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Next-generation sequencing-based characterization of the invasion by anatomical contiguity in a primary osseous diffuse large B-cell lymphoma. Correlation between the genetic profile of the malignancy and the clinical outcome of the patient

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Abstract.

Primary bone lymphoma is now a well-described entity in the World Health Organization (WHO) Classification of Tumors of Soft Tissue and Bone as a malignancy of the lymphoid tissue, with at least one mass within bone, without involvement of supraregional lymph nodes or other extranodal sites. In the current paper, we describe the complete characterization of the mutational landscape of a diffuse large B cell non-Hodgkin's lymphoma (DLBCL) of the tibial plateau. Currently, there is very little data about the genetic landscape of primary osseous lymphomas and about the genetic background of this type of malignancy, resistant to chemotherapy and invading the surrounding tissues. In the current paper, we describe the complete characterization of the mutational landscape of a DLBCL of the tibial plateau. Our data is consistent with already published data, that have shown that MKI67 activation is correlated with lymphoma progression. Along with a high Ki67 index, resistance to chemotherapy occurs with neurogenic locus notch homolog protein 1 (Notch) and KRAS activation.

This is the first molecular characterization for the invasion by anatomical contiguity for a primary bone lymphoma and while we only characterized one case and further deep sequencing
analyses are required, we can explain the clinical dismal evolution of the patient by correlating them with the genetic landscape of this type of lymphoma.

Introduction.

Primary non-Hodgkin lymphoma of the bone (PLB) was described for the first time in 1928 in a cohort of French patients, by Oberling (Oberling, 1928), with the first case series later published as 17 cases of “primary reticulum cell sarcoma of bone” from the National Tumor Registry in 1939 (Jackson and Parker, 1939). PLB is now a well-known lymphoma subtype, described by Chisholm et al (2017) (Chisholm et al., 2017) as a malignancy of the lymphoid tissue with at least one mass within the bone, without involvement of supraregional lymph nodes or other extranodal sites. Messina et al. describe it as a single bony lesion with or without involvement of regional lymph nodes or multiple bony lesions, but without lymph node or visceral disease (Messina et al., 2015). Thus, it excludes lymphomas that have disseminated from lymph nodes or extranodal sites and represent secondary skeletal involvement. Overall, PLB accounts for approximately 3-7% of bone malignancies, less than 1% of all non-Hodgkin lymphomas, and up to 5% of extranodal non-Hodgkin lymphomas (Horsman et al., 2006). An accepted definition of PLB is that of a single bony lesion that persists for longer than 6 months, without evidence of systemic involvement (Franczyk et al., 1989). Patients usually have localized pain, sometimes associated with a soft tissue mass or swelling, as well as the lack of constitutional B symptoms (Alencar et al., 2010). Subik et al. (2014) have reported that proximal tibia lymphoma has an excellent prognosis and occurs predominantly in young patients (median age 22.5 years). Given the excellent prognosis of pediatric PLB reported, the age of the patient, and not the location of the lymphoma (tibia), might influence the prognosis of the PLB (Subik et al., 2014).
Patients and Methods.

Patient.

The current research is based on the description of PBL by Chisholm et al (2017) and assesses the mutational landscape of a DLBCL of the tibial plateau. The paper presents the case report of a 31-year old man that presented to the clinic with a tumoral mass, an Eastern Cooperative Oncology Group (ECOG) grade of 0, LDH value of 570 UI/L (normal value 105-333 UI/L), normal values of serum creatine kinase and no fever, no severe weight loss and no night sweats. The patient first presented to the department of orthopedics with a pathological fracture. The biopsy showed a diffuse large B-cell lymphoma (DLBCL). Thus, the pathology examination showed a malignant lymphoid proliferation, composed of large cells, with a marked pleomorphic status and indistinguishable cell limits. Most cells had an amphiphilic cytoplasm, with a round, large and excentric nucleus, as well as a central nucleolus. The other cells had a basophilic cytoplasm and large nuclei with a vesicular chromatin and two-three visible nucleoli placed near the cellular membrane. The tumoral proliferation associates numerous mitotic events, which are atypical and invade the surrounding soft tissues, as well as the adjacent nervous vascular structures. In the pathology diagnosis algorithm, figures 1a and 1b show the morphology assessment. To have a final diagnosis, immunohistochemistry staining aided the pathology diagnosis. Thus, the malignant cells were positive for CD20 (Figure 1c), PAX5 (Figure 1d), CD10 (Figure 1e), Bcl-2 (Figure 1f), Bcl-6 (Figure 1g), p53 (Figure 1h), CD5 (focal assessment), as well as negative for CD138, TdT and ALK-1. The proliferation index, as assessed by Ki67 was 80%. The final pathology diagnosis was thus a primary osseous DLBCL.

