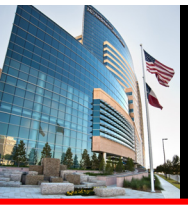

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Secretory human ribonuclease 1 functions as Eph receptor A4 ligand to promote breast tumor initiation

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ABSTRACT

The pancreatic human ribonuclease (hRNase) A superfamily is comprised of eight canonical hRNases, including hRNase1, all of which can be detected in various body fluids, e.g., serum and plasma[1]. Secretory hRNase1 exerts its ribonucleolytic activity to function in extracellular RNA clearance. Moreover, hRNase1 regulates hemostasis, inflammation, and innate immunity, indicating that hRNase1 possesses multiple functions in addition to RNA clearance[2,3]. Focuses on hRNase1 have been extensively investigated the biochemical properties and post-translational modifications, such as glycosylation[4]. **Nonetheless, the biological function of hRNase1 and whether it plays a role in cancer have not yet been completely defined.** Recent studies indicated that hRNase5/ANG serves as a ligand for cell surface RTK EGFR[5] and plexin-B2 receptor[6] in solid and hematopoietic cancers. Another hRNase called hRNase7 has been recently identified as a high-affinity ligand for RTK ROS1 in liver cancer[7]. **Those findings reveal a role of the hRNase A superfamily in tumor progression and an unconventional ligand-receptor relationship between RNase and RTK families[3,8].** Here, we demonstrate that hRNase1, independently its ribonucleolytic activity, enriches the stem-like cell population and enhances the tumor-initiating ability of breast cancer cells. Specifically, secretory hRNase1 binds to and activates the RTK Eph receptor A4 (EphA4) signaling to promote breast tumor initiation in an autocrine and paracrine manner, which is distinct from the classical ligand-receptor ephrin-EphA4 juxtacrine signaling through contact-dependent cell-cell communication. In addition, analysis of human breast tumor tissue microarrays reveals a positive correlation between hRNase1, EphA4 activation, and stem cell marker CD133. Notably, high hRNase1 level in plasma samples is positively associated with EphA4 activation in tumor tissues from the paired breast cancer patients, highlighting the pathological relevance of the hRNase1-EphA4 axis in breast cancer. The discovery of hRNase1 as a secretory ligand of EphA4 to enhance breast cancer stemness suggests a potential treatment strategy for breast cancer by inactivating the hRNase1-EphA4 axis.

BACKGROUND & RATIONALE

Our previous findings demonstrated a RNase catalytic activity-independent role of human RNase 5 (hRNase5), which serves as a *bona fide* ligand of epidermal growth factor receptor (EGFR), a cell surface receptor tyrosine kinase (RTK), to promote oncogenic transformation in pancreatic adenocarcinoma (PDAC), and acts as a serum biomarker for predicting PDAC patient response to a FDA-approved EGFR kinase inhibitor erlotinib, potentially leading to a prognostic test for PDAC patients (**Figure A**)[5]. Recently, we further identified another ligand-receptor relationship between secretory RNase and cell surface RTK families, namely that human RNase 7 (hRNase7) binds to and induces autophosphorylation of ROS1, an orphan RTK with no endogenous ligands identified before. The results clarified that hRNase7 promotes the development of hepatocellular carcinoma (HCC), independently of its catalytic activity through wild-type ROS1 activation, and tumors with activated hRNase7-ROS1 axis are vulnerable to the FDA-approved ROS1 inhibitors such as crizotinib or ceritinib, suggesting that serum hRNase7 may function as a serum biomarker to stratify patients for anti-ROS1 treatment (**Figure B**)[7]. **Together, our studies reveal an unknown but novel role of a secretory RNase serving as a cognate ligand of RTK and a serum biomarker to guide cancer therapy (Figure C)[8].**

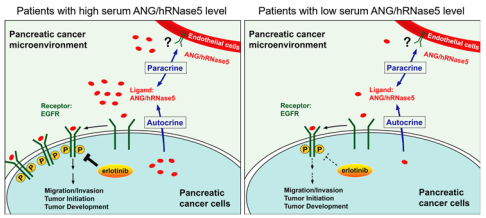

Figure A. A proposed model of elevated hRNase5 as an EGFR ligand in the sensitization to EGFR kinase inhibitor therapy in patients with PDAC. Higher levels of hRNase5 induce its binding to EGFR and activate EGFR signaling, which in turn promotes tumorigenesis and increases erlotinib sensitivity in PDAC patients. ([5]Wang and Lee et al., *Cancer Cell*. 2018 PMID: 29606349)

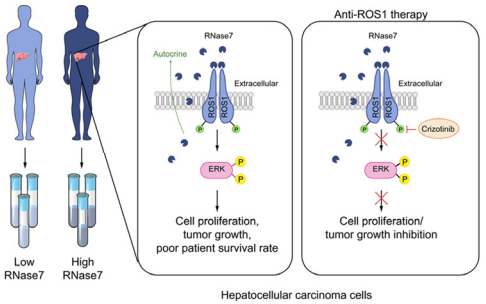

Figure B. A proposed model of elevated hRNase7 as an ROS1 ligand in the sensitization to anti-ROS1 treatment in HCC. RNase7 acts as a ligand by binding and interacting with its receptor ROS1. RNase7-mediated ROS1 activation triggers oncogenic transformation. High plasma RNase7 could be used as a biomarker to identify patients with HCC who may benefit from anti-ROS1 inhibitors. ([7]Liu, Zha, and Zhou et al., *J Hepatol*. 2021 PMID: 33031845)

