DDX24 promotes lymphangiogenesis and lymph node metastasis via AGRN production in cervical squamous cell carcinoma

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To the Editor: Cervical cancer (CC) is the second leading cause of cancer death in women, representing a major global health challenge.[1] Cervical squamous cell carcinoma (CSCC) accounts for 70% of CC cases, and pelvic lymph node metastasis is a critical cause of CC-related death.[2] Thus, understanding the underlying mechanisms of the tumor spread through lymphatic vessels becomes imperative.[3] RNA helicases are involved in almost all aspects of RNA metabolism.[4] DEAD-box helicase 24 (DDX24), one of the least explored DEAD-box RNA helicases, was upregulated in several cancer types.[5] However, the role of DDX24 in CSCC progression and metastasis remains elusive. Here, we investigated the essential role of DDX24 in mediating cancer cell EMT and lymphangiogenesis in the context of CSCC. Our study provides a potential therapeutic target for CSCC lymphatic metastasis.

To survey the distinct expression profile of CSCC, we analyzed two datasets, GSE9750 and GSE7803. Gene Set Enrichment Analysis (GSEA) revealed that DDX24 was robustly upregulated in CSCC tissues [Supplementary Figures 1A–C, http://links.lww.com/CM9/C238]. Given that we previously reported a link between DDX24 and lymphatic malformation,[6] we sought to investigate the role of DDX24 in lymphatic functions. To further verify the protein expression of DDX24 in CSCC, a tissue microarray (TMA) including 75 CSCC cases and 55 adjacent controls was examined by immunohistochemistry (IHC) staining. Intriguingly, the expression of DDX24 was higher in CSCC tissues with lymph node metastasis than in adjacent normal tissues and tissues without lymph node metastasis [Figure 1A]. Moreover, the protein level of DDX24 was positively correlated with the density of lymphatic vessels in CSCC tissues [Figure 1B], which was echoed by the positive correlation between the mRNA levels of DDX24 and LYVE1 [Supplementary Figure 1D, http://links.lww.com/CM9/C238]. Additionally, Kaplan–Meier analysis showed that patients with increased DDX24 expression were associated with shorter overall survival [Supplementary Figure 1E, http://links.lww.com/CM9/C238]. Taken together, these results link DDX24 expression to CSCC lymphatic metastasis.

To investigate the potential effects of DDX24 in facilitating CSCC metastasis, we first performed extensive investigations using the CSCC cell lines MS751 and Siha. Overexpression (OE) and knockdown (KD) of DDX24 were confirmed by both Western blot and real-time quantitative polymerase chain reaction (qRT-PCR) [Supplementary Figures 2A–D, http://links.lww.com/CM9/C238]. Cell invasion and migration assays demonstrated that OE of DDX24 enhanced both the abilities of CSCC cells, whereas KD of DDX24 attenuated both the abilities [Figure 1C and Supplementary Figures 2E–G, http://links.lww.com/CM9/C238]. Furthermore, we detected the suppression of epithelial marker E-cadherin and

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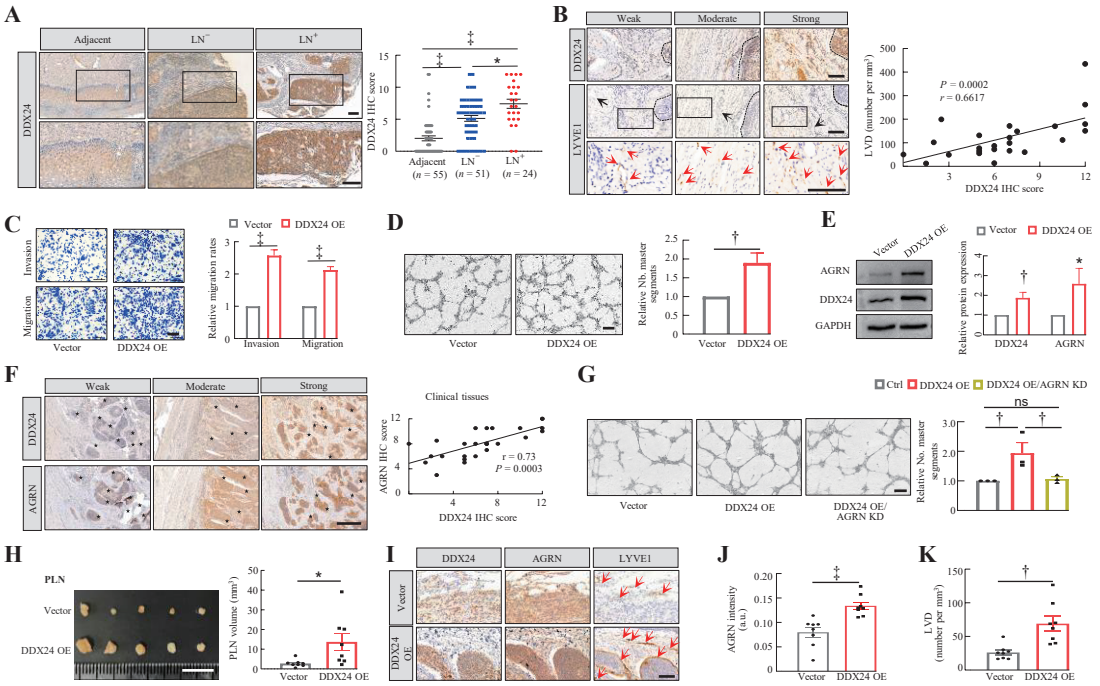