The age-adjusted International Prognostic Index (IPI) score was high-intermediate risk, with an initial 5-year survival expected to be 69% (International Non-Hodgkin’s Lymphoma Prognostic Factors, 1993). Immediately after the initial diagnosis, the patient was investigated using both a PET Scan, as well as computer tomography. Both techniques revealed the absence of metastatic lymph nodes or of the dissemination of the disease in any other organs. The patient received 3 cycles of combination chemotherapy with injectable (inj.) rituximab 375 mg/m2 intravenous (i.v.) on day 1 (d1), inj. cyclophosphamide 750 mg/m2 i.v. on d1, inj. doxorubicin 50 mg/m2 i.v. on d1, inj. vincristine 1.4 mg/m2 i.v. on d1, and tablet prednisone 100 mg on d1–5 (R-CHOP) every 3 weeks. An interim assessment by using magnetic resonance imaging (MRI)
showed a significant reduction in the soft tissue component, when compared to the previous scans. The patient afterwards received 3 more cycles of R-CHOP. The magnetic resonance imaging (MRI) carried out after completion of chemotherapy demonstrated the resolution of the extraosseous component without any significant change in the intraosseous lesion. Following chemotherapy, the patient was eligible for involved field radiotherapy. He received 46 Gy in 23 fractions at 2 Gy per fraction to a localized field covering the pre-chemotherapy volume of the tumoral mass, with adequate margin. On follow-up, the patient showed remarkable symptomatic improvement. After six sessions of this therapy, unfortunately new malignant masses were diagnosed using a positron emission tomography (PET-CT) (Figure 2a), the scintigraphy (Figure 2b) also showed an abnormal mass of the right tibial plateau that extended to the proximal metaphysis before the six sessions of chemotherapy.

A PLB of the tibial plateau is rarely diagnosed and no standard-of-care protocols are published (Giardino et al., 2012). Physicians apply the protocols from classic DLBCL, often with unsuccessful outcomes. After six sessions of this chemotherapy regimen administered to our patient, new malignant masses were diagnosed using a PET Scan. R-CHOP chemotherapy was changed to dexamethasone, cytarabine and cisplatin (DHAP) regimen but 2 months later, the disease relapsed with multiple local malignant nodules and invasion by anatomical contiguity. The treatment plan for the DHAP protocol consisted of the administration of 375 mg/m² rituximab i.v. at day 0, plus 40 mg dexamethasone p.o. at days 1-4, plus 2000 mg/m² cytarabine i.v. infusion over 2 hours at day 2, every 12 hours, plus 100 mg/m cisplatin i.v. infusion at day 1. As the physical status of the patient did not allow further chemotherapy, surgical resection of the limb was the most appropriate, followed by chemotherapy. In figure 3a, we present all the identified mutations and their exact location on the chromosome. Figure 3b shows the resected limb, with clear invasion by contiguity of the primary osseous lymphoma. Figure 3c shows the clinical aspect of the multiple local malignant nodules of the skin.

NGS assay.