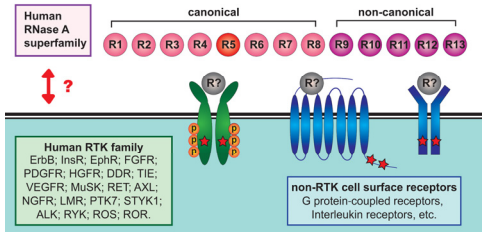

Figure C. A proposed model of the ligand-receptor cognate signaling through a ligand-like function of RNases. The human RNase A superfamily contains 13 known members that are divided into canonical (RNases 1–8) and non-canonical (RNases 9–13) subgroups. The fifth member of the RNase A superfamily, hRNase5/ANG, functions as an EGFR ligand. Identification of the hRNase5/ANG-EGFR axis raises an interesting question of whether other RNase family members may play a ligand-like function, linking the two unrelated protein families, namely RNases and RTKs or non-RTK cell surface receptors. Red stars indicate receptor activation. ([8]Wang et al. *J Biomed Sci*., 2018 PMID: 30449278)

OBJECTIVE

To identify other members of the hRNase A superfamily that contribute to cancer progression in addition to **the hRNase5-EGFR axis in PDAC and the hRNase7-ROS1 axis in HCC**. Among the human RNase A superfamily members, hRNase5 is evolutionarily more close to hRNase1, which has been well documented to harbor the RNase catalytic activity and is critical for the extracellular RNA clearance to achieve homeostasis and host innate immunity. **However, whether hRNase1 plays a role in human cancers remains elusive. Thus, this newly identified ligand-receptor theory prompts us to further study the role of hRNase1 in human cancers.**

RESULTS

hRNase 1 as a ligand of RTK EphA4 activates EphA4 signaling and associates with EphA4

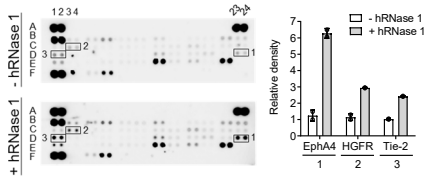

Figure 1. hRNase1 enhances RTK phosphorylation of EphA4 using an unbiased antibody array. Human phospho-RTK antibody array analysis (R&D Systems, #ARY001B) of HeLa cells treated with or without recombinant hRNase1 protein purified from HEK293 cells (1 μg/ml; Sino Biological Inc. #13468-H08H) for 5 min after serum starvation for 3 hr. (*Nat Commun., in press*)

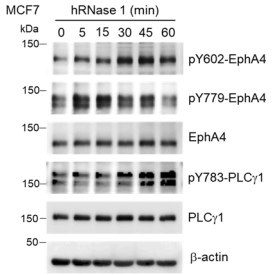

Figure 2. hRNase1 increases phosphorylation of EphA4 and its downstream molecule, PLCγ1, in MCF7 breast cancer cells in a time-dependent manner. WB of MCF7 cells treated with recombinant hRNase1 protein at various time points, blotted the indicated antibodies. (*Nat Commun., in press*)

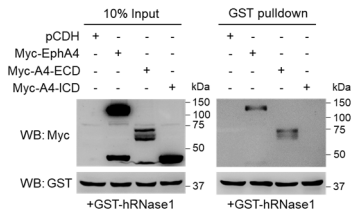

Figure 3. hRNase1 binds to extracellular domain of EphA4. In vitro GST pulldown assay of GST-tagged hRNase1/glutathione magnetic beads incubated with lysate from 293T transfected with the indicated expression plasmids, including full-length, extracellular domain (ECD), and intracellular domain (ICD) of EphA4, and pCDH empty vector. Left, input lysates. (*Nat Commun., in press*)

The biological significance of hRNase1-EphA4 axis in promoting breast tumor initiation

Cell number	KPL4-NEO	KPL4-A4	KPL4-A4-KO-Ctrl	KPL4-A4-KO-R1
1 x 10^5 cells	6/6	6/6	6/6	6/6
1 x 10^4 cells	4/6	6/6	6/6	5/6
5 x 10^3 cells	1/6	3/6	4/6	1/6
2 x 10^3 cells	0/8	2/8	1/8	0/8
TIC frequency	1/16,026	1/5,082	1/4,878	1/12,472

Cell number	BT-549-NEO	BT-549-R1	BT-549-R1-KO-Ctrl	BT-549-R1-KO-A4
1 x 10^6 cells	5/6	6/6	6/6	5/6
1 x 10^5 cells	3/8	6/8	5/8	3/8
1 x 10^4 cells	1/8	3/8	3/8	0/8
TIC frequency	1/351,989	1/52,780	1/69,591	1/412,616

Figure 4. EphA4 positively regulates hRNase1-mediated tumor initiation. Limiting dilution assay of the indicated KPL4 (top) and BT-549 (bottom) breast cancer stable clones. (*Nat Commun., in press*)

Pathological relevance among hRNase 1 expression, EphA4 activation, and CD133 in breast cancer

		phospho-EphA4		
		Low	High	Total
hRNase 1	Low	25	18	43
	High	9	39	48
	Total	34	57	91

p = 0.0002

		CD133		
		Low	High	Total
hRNase 1	Low	31	12	43
	High	23	25	48
	Total	54	37	91

p = 0.032

Figure 5. hRNase1 expression is positively associated with EphA4 activation and a stem cell marker, CD133. Quantification of IHC staining for the correlation between hRNase1 vs. phospho-EphA4-Y779 (left) and hRNase1 vs. CD133 (right) by human breast tumor tissue microarray analysis (Pantomics Inc., #BRC1021). Fisher's exact test. (*Nat Commun., in press*)

CONCLUSIONS

hRNase 1 acts as a first natively secretory ligand of EphA4 to stimulate EphA4 signaling in an autocrine/paracrine manner, leading to upregulation of stem-like cell properties in breast cancer.

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FOOTNOTE

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