Specific Neuropilins Expressions in Alveolar Macrophages among Tissue-Specific Macrophages

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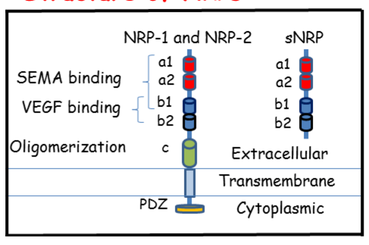
Introduction

Neuropilins (NRPs); 120-130 kDa transmembrane non tyrosine-kinase glycoproteins

Functions; Co-receptor for semaphorins (SEMA) for neuronal genesis and anti-angiogenesis, and vascular endothelial growth factors (VEGF) for angiogenesis

Structure of NRPs



PDZ; Postsynaptic density protein-95/Drosophila disc large tumor suppressor-1/Zonula occludens-1
sNRP; soluble neuropilins

Wild JR, et al. Int J Path 2012;93:81-103

NRPs expression in immune cells

- NRP-1; thymocytes, dendritic cells and regulatory T cells

Wild JR, et al. Int J Path 2012;93:81-103

- NRP-2; alveolar macrophages which is one of tissue-specific macrophages

Jubb AM, et al. Histopathology 2012;61:340-349

Even though NRPs expression on malignant tumors and immune system has studied, a PubMed-based literature query did not yield any articles describing NRPs expression on tissue-specific macrophages.

Objectives

- To detect the expression of NRP-1 and NRP-2 on tissue-specific macrophages in the brain, liver, spleen, lymph node and lung
- To observe NRP-1 and NRP-2 expression on classes of macrophages including alveolar macrophage, bronchial macrophage, interstitial macrophage and intravascular macrophage, and macrophage subsets (M1, M2 and Mox) in lung
- To detect the co-expression of NRP-1, NRP-2 and DC-SIGN on alveolar macrophages

Materials and methods

Diseases (case number)

Normal tissues for tissue-specific macrophages

Brain (n = 5), Lung (n = 5), Liver (n = 5), Spleen (n = 5), Lymph node (n = 5)

Lung tissues

Lung tissue remote to cancer nest (n = 5)

Inflamed lung

Interstitial pneumonia (n = 5), Bronchial pneumonia (n = 4), Organizing pneumonia (n = 4), Lobar pneumonia (n = 1), Epitheloid pneumonia (n = 6)

Lung cancer

Squamous cell carcinoma (n = 15), Adenocarcinoma (n = 15), Small cell carcinoma (n = 3)

Formalin-fixed paraffin-embedded tissues were used in immunohistochemistry, RT-PCR and *in situ*-PCR.

Immunohistochemistry (IHC)

Table 1. Primary antibodies used in IHC

Antibody (clone)	Source	Isotype	Reactivity
Neuropilin-1	Abcam	Rabbit, polyclonal	- Respiratory epithelial cells , endothelial cells and hepatocytes - Hepatocellular carcinoma and lung carcinoma
Neuropilin-1	Santa Cruz	Mouse, IgG1	
Neuropilin-1	Invitrogen	Rabbit, polyclonal	
Neuropilin-2	R&D Systems	Goat, polyclonal	
CD68 (PG-M1)	DAKO	Mouse, IgG1	M1, M2 and Mox macrophages
CD163 (10D6)	NOVOCASTRA	Mouse, IgG1	M2 and Mox macrophages
CD206 (5C11)	Abnova	Mouse, IgG1κ	M2 macrophage
HO-1 (D-8)	Santa Cruz	Mouse, IgG1	Mox macrophage
DC-SIGN	Abcam	Rabbit, polyclonal	M2 macrophage

- Single IHC; To detect NRP-1 and NRP-2 expression on tissue-specific macrophages and on classes of macrophages in lung
- Double and triple IHC; To detect macrophage subsets of NRP-1 and NRP-2 positive alveolar macrophages and co-expression of NRP-1 and DC-SIGN on alveolar macrophages

Reverse transcription-polymerase chain reaction (RT-PCR) and *in situ*-PCR

- **RT-PCR** ; To detect mRNAs of NRP-1 and NRP-2 in normal lung, brain, liver, spleen and lymph node
- ***in situ*-PCR**; To detect mRNAs of NRP-1 and NRP-2 in alveolar macrophage in normal lung

