An Update on the Liposarcoma Genome Project

FALL/WINTER 2019
**Introduction**

Thank you for your support of liposarcoma research and the Liposarcoma Genome Project at the Mass General Cancer Center. Your commitment has ensured that our outstanding team of physician-scientists has the resources necessary to pursue cutting-edge research, which we hope will inform the development of new therapeutic options for this difficult disease and improve outcomes for patients and their families. This progress would not have been possible without your ongoing support and commitment to our mission. We are pleased to provide you with an update on the progress on two arms of research – classifying the molecular profiles of liposarcoma tumors and performing genomic microRNA analysis on liposarcoma tumors – our team has made over the past year.

**Classifying the Molecular Profiles of Liposarcoma Tumors**

*Update from Bradley Bernstein, MD, PhD, Sarah Johnstone, MD, PhD, John Mullen, MD, and Samantha Miller Bevill, PhD*

Liposarcomas (LPSs) are malignant tumors arising from an adipocytic lineage and are the most common subtype of soft tissue sarcoma. The majority of LPS tumors are classified into well-differentiated (WDLPS) and dedifferentiated (DDLPS) forms. WDLPS and DDLPS often coexist within a single tumor and are generally unresponsive to radiation or chemotherapy. In contrast to WDLPS, DDLPS represents an aggressive form of the disease and is associated with frequent metastatic recurrence and disease-specific mortality. Despite this distinction, little is known about how DDLPS is established and maintained – leaving limited therapeutic direction for patients.

Nearly all WDLPS and DDLPS contain supernumerary ring or giant rod chromosomes which are thought to introduce genomic instability and are likely linked to characteristic amplification and overexpression of the oncogenes MDM2 and CDK4. Genomic profiling has identified several genomic alterations that could distinguish WDLPS and DDLPS, such as JUN amplifications. However, these prior analyses were performed on unmatched tumor samples. A common genetic driver of dedifferentiation has yet to be identified. The lack of clear genomic alterations distinguishing WDLPS and DDLPS suggests epigenetic factors may govern the DDLPS transition via regulation of gene transcription.

**Research Aims**

Our team is working to comprehensively define the underlying molecular differences that define high-grade DDLPS relative to WDLPS and normal fat. We have acquired genetic, epigenetic, and transcriptional profiles from patient cohorts at Mass General in collaboration with surgeons and pathologists. To date, two sample cohorts have been established and utilized. The first cohort of patients were diagnosed with WDLPS and DDLPS at the time of presentation. Whole-exome sequencing was performed on formalin-fixed, paraffin-embedded tumor samples of both the WD and DD tumor for each patient in order to quantify genetic mutations and copy number alterations.
between groups. A second cohort of tumor samples has been curated, including fresh tissue from 40 liposarcoma patients that was flash frozen for downstream transcriptional profiling and chromatin mapping. Samples were carefully annotated by pathologists and, when possible, tumors were banked with normal fat from each patient.

Our group had several aims for this research:

**Aim 1: Chart the genomic landscapes of WDLPS and DDLPS**

Copy number alterations were a prominent feature of the whole-exome sequencing dataset, and amplifications in CDK4 and MDM2 were present in all LPS samples. However, there were few additional enriched copy number alterations in WDLPS. In contrast, dedifferentiated tumors displayed statistical enrichments of several amplifications, including the 1p32 region containing JUN (Fig. 1). DDLPS also showed enrichment of many deletions across almost all chromosomes (Fig. 1). Several annotated deletions are published in independent datasets – including 19q13, which is associated with poor prognosis. Whole-exome sequencing also revealed widely heterogeneous mutation profiles among WDLPS and DDLPS tumors, with no recurrent driving mutations. Cumulatively, these results support the genomic heterogeneity of LPS and are consistent with previous studies that failed to define clear genetic driver events that facilitate the evolution of WDLPS to DDLPS.

Copy number alterations enriched in DDLPS samples will be integrated with downstream analyses to uncover the most relevant genetic alterations associated with transcriptional programs and chromatin states that distinguish WDLPS and DDLPS. While these data represent a vital underpinning for our comprehensive LPS analysis, their failure to identify definitive driver genetic lesions support the importance of the current phase of our project focused on transcriptional drivers and epigenetic cell state transitions.

