Investigation on Circulating Neonatal Nav1.5 (nNav1.5) and Its Antibodies in the Blood and Serum of 4T1 Orthotopic Mice Model and Breast Cancer Patients from Hospital Universiti Sains Malaysia (HUSM)

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Abstract

The expressions of Nav1.5 and its neonatal isoform, nNav1.5, in breast cancer metastasis and aggressiveness have been extensively carried out via in vitro studies [1,2]. Brackenbury et al. reported that nNav1.5 is responsible for potentiating the invasive behaviour of MDA-MB-231 human breast cancer cells [3]. In vivo expression of nNav1.5 in human breast cancer biopsy samples was first revealed by Fraser et al whereby it was deduced that the expression of nNav1.5 (sequenced from Nav1.5 positive samples) in the biopsy samples was related to the presence of lymph node metastasis [4]. It is essential to note that these past studies have only focused on detecting nNav1.5 in tissues and cell lines. The immunogenicity of neonatal Nav1.5 (nNav1.5) remains unexplored in the context of breast cancer metastasis. Therefore, the study has aimed to investigate the presence of circulating nNav1.5 and natural antibodies produced against nNav1.5 (anti-nNav1.5-Ab) in the whole blood and serum of 4T1 orthotopic mice model and breast cancer patients.

The research was carried out in two settings: a preclinical experiment using in vivo model and a clinical study involving breast cancer patients. The preclinical research involved a total of 40 female BALB/c mice. The mice were divided into three groups: (i) group 1: control mice (n=20), (ii) group 2: 4T1 orthotopic breast cancer mice (n=17), and (iii) group 3: positive control (n=3). Group 1 received the PBS injection at the third mammary fat pad, whereas group 2 received the 4T1 orthotopic injection at a similar site. nNav1.5 peptide was introduced into group 3 mice to generate anti-nNav1.5-Ab positive sera, as a positive control. After 42 days of tumour development, groups 1 and 2 were sacrificed. Blood samples, target organs and 4T1 tumours were collected. Among the lab analyses conducted include histopathology and cytokine analyses to verify the presence of metastasis within the 4T1 mice, followed by real-time PCR (qPCR) to detect the presence of nNav1.5 antigen in the whole blood and in-house indirect enzyme-linked immunosorbenet assay (ELISA) to detect the presence of anti-nNav1.5-Ab in the serum. On the other hand, the clinical study involved 128 participants: healthy participants (n=64) and breast cancer patients (n=64).
Breast cancer patients were recruited at the Breast Cancer Awareness and Research Unit (BestARi), HUSM, Kubang Kerian, Kelantan. The breast cancer patients were subsequently divided based on their treatment status: pretreatment ($n=32$) and on-going treatment ($n=32$). The pretreatment breast cancer patients were then further categorised based on their stages and subtypes of breast cancer. Approximately 6 ml of blood was withdrawn, and similarly, qPCR and ELISA were conducted to detect the circulating nNav1.5 and anti-nNav1.5-Ab, respectively. Additionally, cytokine analyses were also conducted in the clinical study.

Histopathology (Figure 1) and cytokine analyses showed the establishment of metastasis in 4T1 orthotopic mice. The concentration of vascular endothelial growth factor (VEGF) was significantly higher in the 4T1 orthotopic mice ($P<.001^{***}$). The preclinical study revealed the presence of circulating nNav1.5 antigen in the 4T1 orthotopic mice models. 4T1 mice showed significantly higher absorbance of anti-nNav1.5-Ab than the control group ($P<.001^{***}$). There was a significant negative correlation between the expression of the nNav1.5 antigen and the absorbance of anti-nNav1.5-Ab ($P=.025^*; r=-0.549$). Furthermore, there was an inverse relationship between anti-nNav1.5-Ab and the total metastatic foci ($P=.049^*; r=-0.731$). The clinical study showed very low detection of circulating nNav1.5 antigen. However, the expression of anti-nNav1.5-Ab seems promising. The presence of anti-nNav1.5-Ab was detected in both healthy and breast cancer patients, but the absorbance of anti-nNav1.5-Ab was significantly higher in breast cancer patients ($P<.001^{***}$). Further analysis showed that the pretreatment group portrayed the highest expression of anti-nNav1.5-Ab as compared to the control and on-going treatment group ($P<.001^{***}$). The correlation between cytokine analyses and the expression of anti-nNav1.5-Ab revealed that there was a significant positive correlation between anti-nNav1.5-Ab and interleukin-6 in the pretreatment group ($P=.021^*; r=0.726$), followed by a significant negative correlation between anti-nNav1.5-Ab and VEGF in the ongoing-treatment group ($P=.004^{**}; r=-0.842$). Additionally, the clinical study also showed that breast cancer patients in advanced stages portrayed higher expression of anti-nNav1.5-Ab compared to those diagnosed with early stages of breast cancer ($P=.011^*$). However, the influence of breast cancer subtypes on the expression of anti-nNav1.5-Ab remains insignificant ($P=.759$).
Figure 1. Histology of the target organs retrieved from 4T1 orthotopic and control mice.