Figure 1: DDX24 promotes lymphangiogenesis and lymph node metastasis via AGRN production in CSCC. (A) Representative micrographs of DDX24 expression in tumor-adjacent tissues (Adjacent) and in CSCC without (LN^-) or with (LN^+) lymph node metastasis (Staining method: immunohistochemistry. Scale bar, 100 μm). (B) Representative micrographs and the correlation of LYVE1 positive microvessels with DDX24 expression in CSCC tissues by IHC staining. Black dotted line, tumor; red arrow, LYVE1 positive microvessels; black arrow, blood vessels; black box, enlargement. Scale bar, 200 μm. (C) Representative micrographs and quantifications showing the migration and invasion of DDX24-overexpressed Siha cells compared with empty vector (Vector) groups. Scale bar, 200 μm. (D) Representative images and quantifications of tube formation assay showing migration ability of HLECs cultured with CM derived from the DDX24-overexpressed Siha cells. Scale bar, 200 μm. (E) Western blot analysis showing the expression of AGRN and DDX24 in DDX24-overexpressed Siha cell lysate, respectively. GAPDH, loading control. (F) Representative micrographs and the correlation of AGRN and DDX24 expression in CSCC tissues by IHC staining. Black asterisk, tumor. Scale bar, 200 μm. (G) Representative images and quantifications of tube formation assay showing migration ability of HLECs cultured with CM derived from the Siha cells. Scale bar, 200 μm. (H) Representative photos and quantifications of the volume of PLN in mice. Scale bar, 1 cm. $n = 8$ for each group. (I) Representative micrographs of the expression of AGRN, DDX24, and LYVE1 in the footpad of mice. Red arrow, LYVE1 positive microvessels. Scale bar, 200 μm. Staining method: immunohistochemistry. (J, K) Quantifications of AGRN expression (J) and LVD (K) in the footpad of mice. $n = 8$ for each group. AGRN: Agrin; CM: Conditioned medium; CSCC: Cervical squamous cell carcinoma; DDX24: DEAD-box helicase 24; HLECs: Human lymphatic endothelial cells; IHC: Immunohistochemistry; KD: Knockdown; LN^-: CSCC without lymph node metastasis; LN^+: CSCC with lymph node metastasis; LVD: Lymphatic vessel density; LYVE1: Lymphatic vessel endothelial hyaluronan receptor 1; OE: Overexpression; PLN: Popliteal lymph node. $^*P < 0.05$; $^\dagger P < 0.01$; $^\ddagger P < 0.001$.

the increase of mesenchymal markers such as matrix metallopeptidase 9 (MMP9), N-cadherin, and Slug upon DDX24 OE [Supplementary Figure 2H, http://links.lww.com/CM9/C238]. Conversely, DDX24 deficiency in CSCC cells rendered the opposite effects [Supplementary Figure 2I, http://links.lww.com/CM9/C238]. These data suggest that DDX24 facilitates the invasion and migration ability of CSCC cells by boosting EMT.

In addition to cancer cell EMT, lymphangiogenesis is another crucial player in lymph node metastasis. Therefore, we evaluated the effect of DDX24 in CSCC cells on the functions of lymphatic endothelial cells. Compared to the conditioned medium (CM) of the control group, the CM of DDX24-overexpressing CSCC promoted cell proliferation of human lymphatic endothelial cells (HLECs) as detected by Ki67 expression, whereas silencing DDX24 in CSCC lessened the growth of HLECs [Supplementary Figures 3A, B, http://links.lww.com/CM9/C238]. Additionally, DDX24 in CSCC positively regulated the migration and tube formation of HLECs [Figure 1D and Supplementary Figures 3C–I, http://links.lww.com/CM9/C238]. To further validate DDX24-mediated lymphangiogenesis, we assessed the changes in key molecules involved in lymphangiogenesis. As expected, the levels of VEGFR3, phosphorylated AKT (p-AKT), and phosphorylated ERK1/2 (p-ERK1/2) were upregulated in HLECs stimulated by the CM of DDX24-overexpressing CSCC cells [Supplementary Figure 3J, http://links.lww.com/CM9/C238]. The opposite results were obtained when DDX24 was inhibited [Supplementary Figure 3K, http://links.lww.com/CM9/C238]. Collectively, these data indicate that DDX24 in CSCC cells regulates lymphangiogenesis.

