

The role of the B7x signaling pathway in the progression of neuroendocrine tumors. Ziqiang Yuan¹, Juliet Gardiner¹, Asha Adem¹, Svetlana Bagdasarov¹, Daniel Slegowski¹, Xingxing Zang², Edmund C. Lattime¹, Steven K Libutti¹. ¹Rutgers Cancer Institute of New Jersey, New Brunswick, New Jersey; ²Albert Einstein College of Medicine, Bronx, New York.

Introduction: Cancer immunotherapy is rapidly becoming an important component of treatment for patients with a variety of tumor types. The B7 family, and their receptors the CD28 family, are major immune checkpoints that regulate T-cell function, which makes these pathways very attractive therapeutic targets. A newly characterized member of the B7/CD28 family, B7x, is expressed in a number of tumors and can modulate cancer development and progression by inhibiting T-cell function, thus making it an appealing target for immunotherapy. In the present study, we explored the expression and role of B7x in the development and progression of pancreatic neuroendocrine tumors (PNETs). **Materials and Methods:** *First*, we determined B7x expression in human and murine PNETs. The mRNA and protein expression of B7x in 30 human PNET samples and paired normal tissues were measured using laser capture real-time RT PCR and immunohistochemistry (IHC) assays. Furthermore, we performed correlation analysis between the expression level of B7x and clinical-pathological parameters using Pearson's Correlation. In addition, using our *Men1* knockout (KO) mouse model that manifests PNET β -cell tumors, we evaluated the expression of B7x by IHC. *Second*, we investigated the molecular mechanism of B7x immune checkpoint regulation using our *in vitro* and *in vivo* models. The upregulation of HIF-1 α in neuroendocrine tumors has been reported and hypoxia causes a rapid, dramatic, and selective upregulation of PD-L1. It is important to note that HIF-1 α directly binds to the promoter of PD-L1. Using our *Men1* KO mouse model, we evaluated by IHC the expression of PCNA, HIF-1 α , and B7x in the tumor microenvironment of mice at different time points (4, 6, and 12 months). In addition, using an *in vitro* N134 cell model (a pancreatic β -cell tumor cell line derived from a tumor from *RIP-Tag* mice), we investigated the expression levels of HIF-1 α regulated B7x expression. The expression levels of HIF-1 α and B7x in N134 cells were measured under hypoxic conditions (1% O₂) with and without HIF-1 α siRNA treatment. Furthermore, we tested if the increased HIF-1 α expression can induce B7x transcriptional activation under hypoxic conditions by a luciferase assay. Additionally, we investigated whether HIF-1 α can bind to the B7x promoter in N134 cells by a chromatin immunoprecipitation (ChIP)-PCR assay. **Results:** We have demonstrated that B7x is expressed in 60.5% of human PNETs (stage I: 54.8%, I: 57.5%, III: 75%, and IV: 100%). We have also found a significant correlation between B7x expression with tumor size (R=0.7818, P<0.001) and tumor cell proliferation by Ki67 staining (R=0.8621, P<0.001). In addition, we have demonstrated that HIF-1 α overexpression coincides with the upregulation of B7x in the tumor microenvironment during β -cell proliferation at 6 months (hyperplasia) and at 12 months (insulinoma) but not at earlier time points (4 months) in the absence of β -cell proliferation and not in islets of wild type (WT) mice. To investigate the molecular mechanism of B7x upregulation in the *in vitro* model, when cultured under hypoxic conditions (1% O₂) after 48h and 72h, islet tumor β -cells (N134 cells) that lack B7x expression were found to induce the upregulation of HIF-1 α and B7x. However, the expression levels of HIF-1 α and B7x were not induced in the islet tumor β -cells following HIF-1 α siRNA treatment after 48 hours by western blot. Also, we demonstrated that the B7x promoter transcriptional activity was significantly increased under hypoxic condition by analyzing luciferase activity. Using a ChIP-PCR analysis, we identified that HIF-1 α can bind the B7x promoter in N134 β -cells under hypoxic condition. **Conclusion:** Our results showed that B7x was expressed to a high degree in PNETs and the molecular mechanism of immune checkpoint B7x upregulation may be by the expression of HIF-1 α following relative hypoxia resulting from the rapid growth of tumor cells in the tumor microenvironment. These findings suggest that targeting B7x offers a promising strategy for the immunotherapy of patients suffering from PNETs.