主論文の要旨

Neurocan, an extracellular chondroitin sulfate proteoglycan, stimulates neuroblastoma cells to promote malignant phenotypes

（細胞外コンドロイチン硫酸プロテオグリカンであるNCANは、神経芽腫細胞に悪性な表現型を誘導する）

名古屋大学大学院医学系研究科　総合医学専攻
生物化学講座　分子生物学分野
（指導：門松 健治　教授）
苏 振东

<Background>

Neuroblastoma (NB) , which originates from the sympathoadrenal lineage of the neural crest in humans, is the most common extracranial solid tumor in infancy. The differentiated status of NB cells is closely related to malignancy: the more primitive and neural crest-like histologies cause worse prognoses. The elucidation of molecules and signaling pathways involved in the suppression of neuronal differentiation will thus clarify the mechanisms underlying NB progression and could suggest specific interventions for NB.

The Chondroitin Sulfate Proteoglycan (CSPG) neurocan (NCAN) is a member of the lectican family. NCAN is mainly expressed mainly in nervous tissues. It was reported that NCAN expression was up-regulated in a nerve injury region, and that nerve regeneration and sensory neuron extension were inhibited through protein tyrosine phosphatase receptor sigma (PTPRσ). To the best of our knowledge, the roles of NCAN in cancer have not been investigated.

<Materials and Methods>

Eight cell lines were utilized in this experiment: the human neuroblastoma cell lines IMR32, NB39, TNB1, YT-nu, SH-SY5Y, human embryonic kidney cell line 293T, mouse neuroblastoma cell line Neuro2a and one mouse neuroblastoma sphere cell line. Immunohistochemisty, RT-qPCR, *in situ* hybridization, lentivirus infection (overexpression and NCAN knockdown), western blotting, anchorage-independent growth assay, xenograft mice model, recombinant NCAN truncated proteins, microarray analysis of mRNA and allograft mice model were performed in this experiment.

<Results>

A Kaplan-Meier survival curve analysis using scan as cut-off modus indicated that a high expression of NCAN were closely correlated with poor prognosis based on the public-SEQC/RPM-498 dataset in R2 (http://r2.amc.nl) (Fig. 1A). In order to confirm the clinical data in public database, we performed immunostaining of several clinical sections with anti-NCAN antibody. As shown in Fig. 1B, although NCAN was expressed in all clinical samples in different risk group, its staining tended to be weaker in lower risk, and stronger in higher risk patients.

We further found that NCAN mRNA was significantly increased in the Superior Mesenteric Ganglion of 2–3-week-old TH-*MYCN* hemizygous mice model in both our previous microarray dataset and RT-qPCR results (Fig. 2A and 2B). The IHC results showed that NCAN protein was highly accumulated at the extracellular matrix surrounding tumorigenic neuroblasts (Fig. 2C). The results of the *in situ* hybridization clearly indicated that the tumorigenic neuroblasts observed in the SMG of 2-week-old hemizygotes were intensively positive for NCAN mRNA (Fig. 2D). NCAN mRNA was also ubiquitously

expressed in the neuroblasts occupying the terminal tumor tissue of hemizygotes (Fig. 2E).

We next exogenously expressed NCAN in TNB1, NB39 and YT-nu NB cells, in which the endogenous NCAN protein was almost absent (Fig. 3A), and examined their phenotypes. Surprisingly, all three cell lines changed their morphologies into floating spheres (Fig. 3B). We confirmed the expression of NCAN and secretion into the medium (Fig. 3C). We observed that the NCAN-expressing tumor sphere cells derived from three cell lines showed significantly greater anchorage-independent colony forming ability compared to the venus-expressing cells (Fig. 3D). In addition, the NCAN-expressing tumor sphere cells exhibited a potentiated tumor-forming ability *in vivo* when they were subcutaneously inoculated into nude mice (Fig. 3E).

Because recombinant NCAN could induce the sphere formation of TNB1 cells, indicated that NCAN was truly responsible for the sphere formation (Fig. 4A). The conditioned medium also could induce sphere formation (Fig. 4B). Because NCAN consists of core protein and CS sugar chains, we next addressed the contribution of those components to tumor sphere formation. When TNB1 cells were co-treated with NCAN-containing conditioned medium and chABC to digest CS, the sphere formation was completely abolished (Fig. 4B). When TNB1 cells were treated with either heat-denatured or pre-trypsinized NCAN-containing conditioned medium in which the NCAN core protein is impaired, the sphere formation was also abolished (Fig. 4C, D).

We investigated the comprehensive mRNA expression pattern by a DNA microarray analysis. As a result, among the upregulated genes, the Gene Ontologies (GO) for nucleosome assembly, chromatin assembly, cell cycle and cell division were enriched in NCAN-induced sphere cells (Fig. 5A). Consistently, a gene set enrichment analysis (GSEA) revealed that the cell-cycle gene set was significantly enriched (Fig. 5B). To confirm these results of the DNA microarray, we carried out a RT-qPCR, and we observed that the cell cycle inhibitor CDKN1B was significantly downregulated in NCAN-treated NB39 cells (Fig. 5C). In terms of the downregulated genes in NCAN-treated NB39 cells, the GOs for morphogenesis, development and cell differentiation were enriched (Fig. 5A). The RT-qPCR revealed that some neuronal differentiation markers were significantly downregulated in NCAN-treated NB39 cells (Fig. 5D) and NCAN-overexpressing TNB1 cells (Fig. 5E). TrkA was downregulated only in NB39 cells (Fig. 5D, E). In contrast, putative stemness markers were upregulated in the NCAN-stimulated cells (Fig. 5D, E). The MYC target gene set in GSEA was also significantly enriched (Fig. 5F).

Lastly, we addressed the involvement of NCAN in the formation of tumor spheres derived from TH-*MYCN* mice. Here we confirmed that mouse NCAN was successfully knocked down with two independent shRNA (Fig. 6A, B). We evaluated the tumor sphere formation and gene expression as indicated by the scheme in Figure 6C. The knockdown of NCAN resulted in reductions of both the sphere number and size (Fig. 6D, E). The

putative stemness marker genes were suppressed in these cells (Fig. 6F). As a result of allograft, the control tumor sphere cells developed subcutaneous tumors, whereas the NCAN-knocked down cells could not at all (Fig. 6G).

<Conclusion>

Our present study appears to be the first to address the actual function of NCAN in cancer. The results suggest that NCAN is involved in the tumorigenesis and malignancy of NB. Because NCAN induced both tumor sphere formation and the expression of stemness-related genes, our findings suggest that NCAN may function as a component of the extracellular matrix, which comprises the particular environment for stem cells, i.e., the niche. Taken together, we have identified NCAN as a novel and clinically relevant malignant factor of NB. NCAN could be a potent therapeutic target to kill the malignant cells.