Table 2. Forward and reverse primers used in RT-PCR and *in-situ* PCR

	Forward primer	Reverse primer
NRP-1 (120 bp)	5'-TGAGCCCTGTGGTTTATTCC-3'	5'-CGTACTCCTCTGGCTTCTTG-3'
NRP-2 (257 bp)	5'-GTGGTTCATCTTGACCTTGT-3'	5'-ATTCTTCTTCTGCAACCTCA-3'
GAPDH (137 bp)	5'-GCACCGTCAAGGCTGAGAAC-3'	5'-TGGTGAAGACGCCAGTGGA-3'

Statistical analysis

- Comparison of positive cells among more than 3 groups (lung cancers, inflamed lung and normal lung) was carried out by Kruskal-Wallis test.
- Comparison of positive cells between each 2 groups was carried out by Mann-Whitney test.
- The correlation of NRP-1, NRP-2 and DC-SIGN positive alveolar macrophage was carried out by Pearson correlation method.
- $p < 0.05$ was judged as significance.

Results

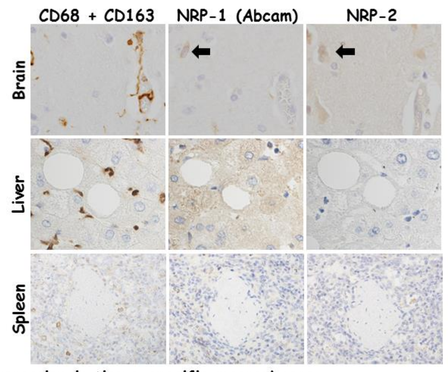


Fig 1. NRPs expression in tissue-specific macrophages
NRP-1 and NRP-2 expression was not observed in tissue-specific macrophages of brain (microglia), liver (Kupffer cells) and spleen (red pulp macrophages). Black arrows indicate the neuronal staining of NRPs in brain. Serial sections were counterstained with hematoxylin. NRP-1, neuropilin 1; NRP-2, neuropilin 2.

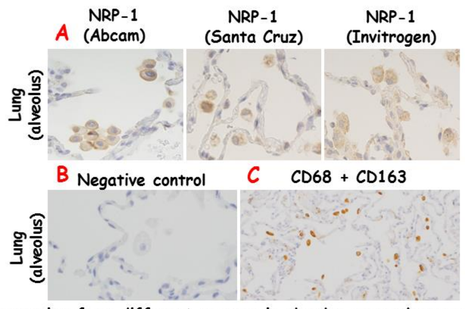


Fig 3. NRPs expression from different sources in alveolar macrophages and, IHC with a cocktail of anti-CD68 and anti-CD163 antibodies in serial section of *in-situ* PCR.
NRPs expression from different sources in alveolar macrophages and, IHC with a cocktail of anti-CD68 and anti-CD163 antibodies in serial section of *in-situ* PCR. (A) Immunohistochemistry showed alveolar macrophages expressing NRP-1 as detected by using 3 different antibodies from Abcam, Santa Cruz Biotechnology and Invitrogen. Serial sections were counterstained with hematoxylin. (B) Isotype or negative control showed no reactivity. It was counterstained with hematoxylin. (C) All alveolar macrophages showed positivity with CD68 and CD163. NRP-1,neuropilin1.

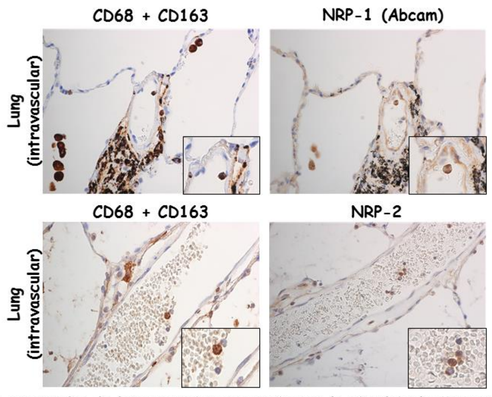


Fig 5. NRPs expression in intravascular macrophages in physiologically normal lung by immunostaining.
Intravascular macrophages (blood monocytes) were recognized by immunostaining with a cocktail of anti-CD68 and CD163 antibodies. NRP-1 and NRP-2 expression was observed in intravascular macrophages. Green arrow indicates NRP-1 expression on vascular endothelium, used as positive control. Serial sections were counterstained with hematoxylin.
NRP-1, neuropilin 1; NRP-2, neuropilin 2.

