Matrix Metalloproteinase 14 promotes lung cancer by cleavage of Heparin-Binding EGF-like Growth Factor

Abstract

Molecularly targeted therapies benefit approximately 15–20% of non-small cell lung cancer (NSCLC) patients carrying specific drug-sensitive mutations. Thus, there is a clinically unmet need for the identification of novel targets for drug development. Here, we performed RNA-deep sequencing to identify altered gene expression between malignant and non-malignant lung tissue. Matrix Metalloproteinase 14 (MMP14), a membrane-bound proteinase, was significantly up-regulated in the tumor epithelial cells and intratumoral myeloid compartments in both mouse and human NSCLC. Overexpression of a soluble dominant negative MMP14 (DN-MMP14) or pharmacological inhibition of MMP14 blocked invasion of lung cancer cells through a collagen I matrix in vitro and reduced tumor incidence in an orthotopic K-RasG12D/+ p53−/− mouse model of lung cancer. Additionally, MMP14 activity mediated proteolytic processing and activation of Heparin-Binding EGF-like Growth Factor (HB-EGF), stimulating the EGFR signaling pathway to increase proliferation and tumor growth. This study highlights the potential for development of therapeutic strategies that target MMP14 in NSCLC with particular focus on MMP14-HB-EGF axis.

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Introduction
Lung cancer remains the leading cause of cancer-related death, resulting in 1.4 million deaths annually worldwide [1]. Non-small-cell lung carcinoma (NSCLC) accounts for 80% of all lung cancers [2]. Despite advances in treatment options prognosis for NSCLC patients remains dismal. Therefore, further molecular analysis of NSCLC is necessary for the development of additional novel and specific targeted therapies for NSCLC.

Matrix Metalloproteinases (MMPs) comprise a family of proteolytic enzymes involved in the degradation of extracellular matrix (ECM) [3]. Of the various MMPs, MMP14, 15, 16, 23, and 24 are the membrane bound. MMP14 (MT1-MMP) is unique as it is the only membrane-bound MMP capable of degrading collagen I, therefore playing a crucial role in cellular migration through ECM. Notably, MMP14 null mice develop abnormalities and die by 4 weeks, suggesting that MMP14 deficiency cannot be compensated by other MMPs [4]. MMP14 is upregulated in many human tumors [5,6] and elevated levels of MMP14 and its substrate MMP2, correlate with poor prognosis and increased metastasis [5].

Activation of MMP14 molecule is regulated by the hemopexin domain (HPX) located between the catalytic domain (Cat) of the enzyme and its transmembrane fragment (TM). HPX domain is involved in recognition of proteolytic substrates of MMP14 and dimerization of HPX significantly increases activity of MMP14, which cleaves and activates pro-MMP2 and pro-MMP13 [7]. This further promotes ECM proteolysis [8,9], and results in enhanced migration, invasion and metastatic dissemination of tumor cells [10,11]. MMP14 localizes at the leading edge of invadopodia in migrating cells via its interaction with glycoprotein receptor CD44 [12,13]. Interaction with CD44 receptor is suggested to induce phosphorylation of the EGFR receptor, and downstream activation of the MAPK and PI3K signaling pathways [14].

Clinical data shows that MMP14 expression is increased in NSCLC compared to normal lung tissue, and MMP14 is associated with poor prognosis [15]. Notably, in lung regeneration, endothelial MMP14 cleaves heparin bound (HB)-EGF, a member of the epidermal growth factor (EGF) family, and the bioavailable EGF activates cell proliferation via the EGF receptor pathway. However, the functional contribution of MMP14 in NSCLC remains poorly understood, and the potential of MMP14 inhibition has not been explored. In this study, we show that MMP14 expression is upregulated in both the epithelial and myeloid compartments of the tumor microenvironment in patients, and in an orthotopic mouse model of NSCLC. Furthermore, we provide mechanistic insights by which MMP14 contributes to NSCLC progression, and demonstrate that blocking the proteolytic activity of MMP14 can effectively block tumor growth.