To further interrogate the factor produced by CSCC cells that facilitates lymphangiogenesis, we analyzed the transcriptomics of CSCC cells after DDX24 KD (GSE229910) and identified that the extracellular matrix-related pathway was most significantly altered [Supplementary Figures 4A, B, http://links.lww.com/CM9/C238]. In terms of specific genes, *FGFR4* and *AGRN* were significantly altered [Supplementary Figures 4C–E, http://links.lww.com/CM9/C238]. Given that the molecule governing the cross-talk between HLECs and CSCC cells was more likely to be a secreted factor, *AGRN*, encoding the extracellular protein agrin, was chosen. To confirm that *AGRN* is regulated by DDX24, AGRN protein levels in both CSCC cell lysate and supernatant were found to be increased upon DDX24 OE and reduced upon DDX24 deficiency [Figure 1E and Supplementary Figures 4F–H, http://links.lww.com/CM9/C238]. Furthermore, the expression of AGRN in CSCC tumor tissues was positively correlated

with that of DDX24 [Figure 1F and Supplementary Figures 4I, http://links.lww.com/CM9/C238]. Importantly, elevated AGRN was associated with poor prognosis [Supplementary Figure 4J, http://links.lww.com/CM9/C238]. To further elucidate the upstream regulator of AGRN, we scanned the *AGRN* promoter sequence for transcription factor (TF) binding sites using the TARP tool.[7] Seven TFs were predicted [Supplementary Figures 4K–M, http://links.lww.com/CM9/C238]. Among them, MZF1 and SP1 were downregulated in MS751 cells upon DDX24 KD [Supplementary Table 1, http://links.lww.com/CM9/C251], and they have been reported to promote cancer progression in CC.[8,9] The above results indicate that AGRN is a downstream target of DDX24 in CSCC.

We next explored whether AGRN in CSCC cells could affect lymphangiogenesis. AGRN-silenced Siha cells were generated [Supplementary Figure 5A, http://links.lww.com/CM9/C238]. Proliferation, migration, and tube formation were significantly suppressed in HLECs treated with the CM of *AGRN*-deficient Siha cells [Supplementary Figures 5B–D, http://links.lww.com/CM9/C238]. In addition, the levels of VEGFR3, p-AKT, and p-ERK1/2 were downregulated in the above HLECs [Supplementary Figure 5E, http://links.lww.com/CM9/C238]. More importantly, the protein level of AGRN was strongly correlated with lymphatic vessel density (LVD) in CSCC tissues [Supplementary Figure 5F, http://links.lww.com/CM9/C238]. To further confirm that AGRN lies downstream of DDX24 in promoting lymphangiogenesis, we took the rescue approach [Supplementary Figure 5G, http://links.lww.com/CM9/C238]. As expected, the elevated proliferation, migration, and tube formation of HLECs induced by DDX24 were largely abolished upon *AGRN* deficiency [Figure 1G and Supplementary Figures 5H, I, http://links.lww.com/CM9/C238]. Taken together, DDX24-mediated lymphangiogenesis is dependent on AGRN.

To further verify the role of DDX24 in CSCC lymphatic metastasis *in vivo*, we generated a doxycycline (dox)-inducible DDX24-overexpressing Siha cell line carrying a luciferase reporter [Supplementary Figures 6A,B, http://links.lww.com/CM9/C238] and inoculated it into the footpads of nude mice. Animals were monitored for lymph node metastasis using the *In vivo* Imaging System (IVIS; Supplementary Figures 6C, D, http://links.lww.com/CM9/C238). Two months after inoculation, both popliteal lymph nodes (PLN) and inguinal lymph nodes (ILN) in the DDX24-overexpressing group were significantly larger than those in the control group [Figure 1H and Supplementary Figures 6E, F, http://links.lww.com/CM9/C238]. The upregulated expression of AGRN and LYVE1 in primary lesions overexpressing DDX24 was validated by IHC analysis [Figures 1I, J]. Notably, both the expression of AGRN and the value of LVD correlated with DDX24 levels [Figure 1K and Supplementary Figures 6G, H, http://links.lww.com/CM9/C238]. In summary, these findings demonstrate that the DDX24/AGRN axis promotes lymphatic metastasis *in vivo*.

Here, we have demonstrated the crucial role of DDX24 in facilitating lymphatic metastasis of CSCC, shedding light on the crosstalk between malignant cells and lymphatic endothelium. Mounting evidence indicates that DDXs are involved in neoplastic transformation.[10] The current results agree with the previous postulation that DDX24 functions as a positive regulator of tumor growth,[5] and demonstrate the mechanism of DDX24-mediated lymph node metastasis and tumorigenesis of CSCC. In summary, the DDX24/AGRN axis serves as a promising prognostic biomarker and therapeutic target for lymphatic metastasis of CSCC [Supplementary Figure 6I, http://links.lww.com/CM9/C238].

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Conflicts of interest

None.

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