**Aim 2: Chart the transcriptional and epigenetic landscape of WDLPS and DDLPS tumors**

Our transcriptional profiling of 22 samples defined a clear continuum of adipocytic differentiation spanning normal fat, WDLPS and DDLPS groups. While WDLPS histologically appears as mature adipocytes, molecular profiling revealed a partial loss of genes associated with adipogenesis. DDLPS diverged more strongly from normal fat,
with an almost complete loss of genes associated with adipocytic differentiation and induction of mesenchymal stem cell markers.

Transcriptional profiling also defined genes statistically dysregulated in DDLPS and WDLPS relative to normal fat. Of the genes identified, 902 were uniquely dysregulated in DDLPS compared to normal fat or WDLPS (Fig. 2A). Pathway analysis defined several potentially targetable pathways – including Aurora kinase signaling, insulin signaling, and transforming growth factor beta (TGFβ) signaling. Closer inspection of the tyrosine kinome revealed TGFBR1 and PTK7 were among the top differentially expressed kinases in DDLPS. Multiple components of TGFβ and PTK7 signaling were upregulated in DD tumors (Fig. 2B). PTK7 promotes non-canonical WNT signaling and subsequent tumor cell invasion and proliferation. High PTK7 expression is associated with poor clinical outcomes in LPS patients. Interestingly, PTK7 signaling in association with WNT5a has been reported to promote TGFβ signaling through increased expression of TGFβ ligands. TGFβ represents a novel therapeutic pathway in DDLPS. We believe this is an attractive pathway to target, given various published roles for TGFβ in promoting a mesenchymal tumor phenotype, tumor cell proliferation, metastasis and immune evasion.

Chromatin profiles have been generated for the activating histone mark H3K27ac across 40 tumors (20 WD and 20 DD). These profiles enable us to identify switches or “enhancers” that activate genes in the respective forms of LPS. Our ongoing data analyses now aim to integrate these enhancer maps with transcriptional profiles in order to explain regulatory programs that differ between WDLPS and DDLPS. These profiles will be particularly helpful in defining what upstream regulators and pathways govern differentiation state in LPS.
Aim 3: Investigating immune components in the LPS tumor ecosystem

The use of immunotherapy for treating soft tissue sarcomas, including LPS, is being tested in combination with chemotherapy (clinicaltrials.gov-NCT03899805). In TCGA datasets, low lymphocyte infiltration was associated with a subset of highly proliferative DDLPS with poor clinical outcomes. However, comprehensive assessments of immune cell content and signaling is lacking in LPS. Cell population predictions derived from our transcriptional datasets defined resident immune cell types in normal fat samples (Fig. 3A). Cell type scores that differed in LPS samples all showed a loss of immune populations (Fig. 3B), indicating an immunosuppressive tumor microenvironment. Our ongoing efforts will define immune cell compositions and activation of immune checkpoint blockades in lymphocytes of LPS samples. These efforts will be enhanced by testing the involvement of pathways defined in Aim 2, such as TGFβ, in modulating the tumor immune environment.

Ongoing Activities and Future Directions

Our efforts to establish comprehensive genomic, epigenetic and transcriptional profiles of LPS have generated a unique and powerful dataset. Our ongoing activities will expand our patient cohort, banked tissue specimens and sequencing datasets. Our current analysis has identified several putative genes and pathways involved in the evolution of WDLPS to DDLPS. We now aim to pursue three new experimental initiatives in order to validate and test their role in LPS biology:

1. Interrogate tissue microarrays to resolve the intra- and inter-tumor heterogeneity and spatial expression of differentially expressed genes in WDLPS and DDLPS. We have generated tissue microarrays from our first cohort of paired WDLPS and DDLPS. We will now evaluate our hypotheses regarding specific pathways and biomarkers activated in LPS by assessing immunohistochemistry for this set of tumors.

2. Define the impact of TGFβ signaling on LPS differentiation and immune evasion. Crosstalk between TGFβ and PTK7/WNT signaling pathways could constitute a critical signaling network that promotes dedifferentiation and metastasis of
DDLPS. Intriguingly, TGFβ also has a role in promoting tumor immune evasion. Inhibition of TGFβ as an immunomodulatory therapy could be a particularly relevant avenue to pursue, especially in the metastatic setting of DDLPS. Our ongoing studies will test the effect of pharmacologic or genetic inhibition of TGFβ/WNT on viability, invasion, adipogenesis marker expression, and immunomodulatory signaling in DDLPS cell lines. Continued access to primary tumor samples will be critical to testing the association of TGFβ pathway activation with immune checkpoints and lymphocyte infiltration in the tumor microenvironment of local and metastatic DDLPS.