Materials and Methods

Mouse Model
All animal work was conducted in accordance with a protocol approved by the Institutional ACUC at WCMC. The HKP1 lung cancer cells was derived from KP tumor lungs [16], and was cultured in DMEM with 10% FBS. 1x10^5 of HKP1 cells were administered via tail vein to C57BL/6 mice (Jackson Laboratory) to generate orthotopic lung cancer and imaged by bioluminescence imaging (BLI) system (IVIS, Caliper Life Sciences).

Tissue Microarrays (TMAs)
For evaluation of MMP14 expression in NSCLC patients we used TMAs derived from 210 lung cancer patients from WCMC. Seventy-four percent of the patients were at stage IA/IB, 8% at stages IIA/IIB, 12% at stage III and 6% at stage IV. Immunohistochemical staining of MMP14 (Clone LEM-2/63.1, Abcam) was performed using the Bond III Autostainer (Leica Microsystems, IL, USA). TMAs were examined in a double blinded manner by two individuals using scale 0 to 3 with score >1 considered positive.

Generation of KP Cell Lines Expressing Dominant Negative FLAG-MMP14

Mouse MMP14 cDNA (Open Biosystems) was cloned into pCDH-EF1-MCS-T2A-copGFP lentiviral vector (System Biosciences). Analogically we generated a dominant negative (DN) form of MMP14-GFP (ΔTMDΔCat MMP14) containing deletions between Tyr112-Gly288 and Ala536-Val582 [17]. For detection, a FLAG tag was inserted between Arg111 and Tyr112. Lentiviral particles were produced in 293 T cells by co-transfection of the MMP14 construct with Lenti Packaging Mix (Advanced Biomedical Materials). GFP positive cells were selected by FACS sorting (LSRII, BD) 5 days after infection. As a positive control for MMP14 inhibition we used NSC405020 small molecule inhibitor at 50uM concentration (Tocris).

Results

MMP14 is Upregulated in Epithelial and Myeloid Compartments in Mouse NSCLC
Using an orthotopic Kras-driven NSCLC mouse model (K-ras^{LSL-G12D+};p53^{flox/flox}), we have evaluated the differential gene expression in epithelial and intratumoral stromal cells by RNA-seq analysis [18]. Interestingly, we found that MMP14 was significantly upregulated both in intratumoral myeloid cells (CD11b+ F4/80+ macrophages, Ly6C+ monocytes, Ly6G+ neutrophils) and EpCam + tumor cells (Figure 1A and Supplementary Table 1). Quantitative RT-PCR analysis (Figure 1B) and Western-blot (Figure 1C) confirmed increased expression of MMP14 in the intratumoral myeloid and epithelial compartments. Immunofluorescence staining of lung sections showed that membrane-specific MMP14 expression was detected in both the epithelial (EpCam+) cells and hematopoietic (CD45+) stromal cells in the tumors (Figure 1D).

Together, these data show that MMP14 expression is markedly increased in NSCLC tumor, which was contributed by both tumor epithelial and stromal cells.

MMP14 is Up-Regulated in Epithelial and Myeloid Compartments in Human NSCLC

The up-regulation of MMP14 expression in tumor tissue was also observed in NSCLC patients. We have performed RNA-seq analysis of individual cell populations isolated from patient-derived tumors compared to matched adjacent non-neoplastic lung tissue [19]. The result showed that MMP14 was specifically upregulated in tumor tissue compared to adjacent lungs (Figure 2A and Supplementary Table 2) with MMP14 expression increased in both intratumoral myeloid cells (CD11b+ CD133+ monocytes, CD11b+ CD33- neutrophils) and EpCam + epithelial cells (Figure 2A). The increase expression of MMP14 was confirmed by qRT-PCR (Figure 2B). Consistently, immunofluorescence staining showed that MMP14 expression was found in both epithelial (EpCam+) cells and hematopoietic (CD45+) cells in the tumors (Figure 2C). With a tissue microarray (TMA) derived from a large cohort of NSCLC patients (n = 210), high MMP14 expression was detected in approximately 68% of patients. Among them, 10% showed
MMP14 staining in both stromal and epithelial compartments (Figure 2, D and E).

The consistent high expression of MMP14 in both mouse lung cancer model and NSCLC patients suggests that MMP14 may play a pivotal role in NSCLC progression.