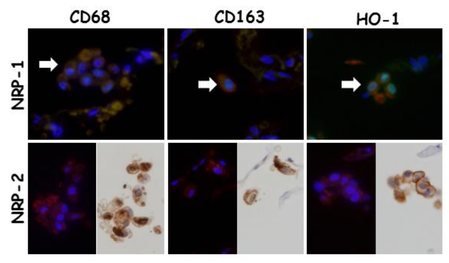


Fig 7. Double immunohistochemistry of lung tissue adjacent to the cancer.
(A) Double IF showed expression of CD68, CD163 and HO-1 (Rhodamine, anti-mouse, red color) on NRP-1⁺ (Fluorescein, anti-rabbit, green color) alveolar macrophages. White arrows showed double positive cells. Single IHC after single IF showed NRP-2+ (Rhodamine, anti-mouse, red color) alveolar macrophages also express CD68, CD163 and HO-1 (LSAB, anti-mouse, brown color). NRP-1, neuropilin 1; NRP-2, neuropilin 2.

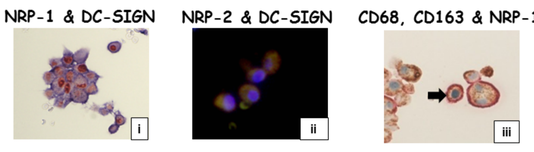


Fig 8. Double and triple immunohistochemistry of lung tissue adjacent to the cancer.
(i) Double IHC showed NRP-1(Red) and DC-SIGN (blue) positive alveolar macrophages. (ii) Double IF showed the co-expression of NRP-2 (Rhodamine, anti-mouse, red color) and DC-SIGN (Fluorescein, anti-rabbit, green color) on AMs. (iii) Triple IHC of CD68 (brown), CD163 (light red) and NRP-1 (light blue) showed triple-positive cells (CD68⁺CD163⁺NRP-1⁺; indicated by *black arrow*) and double-positive cells (CD68⁺NRP-1⁺, CD68⁺CD163⁺ and CD163⁺NRP-1⁺). NRP-1, neuropilin 1; NRP-2, neuropilin 2; DC-SIGN, dendritic cell-specific ICAM-3-grabbing nonintegrin.

Table 4. Comparison of dendritic cell-specific ICAM-3-grabbing nonintegrin (DC-SIGN) expression on alveolar macrophages in lung tissue adjacent to the cancer margin and lung tissue remote to the cancer nest.

Cases/Disease	Number of DC-SIGN-positive cells* (mean± SD)	
Lung tissue adjacent to cancer (n = 5)	32.3 ± 12.9*	a
Lung tissue remote to cancer (n = 5)	8.1 ± 3.1*	

ᵃ, $p < 0.01$ significant between alveolar macrophages and interstitial macrophages by the Mann-Whitney *U*-test. *, average number of positive cells/high-power view fields.

Summary

1. NRP-1 and NRP-2 were specifically expressed on alveolar macrophages among tissue-specific macrophages.
2. Among classes of macrophages in lung, NRP-1 and NRP-2 were mostly expressed on alveolar macrophages. M1, M2 and Mox subsets of alveolar macrophages expressed NRP-1 and NRP-2.
3. The co-expression of DC-SIGN and NRPs was observed.

Conclusion

1. In this study, the expression of both NRPs, specifically in AMs among tissue-specific macrophages, was observed for the first time.
2. This study demonstrated that NRP-1 and NRP-2 are expressed on M1, M2 and Mox macrophages.
3. Furthermore, co-localization of NRPs and DC-SIGN in alveolar macrophages from blood monocytes was described.

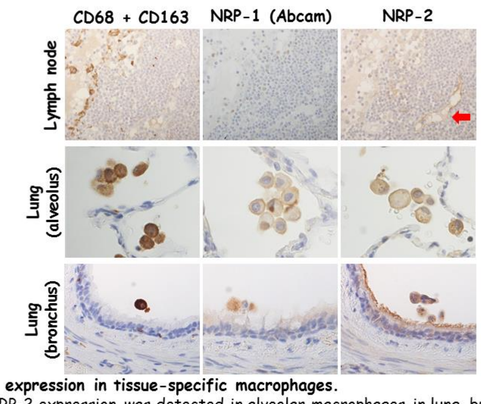


Fig 2. NRPs expression in tissue-specific macrophages.
NRP-1 and NRP-2 expression was detected in alveolar macrophages in lung, but not in lymph node (sinus macrophages). And NRP-1 and NRP-2 also expressed on bronchial macrophages. Red arrow indicates NRP-2 expression on lymphatic vascular endothelium, used as positive control. Serial sections were counterstained with hematoxylin.
NRP-1, neuropilin 1; NRP-2, neuropilin 2.