Tissue samples were collected from the primary bone, as well as from the surrounding areas of invasion by anatomical contiguity and fixed in paraffin. DNA was extracted from 8
sections of 10µm of formalin-fixed, paraffin-embedded tissues using the PureLink Genomic DNA Mini Kit from Invitrogen with the protocol provided by the manufacturer. DNA quantification was performed using NanoDrop (Thermo-Scientific). 20ng of DNA were used in amplicon library synthesis for next generation sequencing. For this, we used the Ion AmpliSeq Library kit (Thermo Scientific) and a custom panel of specific primers for our genes of interest. The custom gene panel contains the following genes: JAK1, NRAS, STAT1, IKZF2, TLR2, BRAF, MYC, ABL1, NOTCH1, MKI67, ATM, FOXN1, KRAS, RBL2, E2F4, TP53, CCL2 and JUND. The panel of genes chosen for the analysis consisted of a mixed panel of the most common genes involved in chronic inflammation and cancer (Cruz et al., 2015; Hermouet et al., 2015; Shalapour et al., 2015). The synthesized libraries were quantified using Qubit 2.0 fluorimeter and Qubit DNA HS kit. We further diluted the libraries at 100pM and used them to synthesize the template for sequencing using the Ion PGM Template OT2 200 kit and the Ion OneTouch 2 instrument. The template was quantified using the Ion Sphere Quality Control kit and Qubit 2.0, for which we obtained concentrations between 10-15%. These templates were then sequenced on the Ion Torrent Personal Genome Machine using an Ion 316v2 Chip and the Ion PGM 200 Sequencing kit. For data analysis, we used the Torrent Suite software and the Ion Reporter v5. For signal processing, base calling and sequence alignment to Hg19 we employed the Torrent Suit software and for variant calling we used the Ion Reporter software. The filters used for analysis on the Ion Reporter software were \( p \) value \( \leq 0.05 \), coverage \( \geq 200 \).

No malignant tissue before initiation of therapy was assessed by NGS. Immediately after the pathology diagnosis, chemotherapy was administered. Usually, primary osseous lymphomas have a good prognosis. Still, for this case, resistance to classic chemotherapy was early and the outcome dismal. Malignant tissue was removed from the tumoral mass after surgical resection and the idea of sequencing this tissue was brought in the internal hematology review board of the Ion Chiricuta Oncology Institute, considering the negative response to therapy. For negative control of the sequencing, we have used normal tissue, surrounding the malignant mass, assessed by pathology slides as having no malignant cell invasion.
Results.

We identified 9 different mutations in 6 genes (ABL1 c.*1596T>C, NOTCH1c.2365C>G, p.Gln789Glu, MKI67c.3278A>G, p.Lys1093Arg, FOXM1, c.847-121insTCA, KRAS c.*679T>C and c.*633T>C and TP53, c.375+369delCCinsTG, c.375+363C>T and c.375+356A>G), (data not shown). The majority of mutations being in the intronic area. The exact percentage of mutations for all the studied samples for each location is presented in Figure 3d. The variant effect of the identified mutations was analyzed, and the results are presented in Figure 3e. The mutations were called with the Ion Reporter software and the filters used are: $p$ value $\leq 0.05$, coverage $\geq 200$.

Discussion.