Dominant Negative MMP14 Inhibits Invasion of Cancer Cells by Suppressing Their Ability to Degrade Collagen I

To obtain insights by which MMP14 contributes to NSCLC, we sought to generate MMP14 loss-of-function in HKP1 lung tumor cells. Given that homodimerization of MMP14 through the hemopexin (HPX) domain is important for its optimal proteolytic activity [17,20], we chose to use a dominant negative (DN) approach. The DN-MMP14 (ΔTMΔCat-FLAG-MMP14) construct lacks the transmembrane and the catalytic domains (Figure 3A). Since the hemopexin (HPX) domain remains intact, the DN-MMP14 is still able to dimerize with wild type MMP14 and block its catalytic function [7] probably by inhibiting ability of MMP14 to bind collagen.

HKP1 cells overexpressing the DN-MMP14 were generated by infecting wild type HKP1 cells with lentivirus carrying DN-MMP14. Western blot analysis of HKP1 cell lysates showed robust expression of DN-MMP14 (40 kDa) as well as DN-MMP14-GFP fusion protein (64 kDa), possibly due to imperfect efficiency of ribosome
skipping. Of note, both DN-MMP14 (40 kDa) and DN-MMP14-GFP (64 kDa) were efficiently secreted, as both bands were detected in the supernatant compartment (Figure 3B). The overexpression of DN-MMP14 did not affect the expression of endogenous MMP14 (64–66 kDa) (Figure 3B). The surface presentation of endogenous MMP14 was not affected either as confirmed by surface biotinylation assay (Figure 3C). Interestingly, DN-MMP14 expressing cells displayed decreased levels of active MMP-2, suggesting that dominant-negative approach blocks effectively MMP14-mediated processing of MMP2 precursor (Figure S3).

Comparison of proliferation rates showed that both control (expressing GFP) and DN-MMP14 expressing HKP1 cells proliferated at similar rate. Interestingly in a serum free media control HKP1 cells retained high proliferation while the proliferation of DN-MMP14 cells was significantly impaired (Supplementary Figure S1). Such observation suggested that MMP14 through its proteolytic
activity can help releasing growth factors that can act in an autocrine manner. Given MMP14 [21,22] is the only membrane MMP able to degrade collagen I, we tested the impact of DN-MMP14 on MMP14 function by collagen I degradation assay. Control (expressing GFP) or DN-MMP14 expressing HKP1 cells were seeded in 6-well plates coated with collagen I and cultured for 72 hr. While the control cells displayed considerable collagen degradation, the DN-MMP14 expressing cells were significantly defective in removing the collagen (Figure 3, D and E). Consistently, the DN-MMP14 expressing HKP1 cells were dysfunctional to invade through the collagen I layer in a transwell assay. They showed a 10-fold decrease in invasion as compared the control cells (Figure 3F). Pharmacological inhibition of MMP14 with a specific small-molecule inhibitor NS405020 decreased invasion even more severely almost totally blocking ability of HKP to migrate through collagen (Supplementary Figure S2).

Together, these results suggest that DN-MMP14 blocks MMP14 function and abrogates tumor cell invasion through the ECM possibly due to its impaired ability to degrade collagen I, which encouraged us to further investigate MMP14 roles in NSCLC in vivo.
**DN-MMP14 Impairs Tumor Growth in the HKP1 Mouse Model of Lung Cancer**

To assess the role of MMP14 in vivo, we employed orthotopic lung tumor model by injecting control or DN-MMP14 expressing HKP1 cells through tail vein into the syngeneic immunocompetent mice. The control HKP1 cells generated massive lung tumor burden in mice as manifested by measuring the lung weight 4 weeks after inoculation. Significantly, the expression of DN-MMP14 led to less lung tumor burden (Figure 4A). H&E analysis of lung sections also revealed significantly bigger tumor area in control mice (18% ± 0.7), as compared to the DN-MMP14 group (0.01% ± 0.5) (Figure 4B). The whole lung images showed massive hemorrhagic tumors in the control lungs, whereas tumors were not visible in lungs with DN-MMP14 HKP1 cells (Figure 4B).