3. Test combinatorial pharmacologic inhibition of differentially expressed pathways in DDLPS cell lines and xenograft models. Our dataset has already identified several potential therapeutic avenues, which we will begin testing in LPS cell lines. We will assemble a focused small molecule library to target pathways and signaling nodes dysregulated in DDLPS (Fig. 4). It is likely that inhibition of a single signaling node will be insufficient to achieve complete growth arrest or induction of tumor cell death. For instance, despite overwhelming evidence for the role of CDK4 in LPS, clinical trials have reported only partial responses to various CDK4 inhibitors as single agents. Therefore, we will also systematically test drug combinations to uncover synergistic relationships. We will advance any exceptional drug combinations to testing in xenograft models of LPS.

In conclusion, the Liposarcoma Genome Project has accumulated a critical body of genomic, epigenomic and transcriptional data for a sizable cohort of LPS tumors. Our analyses of these data support the importance of non-genetic events in driving LPS progression and have nominated genes, pathways and targets with specific roles in dedifferentiation. These candidates are now being validated, and their potential value for understanding, diagnosing and treating LPS is under investigation.
Liposarcoma Genomic MicroRNA Study

Update from Dimitrios Spentzos, MD, John Mullen, MD, Petur G. Nielsen, MD, Edwin Choy, MD, PhD, Ruslan Sadreyev, PhD, Bradley Bernstein, MD, PhD and Sarah Johnstone, MD, PhD

MicroRNAs are important molecules in the cell whose role has only been recognized in the last 10-20 years. They are products of genes (DNA) but, unlike classic genes, they do not produce a protein. Initially they were thought to be “junk,” but we now recognize that just the opposite is true – they play a critical role in regulating many functions in the cell, including protein production from classic genes and classic RNA molecules.

The literature has rapidly evolved on the role of microRNAs in cancer, including some sarcomas. However, little work has been done thus far in liposarcoma. Some profiling studies at other institutions have shown interesting microRNA patterns, but scientists have not yet been able to clearly identify or validate a signature that can explain or predict the progression to more aggressive forms of liposarcoma. With your support, our team at Mass General is working to change this course and develop microRNA markers for this disease.

One reason for developing microRNA markers is that microRNAs are small molecules which are very stable and are thus preserved in archived repositories of old tissues. Our group has performed studies in other sarcomas where microRNA quality was preserved in samples taken all the way back in the 1990s.

The creation of microRNA markers will allow us the opportunity to run very large studies and will also allow us to develop markers and tests that would be feasible and practical for clinical application in the future. Our team at Mass General is taking advantage of our unique tissue resources from patients with liposarcoma – strengthened by the fact that we have a group of patients for whom we have matched serial samples from benign fatty tissue to WDLPS and DDLPS. Our microRNA projects will be an ideal complement to the profiling studies (such as DNA and gene expression) outlined above.

Research and Results

Our team obtained 22 RNA samples that were initially used by the Bernstein Lab for other profiling experiments but were not used for microRNA. These correspond to 11 unique patients, for some of whom two or three matched serial samples were collected. This RNA was sent for microRNA sequencing at the MGH Sequencing Core Facility. All samples were sequenced successfully, with no failures. Sequencing data were imported to our specialized genomic analysis computational platforms for bioinformatics and statistical analysis. We explored several questions related to the clinical course of the disease. These findings – summarized below – are further detailed in Appendix A:

We found that normal fatty tissue, WDLPS and DDLPS have significantly different microRNA patterns of expression (profiles) when the entire set of microRNAs is examined. But WDLPS and DDLPS did show some partial overlap. When we examined a
smaller set of 87 microRNAs with the highest difference in expression levels, we saw a clearer separation between the three types of tissue.

We then applied additional analysis, taking into account that some samples are derived from the same patient (fatty tissue/WDLPS tumor, or WDLPS/DDLPS tumor, or all three). This analysis generated additional lists of markers. These lists were matched with the list of 87 microRNAs described above, and the final common overlap consists of 6 microRNAs. These could possibly be among the most crucial regulatory molecules involved in the progression of WDLPS to DDLPS. These microRNAs are: miR-139-5p, miR-139-3p, miR-150-3p, miR-150-5p, miR-493-5p, and miR-493-3p. Further, we observed that they are all processed from 3 precursor miRNAs, which suggests that these are less likely to be random findings due to examining too many miRNAs at once.

