A Platelet Aggregation-inducing Factor Podoplanin Is Highly Expressed In Metastatic Legions Of Osteosarcoma

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Introduction: Osteosarcoma is the most common primary malignant bone tumor and has a high rate of systemic spread especially to the lungs. Approximately 14% of the patients have lung metastases at diagnosis. Primary metastasis is one of the risk factors, and increases mortality rate. Moreover, multidrug combination chemotherapy for osteosarcoma entails ototoxicity, cardiac toxicity, and secondary malignancies. To resolve these problems, new molecular targeting therapy with high tumor specificity is expected. Podoplanin (PDPN/Aggrus), a platelet aggregation-inducing type I transmembrane sialoglycoprotein, is involved in tumor invasion and metastasis (1). By contrast, podoplanin is also useful as a selective marker of lymphatic endothelium (2). Several studies have reported that osteosarcoma tissues and cell lines such HOS, U-2 OS, and MG63 express high levels of podoplanin. Moreover, podoplanin expression was reported to be involved in poor prognosis of osteosarcoma patients (3). However, the association between podoplanin expression and metastasis of osteosarcoma remains to be clarified because of the lack of high-sensitive anti-podoplanin monoclonal antibodies (mAbs), although many anti-podoplanin mAbs such as NZ-1 and D2-40 have been established. In this study, we established a novel anti-podoplanin mAb, LpMab-7, which possesses high sensitivity against podoplanin. Using LpMab-7, we investigated podoplanin expression in primary and metastatic lesions of osteosarcomas.

Methods: Hybridoma production
BALB/c mice were immunized by i.p. injection of 1 × 108 LN229/hPDPN cells. After several additional immunizations with 1 × 108 LN229/hPDPN cells, a booster injection was given i.p. 2 days before spleen cells were harvested. The spleen cells were fused with mouse myeloma P3U1 cells. The hybridomas were grown in RPMI medium with hypoxanthine, aminopterin, and thymidine selection medium supplement. The culture supernatants were screened using ELISA for binding to recombinant human podoplanin purified from LN229/hPDPN cells.

Epitope mapping using ELISA, Western-blot, and flow cytometry
To determine the epitope of LpMab-7, ELISA was performed for synthetic peptides of podoplanin. Several point mutants of podoplanin were produced and detected using Western blotting and flow cytometry.

Immunohistochemistry (IHC) against osteosarcoma tissues
Tissue specimens from 16 osteosarcoma patients, who underwent surgery at University Hospital of our institute, were used for IHC using LpMab-7, NZ-1, and D2-40. Four pulmonary metastatic specimens were used for additional studies. The study was approved by the institutional ethical committee.
Informed consent for obtaining samples and for subsequent data analyses was obtained from each patient or the patient’s guardian.

**Results:** We first established a novel anti-podoplanin mAb, LpMab-7, by immunizing mice with LN229/hPDPN. We next identified minimum epitope of LpMab-7, and identified it as RIEDL, which corresponds to Arg79-Leu83 of human podoplanin using ELISA, Western-blot, and flow cytometry. Using IHC analysis, LpMab-7 showed high reactivity against osteosarcoma tissues compared with NZ-1 mAb (Fig. 1). Furthermore, LpMab-7 detected podoplanin expressed in metastatic lesions of osteosarcomas (Fig. 2; lower). Of interest, podoplanin expression at metastatic lesions was higher compared with primarily lesions in 3 of 4 cases with lung metastasis (Fig. 2).

**Discussion:** We investigated podoplanin expression by IHC using LpMab-7 mAb against 16 osteosarcoma tissues, four of which have pulmonary metastatic lesions. Although 3 of 4 metastatic lesions showed higher podoplanin expression than primary ones, more cases should be examined to conclude the association between podoplanin expression and osteosarcoma metastasis.

**Significance:** Because LpMab-7 has high sensitivity against podoplanin, LpMab-7 mAb is expected to be useful for molecular targeting therapy and a metastatic marker for osteosarcomas.
Fig. 2. IHC analysis using LpMab-7 against primarily and metastatic lesions of osteosarcomas.

ORS 2015 Annual Meeting

Poster No: 1945