The dynamic monitoring of tumor growth by BLI also revealed that control HKP1 cells rapidly formed tumors, whereas HKP1 cells expressing DN-MMP14 failed to generate tumors (Figure 4C). Further analysis of lung lysates showed that DN-MMP14 expressing tumor cells were present in the lungs at the time of sacrifice (Figure 4D), suggesting that DN-MMP14 cells survived in the lung, but fail to establish tumors. H&E staining of lung tissues showed a lack of detectable tumor nodules in DN-MMP14 mice, whereas large nodules were observed in the control group (Figure 4E, upper panel). The immuno-stainings consistently showed that the residual DN-MMP14 cells expressed the FLAG reporter (Figure 4E, lower panel). These results demonstrate that MMP14 loss-of-function results in impaired lung tumor growth in vivo.

**MMP14 Cleave Heparin-Binding EGF-Like Growth Factor to Activate the Protumorigenic EGFR Pathway**

MMP14 is best known to accelerate tumor progression by cleavage of collagen I and degradation of ECM. Interestingly, MMP14 was recently reported to participate in the lung regeneration by activation of HB-EGF and stimulation of growth of lung epithelial cells [24]. To determine whether the same mechanism can be used by MMP14-expressing cancer cells to promote growth of lung tumors, we re-analyzed our RNA sequencing data to identify known substrates of MMP14. HB-EGF, an important factor in survival signaling [23] and lung regeneration [24], was upregulated in HKP1 tumor epithelial cells, as well as monocytic and neutrophil populations within the tumor microenvironment (Figure 5A). MMP14 is known to cleave off 20 amino acids at the N-terminus of HB-EGF to generate heparin-independent growth factors: mHB-EGF (membrane-bound) and sHB-EGF (soluble) (Figure 5B) [25,26].

To evaluate processing of HB-EGF in the presence and absence of functional MMP14, we analyzed both the intracellular and extracellular fractions of HKP1 cells by Western blot. Control HKP1 cells expressed a 23 kDa unprocessed HB-EGF isoform in the cellular fraction, while the extracellular fraction expressed a distinct pattern of multiple isoforms (Figure 5C). Although DN-MMP14 HKP1 cells also expressed the 23 kDa unprocessed HB-EGF isoform in the cellular fraction, there was a marked decrease in the ~18 kDa and the ~9 kDa isoforms from the extracellular fraction (Figure 5C). Cleavage patterns of HB-EGF revealed that the 18 kDa and 9 kDa isoforms correspond to membrane-bound and soluble active HB-EGF respectively, consistent with previous observations [25,26]. In accordance with the HB-EGF cleavage patterns, DN-MMP14-expressing HKP1 cells showed impaired EGFR signaling, as determined by reduction in the phosphorylated EGF receptor (p-EGFR) and its downstream signaling components including ERK1/2 (Figure 5D) and Supplementary Figure S5A. Consistent with our in vitro data, HKP1 tumors also exhibited impaired EGFR signaling (Figure 5E and Supplementary Figure S5B). Immunofluorescence staining of HKP1 tumors showed increased p-EGFR in the GFP+ nodules indicating that the EGFR pathway was activated in these tumors (Figure 5F). Together, these results suggest that MMP14-mediated cleavage of HB-EGF, results in the activation of the protumorigenic EGFR pathway in NSCLC.

**Discussion**

Clinical studies have shown that MMP14 expression is elevated in NSCLC cells, and confers poor prognosis in NSCLC patients [14,15]. However, the contribution of MMP14 in NSCLC has not been widely studied, and the therapeutic potential of MMP14 inhibition remains unexplored. By analyzing specific cellular compartments in tumor tissue, we have demonstrated that MMP14 expression is elevated in both tumor epithelial cells and myeloid cells of mouse and human NSCLC.

Our study involved use of dominant negative MMP14 (DN-MMP14) that lacked the transmembrane and catalytic domains of the mature enzyme but contained intact hemopexin domain. Such construct has been reported to inhibit activity of MMP14 by dimerizing with wild-type enzyme [7]. In our study HKP1 lung cancer cells expressing DN-MMP14 were impaired in their ability to degrade collagen I, associated with decreased invasiveness and marked suppression of tumor growth. While most of the synthesized DN-MMP14 appeared to dimerize with the wild-type MMP14 on the surface of the lung tumor cells in an autocrine fashion, the secreted form in the extracellular milieu could also effectively target MMP14 on the stromal cells, thus inhibiting role of stroma-derived MMP14 on tumor progression.