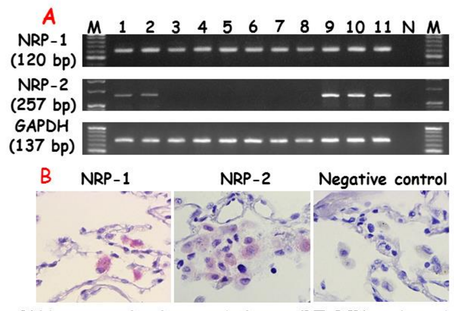


Fig 4. NRPs mRNAs expression in normal tissues (RT-PCR) and on alveolar macrophages in physiologically normal lung (*in situ*-PCR).
(A) By reverse transcriptase polymerase chain reaction (RT-PCR), NRP-1 mRNA was expressed in normal brain (*lanes 1, 2*), liver (*lanes 3, 4*), spleen (*lanes 5, 6*), lymph node (*lanes 7, 8*) and lung (*lanes 9, 10 and 11*). NRP-2 mRNA was expressed in normal lung and brain but was not expressed in liver, spleen and lymph node. N represents the negative control, and M represents the 20 base-pair DNA ladder. (B) NRP-1 and NRP-2 mRNAs of alveolar macrophages in physiologically normal lung (remote to the cancer nest), as observed by *in situ*-polymerase chain reaction (*in situ*-PCR). NRP-1, neuropilin 1; NRP-2, neuropilin 2; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

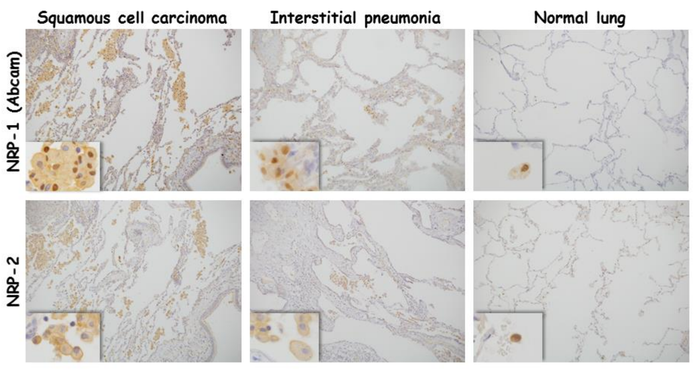


Fig 6. Immunohistochemistry of NRPs expression on alveolar macrophages in lung tissue (adjacent to the cancer margin, inflamed and physiologically normal)
NRP-1 and NRP-2 expressed on alveolar macrophages in lung tissue adjacent to cancer (squamous cell carcinoma), inflamed lung (interstitial pneumonia) and physiologically normal lung (remote to the cancer nest). NRP-1, neuropilin 1; NRP-2, neuropilin 2.

Table 3. Comparison of neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) expression in alveolar macrophages and interstitial macrophages in lung tissue remote to the cancer nest (physiologically normal lung) (n = 5).

Classes of macrophage	Number of NRP-1+ cells* (mean± SD)		Number of NRP-2+ cells*	
Alveolar macrophage	9.2 ± 3.8	a	8.9 ± 3.9	a
Interstitial macrophage	0.0 ± 0.0		0.0 ± 0.0	

ᵃ, $p < 0.01$ significant between alveolar macrophages and interstitial macrophages by the Mann-Whitney *U*-test.
*, average number of positive cells/high-power view fields.

Table 4. Comparison of neuropilin-1 (NRP-1) and neuropilin-2 (NRP-2) expression on alveolar macrophages in lung cancer adjacent to the cancer margin, lung inflammation and lung tissue remote to the cancer nest (physiologically normal lung).

Disease (case number)	Number of NRP-1⁺ cells* (mean± SD)		Number of NRP-2⁺ cells* (mean± SD)	
Adenocarcinoma (n = 15)	38.3 ± 8.9	b a	37.8 ± 9.2	b a
Squamous cell carcinoma (n = 15)	46.7 ± 9.2	a a	48.1 ± 10.7	a a
Inflamed lung (n = 22)	25.1 ± 9.1	a	24.5 ± 12.1	a
Normal lung (n =5)	9.2 ± 3.8		8.9 ± 3.9	

ᵃ, $p < 0.01$; ᵇ, $p < 0.05$ by Mann-Whitney test
*, average number of positive cells/high power view fields

Future research plan

- Further investigations such as

(a) factors influencing the expression of NRP-1 and NRP-2,

(b) status of inflammatory response during inactivation of these factors, and

(c) functional relation of these factors to DC-SIGN in M2 macrophages are suggested for future research.

COI Disclosure

Presenter: Naing Ye Aung

Competing interests; No existence of competing interests has been declared.