The clinical evolution of primary tibial lymphomas is often unpredictable. Cytogenetic abnormalities cannot entirely explain the genetic background for the clinical heterogeneity of primary bone lymphomas and additional information comes from the detailed definition of the molecular correlates with chromosomal aberrations. Recently, the improvements in next generation sequencing (NGS) technologies have provided a novel opportunity to examine the genome of the mature B-cell malignancies. This has further allowed for previously unknown genomic alterations to be identified, as are the mutations of NOTCH1, SF3B1 (splicing factor 3B subunit 1) and BIRC3. Although the pathogenesis of osseous lymphomas is only partially understood, recent data sheds more light on the molecular pathways involved in disease progression. Such advances in the understanding of the biology of the clinical stage are enabled by developments in the field of novel genome-wide molecular techniques, particularly NGS, often involving regulatory genes such as TP53, KRAS, NOTCH1 and KRAS resulting in chemoresistance. Our study is one of the first to report molecular changes following chemotherapy for primary osseous lymphomas, based on the data obtained from the clinical outcome of the case and correlated with genomic data. The single-case analysis is the main weakness of the study, as it requires the enrollment of a large cohort of osseous lymphomas cases to validate or invalidate our gene panel as a prognostic factor for the evolution of primary osseous lymphomas towards a more aggressive, chemotherapy-refractory clinical entity. Our
data is consistent with already published data, that have shown that MKI67 activation is correlated with lymphoma progression (Hoster et al., 2016; Kridel et al., 2016). In a large, international, multicentric study of the European Mantle Cell Lymphoma Network, Hoster et al. (2016) have proven that the Ki-67 index is a strong independent prognostic factor. The Ki-67 index was investigated in 508 out of 832 lymphoma cases and the blastoid cytology with an inferior overall survival independent of the Mantle Cell Lymphoma International Prognostic Index (MIPI), but not independent of Ki-67. Considering that mantle cell lymphomas are rather aggressive B-cell malignancies with an important variability in individual outcome, they concluded that Ki-67 is superior to cytology or even to growth patterns as a prognostic factor. The data is in accordance with the mutational status of our case, diagnosed with a B-cell lymphoma, and thus confirming that Ki-67 plays an important role in disease progression and dissemination. Along with a high Ki67 index, resistance to chemotherapy occurs with Notch and KRAS activation (McKinney et al., 2017; Tan et al., 2017). In DLBLC, 8% of cases have mutations of NOTCH2, gene that is highly mutated also in autoimmune disorders and diseases that are correlated with a chronic state of inflammation (Kuksin et al., 2015). Regarding the NOTCH mutation, c.2365C>G is considered a pathogenic mutation both by the FATHMM and PolyPhen2 predictions, found in the Calcium binding EGF- domain. We can conclude that this is a mutation that can affect the protein's function. The MKI67 c. 3278A>G mutation is a pathogenic mutation as predicted by the PolyPhen 2 score, as this mutation is in the forkhead-associated domain of the Ki-67, where the NIFK protein binds, a protein that plays an important role in mitosis and cell cycle progression. The 3-UTR mutations c.*633T>C, also known as c.512T>C rs9266, are classified by the ClinVar database as likely benign. The other intronic mutations and 3-UTR mutations are new mutations, that are not found in any database. NOTCH pathway was proven to be important in lymphomagenesis (Weng et al., 2004), mostly due to the chronic antigenic stimulation and inflammation. This pathway is also recurrently mutated in DLBLC, associated with a hepatitis C infection and a shorter overall survival (OS) (Arcaini et al., 2015). Whereas Karube et al have used an in-silico drug discovery analysis for the cases with NOTCH alteration (Karube et al., 2017), the data is in accordance to our patient, that also has these very genes mutated and a negative clinical outcome. Nevertheless, it is true that it may actually be misleading to link intronic variants and non-Hodgkin's lymphoma pathobiology. Even though the current paper is a case report and it should be considered as such, it presents a
new way to look at the pathophysiology of primary osseous lymphomas and it provides some insight into how a DLBCL may evolve during the clinical course of the disease, from diagnosis to resistance to chemotherapy and relapse. The mutations identified by the NGS are linked to the phenotypic behavior of the lymphoma and are also in concordance to the results of the immunohistochemistry staining, as provided by the pathology report.

The scintigraphy carried out showed an abnormal uptake at the distal end of the right tibia, where the tibia relapsed. Even if it is most likely that this was a new lesion that arose after chemotherapy, there is a small possibility that this was actually a lymphomatous lesion before chemotherapy. Still, this hypothesis does not affect the conclusions of our case report and the genes involved in cancer progression, as identified by NGS, might have defined a cancer stem cell sub-population, no matter the lesion identified on the scintigraphy. Cancer stem cells are responsible for both the relapse of a malignancy, as well as for resistance to therapy, as previously shown by our department (Tomuleasa et al., 2010a, 2010b; Florian et al., 2011; Susman et al., 2012; Carmignani et al., 2014; Frinc et al., 2014). To clearly define the lesion, clonality assays are needed, which for now surpassed the purpose of the manuscript.

A weak point of the manuscript is that we did not assess the genetic profile of the patient at diagnosis and linking the presence of the mutations to the clinical presentation of the patient is biased. Still, it is well known in the field that KRAS is not associated with lymphoma progression, as is the case of most hematological malignancies. For MKI67 and NOTCH1, the presence of these mutated genes was linked to the progression of other hematological malignancies, as well as lymphomas. Their mutation is not a pathogenic mutation and is of potential clinical importance when associated with other extrinsic factors, as is infection of hepatitis C virus (Mationg-Kalaw et al., 2012; Karube et al., 2014; Arcaini et al., 2015; Witkowski et al., 2015). Thus, even if we admit that one of the weaknesses of our study is the lack of evaluation for the previously described gene mutation at diagnosis, we state with all responsibility that the clinical manifestations of the case are linked to the acquisition of the presently-described mutational status for the malignancy.

