Identification of Circulating miRNA Signatures in Osteosarcoma Patients

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Introduction: Osteosarcoma is the most common primary bone malignancy arising in children and young adults. Along with the development of multi-agent chemotherapy and surgical techniques, patient prognosis has gradually improved over the past 30 years. However, osteosarcoma patients who show a poor response to chemotherapy or who have multiple pulmonary metastases have a poor prognosis, with an overall survival rate of <50% and <30%, respectively. Thus, early detection of recurrent or metastatic disease or early decision making according to tumor response to chemotherapy could improve patient prognosis. Although the discovery of novel biomarkers to detect tumors, predict their drug sensitivity, and monitor them is one of the most important challenges that must be overcome, there are no useful biomarkers for these purposes.

microRNAs (miRNAs) are a class of small noncoding RNAs that play a central role in the regulation of mRNA expression. Despite their small size, these endogenous noncoding RNAs have an enormous effect on gene expression and regulate a variety of physiological and pathological processes. Over the past several years, it has become evident that dysregulation of many types of miRNAs has been associated with the initiation and progression of human cancers. A number of many studies have indicated that miRNAs can act as either oncogenes or tumor suppressors. The recent discovery of miRNAs as novel biomarkers in human serum or plasma has represented a new approach for the diagnostic screening for malignant diseases. To date, however, little has been known about the circulating miRNAs from osteosarcoma and no trial has been reported based on genome-wide miRNA profiling. In this study, we investigated whether the serum miRNA levels could be used as novel biomarkers for osteosarcoma by comparing findings in osteosarcoma (OS) patients, non-osteosarcoma (non-OS) patients, and healthy controls.

Methods: The serum samples were collected from OS patients, age-matched non-OS patients and healthy volunteers. Peripheral blood was obtained from each patient and immediately subjected to isolation of cell-free nucleic acids to prevent contamination by cellular nucleic acids. Total RNA was extracted from patient serum using a miRNeay Serum/plasma Kit. Microarray analysis was performed using the extracted RNA, and the miRNA levels in the serum of OS patients was compared with those of non-OS patients and/or healthy volunteers. The miRNA candidates were evaluated using the serum samples of the additional patient set. The expression levels of extracted miRNA were confirmed in human OS cell lines (SaOS2, U2OS, HOS, 143B), human mesenchymal stem cell (hMSC), and their culture media. The Mann-Whitney test was used to compare differences in serum miRNA concentrations and miRNA ratios between the tumor group and healthy group. A p-value of 0.05 was considered significant. This study was approved by the Institutional Review Board of National Cancer Center Hospital and Okayama University Hospital.
**Results:** Marked upregulation of serum miRNAs in osteosarcoma patients compared with controls:
The miRNA expression profile of the serum samples was examined using a microarray analysis of 14 OS patients, 10 age-matched non-OS patients, and 10 healthy controls. The microarray was only used to identify candidates for further analysis. The expression levels of eight miRNAs were markedly upregulated in OS serum samples compared with non-OS and healthy controls. The miR-X expression level was the most markedly and significantly upregulated in the serum from OS patients (p = 0.012). Accordingly, miR-X was selected as a candidate for a further analysis.

Identification of upregulated miRNA in the serum of osteosarcoma patients:
Following this miRNA screening, we evaluated the serum expression level of miR-X in a total of 14 OS patients, 14 age-matched non-OS patients, and 8 healthy controls by qRT-PCR. The miR-X expression level in the serum from OS patients was significantly higher than in that from controls (p = 0.00052). There were no significant differences in the expression levels of miR-X between age-matched non-OS patients and healthy controls. Receiver-operating characteristic (ROC) curve analysis revealed that the serum levels of miR-X were useful biomarkers for differentiating patients with OS from patients with non-OS and healthy controls.

Marked decrease of serum miRNA levels after surgical resection of primary tumors:
To evaluate whether the serum expression level of miR-X could be used to monitor tumor dynamics, we compared the miR-X expression levels between pre- and postoperative samples of two patients obtained from OS patients. In both cases, the miR-X expression levels of the postoperative samples were markedly lower than those of the preoperative samples.

Validation of the miRNA expression in osteosarcoma cells and cell culture media:
We determined whether miR-X act as secretory miRNAs and are excreted into the culture media by OS cell lines. We first evaluated miR-X expression in human OS cell lines (SaOS2, U2OS, HOS, and 143B) and human mesenchymal stem cells (hMSC) by real-time qRT-PCR. Expression of miR-X was significantly higher in all OS cell lines than in hMSC. We next identified that miR-X expression in the culture media from all OS cell lines increased with time (24 and 48 hours) and with increasing numbers of tumor cells. These data suggested that miR-X is a secretory miRNA derived from OS cells.

**Discussion:** Although the mechanism underlying miRNA secretion is still being elucidated, the circulating miRNAs present in the blood have been shown to be remarkably stable, even in the RNase-rich environment of the blood. Furthermore, the miRNA levels were found to be independent of the subject age and sex. In this study, we confirmed that there is no significant difference in the expression of candidate miRNAs that were identified by microarray analysis between age-matched non-OS patients and healthy controls. Considering the emerging evidence that intracellular miRNAs may be released into the circulation during processes accompanying cellular destruction or pathological injury, circulating miRNAs have the potential to be used as novel biomarkers.

It has been unclear whether miRNA expression in the tumor cells reflect those in the blood of the patients. To date, no more than 10 upregulated miRNAs in OS have been identified as oncomiR in these seven years. In the present study, we performed genome-wide miRNA profiling and identified 8 miRNAs whose expression was markedly different in the serum of patients with OS compared with healthy controls. Although several miRNAs corresponded to the reported ones, miR-X, that has not been identified by the analysis dealing with tumor specimens, was the definitive miRNA that mostly reflected tumor burden. These results suggested that not all oncomiRs in OS cells and tissues associate with
those in the blood of the patients. Evaluating the serum miR-X expression may thus have important clinical implications for risk stratification and the planning of post-therapeutic surveillance. Collectively, miR-X could be a non-invasive biomarker for OS.

**Significance:** This is the first report of circulating miRNA that was identified by genome-wide miRNA profiling using the serum of the patients. The novel miRNA expression signature identified in this study had sufficient efficacy for development into blood-based biomarkers for osotosarcoma detection and monitoring.

*ORS 2015 Annual Meeting*

*Poster No: 1071*