporter of key molecules and regulator of the subcellular distribution of their (3,9,10). Exportin-1 targets cargo proteins that carry a leucine-rich NES, which is required for the binding of p33^{ING1b} to exportin-1 (11). p33^{ING1b} contains NLS and plant homeodomain (PHD) motifs, which are involved in the subcellular activities of p33^{ING1b} (7,8). The NLS motif of p33ING1b carries a leucine-rich stretch at both the N-terminus and C-terminus regions (UniProtKB - Q9UK53) and is transferred from the cytoplasm to the nucleus by importins (29). Van der Watt et al. reported that the expression of the tumor suppressor proteins p27^{Kip1}, p53, p21^{Cip1}, and p18 was considerably increased and that their subcellular location was shifted in exportin-knockdown cervical cancer cells (12). The altered expression and nuclear accumulation of p33^{ING1b} after exportin-1-inhibition in their study, together with the finding in the present study of a formerly unreported motif, most likely NES, in p33^{ING1b}, provide support for a potential interaction between exportin-1 in mediating the export of p33^{ING1b}. However, we did not investigate the interaction of p33^{ING1b} and exportin-1 in our study. Consistently, Gong et al. (8) demonstrated that inhibition of 14-3-3η in cancer cell lines (HEK293 and HeLa) resulted in a better tumor response and mediated cytoplasmic accumulation of p33^{ING1b}. Cytoplasmic p33^{ING1b}-mediated control of cell viability and proliferation and nuclear retention of p33^{ING1b} after exportin-1-inhibition point to the critical role of p33^{ING1b} in enhanced apoptosis in HNSCC cells (5-8). To our knowledge, the current study is the first to report nuclear entrapment of p33^{ING1b} in HNSCC cells in which exportin-1 was inhibited by LMB.

In accordance with the findings of other studies (5-8,30), the results of the current study revealed that LMB-induced inhibition of exportin-1 suppressed cell viability and enhanced caspase 3/7 activity in HNSCC cells. As reported previously, exportin-1 recognizes numerous tumor suppressor proteins, and shifts their subcellular locations (12). Therefore, it seems likely that LMB-induction of nuclear accumulation of p33^{ING1b} and other tumor suppressor proteins would result in an increase in tumor suppressor activity in cancer cells. As p33^{ING1b} only partially mediated the apoptotic effect induced by LMB, it is likely that p33^{ING1b}-independent mechanisms are also involved in cell death induced by exportin-1 inhibition.

Previous studies showed that exportin-1 played an important role in nuclear export of macromolecules belonging to pathways involved in the metastatic behavior of tumors (11-13). The present study revealed marked induction of exportin-1, particularly in metastatic HNSCC cells. These data are consistent with the findings of previous reports (12-20). Previous research reported that overexpression of exportin-1 was associated with tumor progression and that exportin-1 expression was a prognostic factor in human cancers due its role in the regulation of mitosis (12). A number of studies confirmed the prognostic value of exportin-1 expression in other types of cancers, such as cervical squamous cell carcinomas (12), ovarian cancer (13), osteosarcomas (14), pancreatic cancer (15), esophageal cancer (16,17), gliomas (18), gastric cancer (19), and multiple myelomas (20). Yang et al. (16) reported that knockdown of exportin-1 induced apoptosis in esophageal cancer cell lines (ECA109, TE1, TE8, and KYSE30). Similarly, van der Watt et al. (17) showed that inhibition of exportin-1 triggered cell death in esophageal cancer lines (WHCO5 and KYSE30). To the best of our knowledge, this is the first study to report elevated expression of exportin-1 in UT-SCC-74A and UT-SCC-16A (primary HNSCC lines) and UT-SCC-74B and UT-SCC-16B (metastatic lymph node tumor cells) (21). Head and neck cancer accounts for more than 90% of all cases of squamous cell cancer. Of about 300 HNSC cell lines that have been established, the majority originated from the oral cavity (31). However, these cell lines represent only general characteristics of head and neck tumors. As tumor cell lines may have various genetic and phenotypic differences, different lines should be studied in further research.

Current therapies for HNSCC are limited and associated with high rates of relapse and metastasis, highlighting the urgent need for alternative therapeutic modalities (2,3). The development of new exportin-1 inhibitors, such as LMB, underlines the potential utility of this protein as a possible therapeutic target in cancer (32). Previous research reported that aberrant function of exportin-1 led to dysregulation of cell growth (33). Therefore, targeting exportin-1 is an encouraging therapeutic strategy for HNSCC. We demonstrated that LMB-induced inhibition of exportin-1 mediated the cellular vi-

ability, cell proliferation, and cell migration capabilities of HNSCC cells. These results provide support for the idea that targeting exportin-1 can overcome resistance mechanisms in HNSCC cells. They also strengthen the evidence for the role of exportin-1 as a drug target.

LMB covalently binds to and inhibits exportin-1 at a specific cysteine residue (21). van der Watt et al. (12,17) reported that LMB treatment at nanomolar concentrations was an effective antiproliferative agent against cancer cells. Similarly, in the present study, LMB treatment of primary and metastatic cells at concentrations of 10 and 20 nM LMB, respectively, suppressed the proliferation of HNSCC cells in vitro. Dose differences in the effects of LMB were supported by Kuusisto et al. (34). They reported that exportin-1's inhibitor concentration increased for export machine-inhibition due to nuclear export activity in transformed cells as compared with than in nontransformed cells. Surprisingly, in the present study, increasing the LMB dose from 0.5 to 40 nM did not enhance the suppression of cell growth, emphasizing the high specificity and effectiveness of LMB at low concentrations.

The results of the present study revealed the participation of multiple cellular networks. The findings provide a basis for the utilization of LMB as a treatment for HNSCC cells and other malignant tumor types displaying severe exportin-1-dependance. In addition, the results shed light on alterations in the expression and localization of $p33^{ING1b}$ induced by LMB-mediated inhibition of exportin-1. Information is lacking on the nature of the molecular interactions between exportin-1 and $p33^{ING1b}$. Therefore, further functional studies using novel experimental methods, human tissue samples, and large and diverse HNSCC lines are necessary to shed light on the molecular mechanisms of HNSCC.

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