Description: (a) Signs of metastasis were not detected in the heart resected from control mice at 10X magnification. (b) The presence of metastasis on the heart (highlighted by the arrow), resected from the 4T1 mice at 10X magnification. (c) The presence of spindle-shaped cancer cells was seen at the heart of 4T1 mice, similar to those of the primary tumour at 40X magnification. (d) Signs of metastasis were not detected in the kidney resected from control mice at 10X magnification. (e) The presence of invasion of metastatic cells at the kidney (highlighted by the arrow), resected from the 4T1 mice at 10X magnification. (f) Under 40X magnification, the tumour cells exhibited a mixed presence of hyperchromatic and vesicular nuclei (pleomorphic) as well as eosinophilic cytoplasm observed. (g) There was an absence of any signs of metastasis in the lungs resected from control mice under 10X magnification. The alveolar walls were one-cell thick. (h) Lung tissue-section showed infiltration by malignant tumour cells that appear in clusters (highlighted by the arrow). The alveolar walls were infiltrated with tumours cells causing the alveolar space to reduce in size. There were marked disturbance of lung architecture with abnormal thick-walled and collapsed alveoli (10X magnification). (i) Cytological features exhibited moderate polymorphic irregular nuclei (a mixture of hyperchromatic and vesicular nuclei) under 40X magnification. (j) There was an absence of any signs of metastasis in the liver resected from control mice under 10X magnification. (k) Clusters of tumour cells (highlighted by the arrow) seen in the liver sections retrieved from 4T1 mice group under 10X magnification. (l) These clusters were prominent at the edges of the lobes along with the endothelial cells of the blood vessels. The clusters consisted of tumour cells with a mix of hyperchromatic and vesicular nuclei under 40X magnification. (m) There was an absence of any signs of metastasis in the spleen resected from control mice under 10X magnification. (n) There was an invasion of tumour cells...
within the spleen retrieved from the 4T1 mice, portrayed as a “foggy” cluster (highlighted by the arrow) under 10X magnification. (o) Giant tumour cells (marked by black arrows) were observed under 40X magnification.

The use of whole blood and serum is a depiction of liquid biopsy [5]. Liquid biopsy is a less invasive method to detect the progression of breast cancer [6]. The presence of anti-nNav1.5-Ab highlights the immunogenicity of nNav1.5 and uplifts the novelty of the present study. The preclinical study displayed the consistency in the expression of circulating nNav1.5 and anti-nNav1.5-Ab with the presence of breast cancer metastasis in 4T1 orthotopic mice. The clinical analysis on the other hand, emphasises the role of anti-nNav1.5-Ab as an immunosurveillance marker to monitor treatment efficacy and identify breast cancer stages. In conclusion, the presence of circulating nNav1.5 antigen and its antibodies were detected in the blood and serum of 4T1 orthotopic mice model and breast cancer patients from Hospital Universiti Sains Malaysia (HUSM). The implementation of a simple liquid biopsy to detect the presence of circulating metastatic biomarkers may remove the fear of invasive procedures among Malaysian women.

Keywords
Breast cancer, Immunogenicity, nNav1.5

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Reference