**RPN2 Gene Confers Osteosarcoma Lethal Phenotypes and Determines Clinical Prognosis**

Tomohiro Fujiwara¹², Toshiyuki Kunisada¹, Ken Takeda¹, Yutaka Nezu², Aki Yoshida¹, Koji Uotani¹, Kazuhisa Sugiu¹, Toshiki Omori¹, Takehiro Uehara¹, Yasuaki Yamakawa¹, Akira Kawai³, Takahiro Ochiya², Toshifumi Ozaki¹.

¹Department of Orthopaedic Surgery, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan, ²Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, Tokyo, Japan, ³Division of Musculoskeletal Oncology, National Cancer Center Hospital, Tokyo, Japan.


**Introduction:** Drug resistance and metastasis are lethal characteristics of tumors. Recent study showed that downregulation of ribophorin II (RPN2), which is part of the N-oligosaccharyl transferase complex, efficiently induced apoptosis in drug-resistant human breast cancer cells in the presence of docetaxel. Silencing of RPN2 decreased membrane localization of P-glycoprotein through a reduction of glycosylation status, and restored sensitivity to docetaxel. These results indicated that regulation of RPN2 expression contributes to a more effective response to docetaxel-based chemotherapy. However, it has been unclear whether these mechanisms would be effective in other cancers, including neoplasms of mesenchymal origin. In the present study, we investigated whether the level of RPN2 expression affected malignant phenotypes of osteosarcoma cells such as cell proliferation, drug sensitivity, sphere formation ability, and cell invasion in vitro, as well as tumor growth and metastatic ability in vivo.

**Methods:** The study protocol for obtaining clinical information and collecting samples was approved by the Institutional Review Board of our institutes. Immunohistochemistry (IHC) staining and qRT-PCR was performed on clinical samples from 35 osteosarcoma patients, and the clinicopathological correlation with RPN2 expression was evaluated. The expression levels of RPN2 were analyzed in several osteosarcoma cell lines. We then established stable clones of a highly metastatic 143B, expressing shRNA against RPN2 (143B-shRPN2) and control shRNA (143B-shNC). Tumor cell phenotype such as cell proliferation, invasion, and drug sensitivity were compared between these cell lines. Finally, mice transplanted with 143B-shRPN2 and -shNC cells were evaluated for tumor growth, metastasis, and their survival. Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

**Results:** High expression of RPN2 in osteosarcoma biopsies is significantly correlated with poor patient survival:

We evaluated tissue samples from 35 osteosarcomas obtained by diagnostic incisional biopsy of primary osteosarcoma. Immunohistochemically, RPN2 protein was moderately to strongly expressed in all of these specimens, and localized in the cytoplasm. We next performed qRT-PCR using cDNA obtained from these osteosarcoma patients and evaluated the clinicopathological features according to the expression of RPN2 in the same cohort set. The area under the receiver-operating characteristic (ROC) curve was 0.838, and Kaplan-Meier analysis showed that high levels of RPN2 expression were associated with significantly worse overall survival rates (log-rank test, P = 0.002) and disease-free survival rates.
RPN2 regulates drug response and invasiveness of osteosarcoma cells:
We found higher expression of RPN2 in 143B, a highly metastatic osteosarcoma cell line, than in SaOS2 or HOS, which are poorly metastatic osteosarcoma cell lines. We then established stable clones of 143B expressing short hairpin RNA (shRNA) against RPN2 (143B-shRPN2) and control shRNA (143B-shNC). After 48 hours of doxorubicin, docetaxel, and methotrexate treatment, we found substantial cell death in 143B-shRPN2 relative to the control 143B-shNC. In comparison with control 143B-shNC cells, 143B-shRPN2 formed fewer and smaller spheres in a serum-free, growth factor-supplemented, anchorage-independent environment and showed less invasive phenotype than 143B-shNC cells.

RPN2 expression in osteosarcoma cells is induced by doxorubicin treatment:
We found that expression of mRNA for both RPN2 and multidrug resistance gene 1 (MDR1) in 143B cells was markedly and dose-dependently induced by doxorubicin after 48 hours of treatment. These data indicated that the cells surviving after doxorubicin treatment expressed a high amount the MDR1 and RPN2 gene products, suggesting that the development of drug resistance might correlate with induction of MDR1 and RPN2 gene expression in osteosarcoma cells.

RPN2 silencing contributes to the inhibition of tumor growth and lung metastasis formation:
143B-shRPN2 (n = 5) and 143B-shNC (n = 5) were orthotopically implanted into the right proximal tibia of 4- to 6-week-old athymic nude mice. We found that the primary tumor growth of 143B-shRPN2 was less than that of 143B-shNC. After 3 weeks of orthotopic transplantation, there was significantly lesser lung metastasis in 143B-shRPN2-bearing mice than in 143B-shNC-bearing mice. All the mice were evaluated for survival, and 143B-shRPN2-bearing mice showed longer survival than 143B-shNC-bearing mice (log-rank test, P = 0.020), suggesting that decreased RPN2 expression conferred a survival advantage on osteosarcoma-bearing mice.

Discussion: In the present study, we have shown that RNA interference for RPN2 suppresses cell proliferation, sphere formation ability, and invasiveness, and increases the sensitivity of cancer cells to a wide range of chemotherapeutic drugs including doxorubicin, methotrexate, and docetaxel in vitro. Since osteosarcoma patients who show a poor response to these drugs have a poor prognosis, silencing of RPN2 in osteosarcoma tissue would improve prognosis by sensitizing the cancer cells to these drugs. Interestingly, silencing of RPN2 could sensitize osteosarcoma to docetaxel, which might also be effective for patients with recurrent or progressive osteosarcoma. Although the molecular mechanisms responsible for regulation of invasiveness via RPN2 protein are unclear, previous reports have demonstrated that N-linked glycosylation correlates with cell invasion or metastatic phenotypes. Additionally, we found that RPN2 silencing contributed to inhibition of tumor growth and lung metastasis formation in vivo. Considering with the evidence of breast and esophageal cancers, the RPN2 gene may represent a novel target for RNAi therapeutics against a wide range of malignant neoplasms, including osteosarcoma.

Our human study demonstrated that high expression of RPN2 in biopsy samples of osteosarcoma was significantly correlated with prognosis. This result indicated that silencing of RPN2 may contribute to sensitization of cancer cells to chemotherapeutics in all patients, since RPN2 was found to be strongly expressed in biopsy specimens from all patients. In recent years, RNA interference (RNAi) therapeutics, most notably with lipid nanoparticle-based delivery systems, have advanced to the human clinical trial
stage. Our preclinical trial of RPN2 silencing suggests that it would be worth evaluating the efficacy of siRNA administration, which is our next research goal. In fact, a clinical phase I study of siRNA targeting RPN2 is now underway at our institution, and it is anticipated that this will yield novel information on treatments for solid cancers.

**Significance:** This is the first report of elucidating the role of RPN2 in sarcomas, which reduce lethal phenotypes including multidrug resistance and invasiveness via regulating glycosylation status. This study indicates that the RPN2 gene may represent a novel target for RNAi therapeutics against osteosarcoma. A clinical phase I study of siRNA targeting RPN2 is now preliminary stages, anticipating that this will yield novel information on treatments for malignant neoplasms.

*ORS 2015 Annual Meeting*

*Paper No: 0045*