Identification of neurofibromatosis type 1 (NF1) is a hereditary syndrome characterized by the development of benign nerve sheath tumors. This condition is associated with an increased risk of developing malignant peripheral nerve sheath tumors (MPNST). MPNSTs have a very poor prognosis as they do not respond to standard chemotherapeutic or radiation therapy and the only effective treatment appears to be surgery upon early diagnosis. There is an urgent need thus to develop non-invasive methods for the screening and early detection of NF to MPNST transition, as well as monitoring of disease progression and therapeutic outcome. Exosome-based diagnostics represents a cutting-edge frontier of cancer research and clinical management. Exosomes are 50-200 nm vesicles released by all living cells that act as an export pathway for cellular proteins and RNAs (mRNAs and miRNAs), mediating intracellular communication and bio-molecule exchange in parent cells microenvironment and at distance. Exosomes can be found in easily accessible biological fluid such as blood and are considered early sentinel of alterations in cell and tissue homeostasis and metabolism thus becoming an appealing source for identification of novel disease-relevant biomarkers. Our work was directed towards the identification and distinction of neurofibroma (NF)- and MPNST-specific exosome associated markers. We performed a comparative analysis of up to 50 potential biomarkers falling into diverse ontological categories using exosomes purified from conditioned supernatant from a selection of MPNST, plexiform NF and normal NF cell lines, obtaining intriguing results: (i) exosomes originating from different cell models displayed differential expression patterns in particular for biomarkers notoriously indicative of malignant progression of MPNST and Plexiform NF from Dermal NF. (ii) Utilizing FACS, WB and ELISA assays we have identified at least 3 exosome associated protein markers which distinguished between MPNSTs and benign NFs in vitro; 3 markers which were mostly associated to MPNSTs providing clear resolution from Dermal NFs while some Plexi NFs did display these proteins and other 3 promising candidate markers which should be further investigated. (iii) Molecular analysis of the same samples showed 6 miRNA and 2 mRNA markers which have a very appealing performance for accurately distinguishing exosomes originating from malignant neurofibromatosis. Overall, in the battery of preliminary experiments in in vitro models, we have found 8-11 specific candidate markers paving the way to test and validate them in plasma samples obtained from murine xenograft and transgenic models, and NF clinical samples with the ultimate aim of developing a non-invasive, sensitive and specific multimarker assay for identification of NF1 patients with risk of MPNST development and early detection of cancer. Our primary objective is the pilot characterization of protein and mRNA/miRNA profiles in exosomes released from neoplastic Schwann cells isolated from plexiform neurofibromas and from MPNST cell lines. While the overall challenge of identification of molecular changes indicative of neurofibroma to MPNST progression will further benefit from ongoing gene mutation screenings, we will initially focus on assessment of candidates that have emerged from recent studies as well as specifically profiling the mRNA/miRNA cargo of model exosomes.

**Work flow 1: Exosome purification and FACS/WB/ELISA analysis**

**FACS comparative analysis**

We have screened purified exosomes derived from 7 MPNST, 3 Plexiform NF and 3 Dermal Neurofibroma cell lines for the presence and expression level of a panel of 35 protein targets. Targets were chosen following protein/protein alterations reported to be associated to neurofibroma to MPNST transition, or known to be correlated to cancer exosomes (literature and HBM data).

- Consistent variability was observed within MPNST and plexiform neurofibroma samples; i.e. 0309.283 Plexi cell line derived exosomes showed a very aggressive FACS profile similar to MPNST cell lines exosomes (data not showed), a patient follow up would be very interesting to understand if this specific cell line had already started a NF to MPNST transition and we were able to find it out at a very early stage. Dermal neurofibromas were very different from both MPNST and plexiform samples addressing for a more benign profile. On the left are reported representative FACS results of the markers able to differentiate MPNST from Dermal NF and, to a lesser extent, Plexi NF.

**Western Blot analysis**

NEK2, HGF/PTP1, CDKN1A, PDGFRbeta, HIF1alpha, TMSF54, HSP90 and Alix as loading control. CDKN1A and PDGFRbeta were not visible at the tested conditions (adjustment of conditions and selection of suitable antibody needed for further experiments), over-expression of HGF/PTP1 and HIF1alpha in MPNSTs exosomes, as well as 70 KDa Tucap isoform, was observed while NEK2 and HSP90 remained as the most abundant marker proteins, clearly distinguishing MPNSTs from normal NFs.

**Conclusions and final remarks**

Exosomal markers (protein and mRNA) which specifically distinguish between MPNSTs and Benign Neurofibroma (Dermal and to a lesser extent Plexi NF) have been found utilizing different assays: FACS, WB, ELISA and qRT-PCR.

- A better profiling of small RNA population is required to identify ideal reference gene for quantitative analysis and to test other potential miRNA, either described in literature (e.g. Sedani et al. Human Genomics 2012, 6:23), or newly defined by completion of miRNA array mediated profiling of exosomes from in vitro neurofibromatosis and MPNSTs.

- A strong effort has been to perform validated in quantitative assays the most interesting protein markers; in vitro preliminary results are very promising and should be confirmed in biological sample obtained by xenograft MPNSTs in comparison with Plexi Neurofibroma mouse models.

Overall, in the battery of preliminary experiments in in vitro models, we have found 8-11 specific candidate markers with the ultimate aim of developing a non-invasive, sensitive and specific multimarker assay for identification of NF1 patients with risk of MPNST development and early detection of cancer.