MMP14 promotes metastatic dissemination of cancer cells by cleaving components of ECM, including type I and III collagen, fibronectin I and II and laminin 1 and 5 [9,10]. Following dimerization, MMP14 cleaves and activates proMMP2 and proMMP13, which enhances ECM proteolysis [7,8]. In our study, HKP1 cells expressing DN-MMP14 displayed reduced levels of active MMP2 and exhibited reduced invasion through collagen I layer, suggesting that MMP14 loss-of-function impacts tumor cell invasion through the ECM. Moreover, cells expressing DN-MMP14 were impaired in their ability to degrade collagen I, suggesting that the presence of active MMP14 may be essential for effective ECM remodeling. Furthermore, pharmacological blockade of MMP14 activity also inhibited invasion through collagen I confirming specificity of the DN-MMP14 approach. Interestingly, although our HKP1 DN-MMP14 cells did not contain deficiencies in other metalloproteinases, including ADAM10 (Supplementary Figure S4), specific inhibition of MMP14 was sufficient to produce such an overt phenotype. These observations underline the importance of MMP14 in NSCLC progression and make it an attractive therapeutic target.

Our results suggest that besides collagen degradation and ECM remodeling MMP14 can also contribute to lung cancer progression by releasing HB-EGF and activating EGFR signaling. The membrane-bound HB-EGF is not only a precursor of the soluble form, but also independently activates signaling pathways [27,28] being a potent factor in survival signaling [23]. An important co-factor of HB-EGF is heparin, which is required to unmask its
EGF-like domain in vitro [29] and heparin sulfate proteoglycans (HSPG), abundant in the ECM, have been suggested to fulfill this role in vivo [30]. However, MMP14 has been shown to diminish the heparin requirement, as cleavage of the N-terminal heparin-binding domain makes HB-EGF heparin-independent [24,25]. Our data suggest that NSCLC co-opts MMP14-mediated HB-EGF cleavage,
rendering heparin-independent, similar to a phenomenon observed in lung regeneration [24]. The activation of HB-EGF is likely to promote proliferation and survival signaling through the EGFR pathway, as HB-EGF binds EGFR with higher affinity than EGF, and is therefore a potent inducer of tumor growth [31]. Consistently, our results have shown that impaired proteolytic
activation of HB-EGF by MMP14 correlates with impaired EGFR pathway activation.

Our findings that the MMP14/HB-EGF/EGFR axis is involved in NSCLC progression underscore the potential of this pathway in the development of targeted therapies. Previous studies have assessed the potential of either HB-EGF or MMP14 as therapeutic targets in many types of cancer [32–35]. One drawback of the first generation of MMP14 inhibitors was that they targeted the catalytic site of the enzyme, demonstrating affinity not only to MMP14 but also to other MMPs and non-MMP proteases. These off-targeting effects often led to non-specific and severe toxic effects. In this study, we have evaluated the approach of DN-MMP14, which targets the hemopexin domain of MMP14. This allows for selective binding and potentially eliminates off-target effects. Expression of DN-MMP14 resulted in effective inhibition of NSCLC growth, thereby suggesting that blocking the hemopexin domain comprises an attractive therapeutic strategy. Our results is consistent with the previous report showing that the synthetic peptides targeting the hemopexin domain of MMP14 inhibited MMP-14-dependent cell migration, slowed growth of breast tumors, and did not impact other MMPs [13].

Altogether, our study suggests that MMP14 plays an important mechanistic role in NSCLC progression, by supporting cancer invasiveness, promoting collagen degradation, and releasing HB-EGF, which accelerates lung tumor progression. Inhibiting the MMP14- through targeting hemopexin domain of MMP14 may constitute a novel approach in lung cancer therapy that would significantly improve therapeutic outcome in NSCLC patients.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.neo.2016.11.005.

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