We then downloaded and processed independent data from other groups that have made microRNA data publicly available recently, including one dataset from MD Anderson Cancer Center and two separate datasets from Rotterdam, Netherlands. These datasets demonstrated that the microRNA signature separates the different histologic types in a statistically significant manner. We found in the Mass General samples that these microRNA signatures were also able to distinguish primary from recurrent LPS tumors.

Because our internal Mass General sample cohort was small (11 patients), we found another set of data from Memorial Sloan Kettering Cancer Center, which included 92 patients and also provided clinical follow up for these patients. These samples, however, were not analyzed for microRNAs at Memorial Sloan Kettering, and thus we decided to use gene expression data that were available. We first focused on identifying the genes that are targeted by the microRNAs that we discovered in the Mass General samples. We then reasoned that if the microRNAs are important in long-term prognosis, their target genes may be too, and we used the target genes as surrogates for the microRNAs. We discovered that the expression patterns of the target genes strongly predicted which patients will develop future metastasis. A more tailored signature, comprised of 10 miRNAs that were specifically associated with relapse in the Mass General data, also powerfully predicted eventual metastatic relapse in the Memorial Sloan Kettering patient cohort, reinforcing this analysis.

Overall, these efforts led us to four significant conclusions about using microRNA analysis for liposarcoma:

1. A microRNA signature can distinguish normal fatty tissue, lipomas, well-differentiated liposarcomas, and dedifferentiated liposarcomas.
2. This microRNA signature is largely reproducible in several external datasets despite the substantial laboratory, technical and other differences between the studies performed at different institutions.
3. There is a microRNA signature that predicts liposarcoma relapse, which is partly overlapping with the signature that distinguishes the different tumor types.
4. There is indirect evidence suggesting that these microRNA signatures may predict eventual metastasis in both well-differentiated and dedifferentiated liposarcoma.

**Ongoing Activities and Future Directions**

Energized by these results, our group is planning several areas of investigation to build on the data we have generated so far. Further support would help us prioritize this research and allow us to launch new projects, with the ultimate goal to develop useful markers for clinical management and, ultimately, novel therapies for patients with liposarcoma. Over the next five years, we hope to explore the following avenues of research:

1. Perform enhanced statistical analysis (“meta-analysis”) of the 3 validation datasets to find the optimal signature with a larger sample size.
2. Perform an analysis of mRNA sequencing data, representing coding genes (genes that produce proteins) and, more specifically, the genes that are targeted by the microRNAs. We will use matched data from our Mass General cohort, generated by the Bernstein Lab.
3. Identify key cancer pathways regulated by the microRNAs.
4. Expand the Mass General cohort to at least 100 frozen specimens and prove that the microRNA analysis can be done in paraffin-embedded archived tissue, which would dramatically expand the applicability of the findings to the wider population and other hospital settings.
5. Perform survival analysis on our Mass General cohort.
6. Analyze the predictive value of the microRNA profiles for response to chemotherapy and radiotherapy.
7. Perform integrative pharmacogenomic analysis with sophisticated bioinformatics methodology, using the microRNAs as markers to identify novel possible drug treatments beyond chemotherapy. This will be done with intensive machine learning algorithms and publicly available cell line drug response data.
8. Test novel drug hypotheses on patient derived PDX mouse models.
10. Perform DNA methylation analysis (found to be predictive of survival in one of the published datasets group in DDLPS). DNA methylation is a chemical modification of a gene that renders it usually inactive. MicroRNAs are thought to be heavily regulated by DNA methylation.
11. Perform analysis on other liposarcoma types (such myxoid, round cell, and pleomorphic). We believe that this will help us understand why myxoid liposarcoma, for example, is more sensitive to radiation and/or chemotherapy than other forms of LPS. This course of research might help us generate ideas on how to design better therapies for the more classic types of liposarcoma.
The Impact of Your Philanthropy

Philanthropic funding from your family has continued to be vital to advancing promising liposarcoma research in a short period of time. Your commitment has made our work possible and helps support our efforts to better understand and treat this disease.