Still, for non-Hodgkin's lymphoma patients, the International non-Hodgkin's Lymphoma Prognostic Factors Project has developed a scoring model to predict the outcome based on the patient's clinical characteristics before treatment (International Non-Hodgkin’s Lymphoma
Prognostic Factors Project, 1993). Our patient had just one risk factor, thus having an index of 1, corresponding to the low factor group. Still, the IPI is a scoring system developed three decades ago, when state-of-the-art assays as is the case of NGS were not available. This is the intriguing aspect of our case report, for whom the genetic background and characteristics have proven to be superior to classic clinical prognostic systems regarding the clinical outcome and response to treatment.

**Conclusion.**

Currently, there is very little data on the genetic landscape of primary osseous lymphomas – DLBCL subtype, while the entity becomes resistant to chemotherapy and invades the surrounding tissues. In the current paper, we describe the genetic mutations for this type of malignancy and provide a novel insight into its invasion and metastasis.

This is the first molecular characterization for the invasion by anatomical contiguity for an osseous DLBCL. Still, the major drawback of the paper is that it uses only one patient and should be regarded as a case report, with all its subsequent limitations. We correlated the results of the sequencing with the clinical evolution of the patient, by linking our mutations with the potential role of the mutated genes in cancer features such as invasion potential or resistance to chemotherapy, after citing the appropriate papers. While we only characterized one case and further deep sequencing analyses are required, we can explain the clinical dismal evolution of the patient by correlating them with the genetic landscape of this type of lymphoma. Still, the mutation profile of the hereby described case is hardly unique when compared with other DLBCL, in particular relapsed cases. Thus, while the presently described data is interesting regarding primary bone lymphomas, a firm conclusion is premature and should await study of additional cases.
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Conflict of interest:

All authors have approved the manuscript and no potential conflict of interest is reported.

References.


FIGURES

Figure 1. Pathology diagnosis of DLBCL. a) DLBCL of bone 100x HE staining. Lymphoma cells massively invade soft tissues adjacent to the bone-adipose tissue on the left side (star), perineurial involvement (arrow) and perivascular (arrow) on the right. b) DLBCL of bone 400x HE staining. Diffuse infiltration of highly atypical lymphocytes with frequent mitotic figures. Figure 1a shows a low power magnification, whereas Figure 1b shows a high power magnification. The morphology assessment was afterwards completed by specific immunohistochemistry stainings, that have helped to establish a final diagnosis c) DLBCL of bone 400x CD20 staining. Diffuse and intense staining of the tumoral cells. d) DLBCL of bone 400x CD10 staining. Malignant cells are consistently positive for CD10. e) DLBCL of bone 400x Ki67 staining. High proliferative index, consistent with the diagnosis of DLBCL. f) DLBCL of bone 400x p53 staining. Diffuse staining of the majority of tumoral nuclei. g) DLBCL of bone 400x PAX5 staining. Strong expression of PAX5 in the nuclei of tumoral cells. A group of small reactive T cells is apparent in the upper-right corner.

Figure 2. a) PET Scan of the primary tibial DLBCL; b) scintigraphy

Figure 3.

A) Exact location on the chromosome of the identified mutations. A. all the chromosomes and the identified mutations on each chromosome; B) the exact location of mutations identified on chromosome 9; C) the exact location of the mutations identified on chromosome 10; D) the exact location of the mutations identified on chromosome 12; E) the exact location of the mutations identified on chromosome 17.

B) Section in the tibia of the patient after surgical excision showing the invasion by anatomical contiguity. Letters b to e represent the exact tissues selected for NGS and further described in the
manuscript. The section of the patient's foot was selected after the surgical excision of the right foot. By using a macroscopic selection of the invaded tissue, we were able to select malignant tissue, thus avoiding necrotic areas. The selected tissues were checked for quality control by two certified pathologists independent from one another.

C) Clinical appearance of the invasion by anatomical contiguity. We can clearly notice the clinical evolution towards self-amputation, as well as the permeation nodules on the surface of the skin. These represent cutaneous dissemination of the malignancy.

D) The percentage of identified mutations per location.

E) Main mutations identified and responsible for the invasion by anatomical contiguity.