While we have made great progress over the past few years, there is still much more we hope to accomplish. Looking to the future, the team is optimistic that this research will allow us to improve treatment options and outcomes for patients with liposarcoma. We are fortunate to have your support, and we hope that you will continue to partner with us as we get ever closer to conquering liposarcoma.
Appendix A

Methods and Results of the Liposarcoma Genomic MicroRNA Study

1. Normal fatty tissue, WDLPS and DDLPS have significantly different microRNA patterns of expression (profiles), when the entire set of microRNAs is examined. But WDLPS and DDLPS do show some overlap (only partial). The heatmap below shows the similarity of the samples based on the expression patterns of the microRNA signature. Rows represent microRNAs, and columns represent individual specimens. Red color denotes increased expression (relatively), and blue color denotes decreased expression (relatively) when comparing the different types of tissues and tumors. Orange is fatty tissue, dark blue is well differentiated liposarcomas, and pink is de differentiated liposarcomas.

2. With a smaller set of 87 microRNAs with the highest difference in expression (level), there is a clearer separation between the three types of tissue.

3. We then applied additional analysis, taking into account some samples are derived from the same patient (fatty tissue/WDLPS tumor, or WDLPS/DDLPS tumor or all three of them). This analysis generated additional lists of markers. These lists were matched with the list of 87 microRNAs described above, and the final common overlap consists of 6 microRNAs. These could possibly be among the most crucial regulatory molecules involved in the progression to the aggressive tumor types. These microRNAs are: miR-139-5p, miR-139-3p, miR-150-3p, miR-150-5p, miR-493-5p, and miR-493-3p (shown in the chart below). Further, we observe that they are all processed from 3 precursor miRNAs, which suggests that these are less likely to be random findings due to examining too many miRNAs at once.
4. We then downloaded and processed independent data from other groups that have made microRNA data publicly available recently. These were one dataset from MD Anderson Cancer Center and two separate datasets from Rotterdam, Netherlands. Again, red color shows increased expression, and blue color decreased expression. Orange is fatty tissue, dark blue is well differentiated liposarcomas, and pink is dedifferentiated liposarcomas. The very strong p values demonstrate that the microRNA signature separates the different histologic types in a statistically significant manner.
5. We then found that these microRNA signatures also distinguish primary from recurrent samples in the Mass General samples. In the heatmap below, rows correspond to microRNAs: red color denotes increased expression, blue color denotes decreased expression. Columns correspond to tumor specimens: Pink is primary dedifferentiated liposarcoma, orange is primary well differentiated liposarcoma, dark blue is recurrent dedifferentiated liposarcoma, and yellow (yellow), is recurrent well differentiated liposarcoma.
6. Because our internal Mass General sample cohort is still small (11 patients), we found another set of data from Memorial Sloan Kettering Cancer Center, which included 92 patients and provided follow up to the patients’ clinical course. These samples, however, were not analyzed for microRNAs at Memorial Sloan Kettering, and thus we decided to use gene expression data that were available. We first focused on identifying the genes that are targeted by the microRNAs that we discovered on the Mass General samples. We then reasoned that if the microRNAs are important in long term prognosis, their target genes may be too, and we used the target genes as surrogates for the microRNAs. We discovered that the expression patterns of the target genes strongly predicted which patients will develop future metastasis. The heatmap below shows the similarity of the samples based on the expression levels of the microRNA signature. Rows correspond to microRNAs: red color denotes increased expression; blue color denotes decreased expression. Columns correspond to tumor specimens: pink is dedifferentiated liposarcoma, orange is dedifferentiated liposarcoma with eventual metastatic relapse, dark blue is well differentiated liposarcoma, and yellow (yellow), is well differentiated liposarcoma with eventual metastatic relapse.

The dense grouping/clustering of orange and yellow labeled samples in two of the three main branches of the tree suggests that the microRNA signature is predicting the
probability of metastatic relapse. This is further shown in the formal Survival Analysis of the patients in the different branches of the trees shown on the side.

7. A more tailored signature consisting of 10 miRNAs that are specifically associated with relapse in the Mass General data also powerfully predicts eventual metastatic relapse in the Memorial Sloan Kettering patient cohort with a similar analysis as in #6, above. Colors in the heatmap below have the same meaning as in the heatmap in #6.

Again, the dense grouping/clustering of samples labeled yellow and orange in one of the two main branches of the tree suggests that the microRNA signature predicts the probability of eventual relapse, as also exemplified in the related Survival Analysis shown on the side.