

CHARLES UNIVERSITY

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DISSERTATION

**THE DIAGNOSTICS OF RARE SOFT TISSUE AND SKIN
TUMORS USING THE HISTOLOGICAL,
IMMUNOHISTOCHEMICAL, AND MOLECULAR BIOLOGICAL
METHODS**

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ABSTRAKT

Disertační práce je výsledkem doktorského výzkumu Antoniny Kalmykové, prováděného na Univerzitě Karlově v Praze, na Lékařské fakultě v Plzni, v letech 2019 až 2025. Hlavním zaměřením výzkumu byly vzácné nádory měkkých tkání a kůže. Ve své práci využívala různé vyšetřovací metody, včetně morfologie, imunohistochemie a molekulárně genetických technik, s cílem rozšířit a/nebo zlepšit stávající klasifikační a diagnostické přístupy.

Disertační práce se skládá ze dvou částí. První část se zaměřila na vzácné nádory kůže a obsahuje tři články. V první studii jsme se soustředili na komplexní klinickou, morfologickou a molekulární charakteristiku melanocytárních nádorů inaktivovaných genem *BAP1* (BIMT). Během tohoto výzkumu jsme rozšířili morfologické spektrum BIMT a identifikovali nové mutace genu *BAP1*. Zdůraznili jsme potřebu dalšího výzkumu pro správné vymezení BIMT a identifikaci možných morfologických nebo molekulárních prognostických faktorů.

Ve druhé publikaci jsme popsali nově identifikovaný nádor asociovaný s fúzí *MITF::CREM* a shrnuli naše nálezy společně se dvěma dříve publikovanými případy. Naše výzkumy rozšířily morfologické charakteristiky této vzácné jednotky. Uvedli jsme, že molekulární potvrzení je nezbytné pro definitivní diagnózu a že jsou zapotřebí další studie k určení přesného biologického chování.

Třetí článek je přehledovou studií zaměřenou na hlavní změny v kapitole o mezenchymálních nádorech v nové 5. edici WHO klasifikace kožních nádorů, především se zaměřením na nové přidané jednotky. Mezi tyto jednotky patří nádory s melanocytární diferenciací se změnami *CRTC1::TRIM11*, *ACTIN::MITF* a *MITF::CREM*, fibroblastické nádory s přestavbou *EWSR1::SMAD3*, povrchové CD34-pozitivní fibroblastické nádory a vřetenobuněčné neoplazie s přestavbou genu *NTRK*. V článku byly diskutovány morfologické rysy, imunohistochemické charakteristiky, molekulární profil a diferenciální diagnostika.

Druhá část disertační práce se zaměřila na vzácné nádory měkkých tkání a obsahuje čtyři články. První článek se věnoval vzácnému nádoru měkkých tkání s indolentním průběhem: zánětlivému leiomyosarkomu (ILMS). Zkoumali jsme imunohistochemický profil a analyzovali údaje z dlouhodobého sledování s cílem pochopit klinické chování tohoto nádoru. Výsledky molekulární studie a imunohistochemické charakteristiky ukázaly, že ILMS není podtypem leiomyosarkomu. Všechna tato zjištění poprvé naznačila, že ILMS pochází z primitivnější myogenní linie s hladkou, ale převážně kosterní svalovou diferenciací. Toto bylo potvrzeno následnými studiemi a nakonec vedlo k redefinici klasifikace tohoto nádoru z ILMS na neoplazii s kosterně svalovým imunofenotypem v připravované WHO klasifikaci nádorů měkkých tkání.

Ve druhém článku jsme popsali vzácný fenomén transformace biphenotypického nosního sarkomu (BSNS) s fúzí *PAX3::MAML3* na vysoce maligní rhabdomyosarkom (RMS). Z tohoto důvodu jsme doporučili pečlivé klinické sledování a důkladné histologické zpracování každého případu BSNS, stejně jako molekulární profilování všech typů RMS v oblasti nosu, aby se předešlo možným diagnostickým chybám.

Ve třetí studii jsme se zabývali doposud největší sérií případů sarkomů s přestavbou *EWSR - PATZ1* (EPS), se zaměřením na detailní klinicko-patologickou analýzu. Rozšířili jsme známé morfologické spektrum a definovali specifické histologické a imunohistochemické znaky těchto nádorů. V některých případech jsme také popsali indolentnější klinický průběh, než byl dříve uváděn, což naznačuje, že určitá podskupina EPS může mít příznivější prognózu, než se původně předpokládalo – toto zjištění bylo následně potvrzeno. Další výzkum je však nutný k identifikaci potenciálních morfologických nebo molekulárních prognostických faktorů, které mohou ovlivnit klinický vývoj.

Ve čtvrté studii jsme se zaměřili na komplexní klinicko-patologickou, molekulární a metylační analýzu mezenchymálních nádorů s aberacemi genů *NTRK* a jiných kináz – jednu z největších sérií dosud. Bylo potvrzeno, že morfologické spektrum, definované buněčností a stupněm atypie, koreluje se změnami počtu chromozomálních kopií a prognózou. Bylo identifikováno několik nových fúzí, včetně *PWWP2A::RET*, *NUMA1::RET*, *ITSN1::RAF1* a *CAPZA2::MET*. Navíc byl v této sérii objeven a publikován první případ nádoru děložního čípku s mutacemi *BRAF* a *EGFR*. Sekvenování DNA ukázalo, že sekundární molekulární změny jsou u těchto převážně fúzí kináz řízených nádorů vzácné. Nejdůležitější však je, že jsme prokázali, že metylační profil tzv. vřetenobuněčných nádorů s přestavbou *NTRK* se ve velké míře překrývá s profilem infantilního fibrosarkomu, což je v současné klasifikaci WHO samostatná jednotka – tím jsme podpořili názor, že všechny tyto neoplazie jsou úzce příbuzné a pravděpodobně představují morfologické spektrum jediné jednotky.

SUMMARY

The dissertation is the result of Antonina Kalmykova's doctoral research conducted at Charles University in Prague, at the Faculty of Medicine in Pilsen, from 2019 to 2025. The primary focus was on rare soft tissue and skin tumors. In her work she used various investigative methods, including morphology, immunohistochemistry, and molecular genetic techniques, with the aim of extending and/or improving existing classification and diagnostic approaches.

The dissertation consists of two parts.

The first part focused on rare skin tumors and included three articles.

In the first study, we focused on comprehensive clinical, morphological, and molecular characteristics of BAP1-inactivated melanocytic tumors (BIMT). During this research, we extended the morphological spectrum of BIMTs and identified novel BAP1 mutations. We stressed the necessity of further investigations to correctly define BIMTs and identify possible morphological or molecular prognostic factors.

In the second paper, we reported newly identified *MITF::CREM* fusion-associated tumor and summarized our findings together with two previously published cases. Our research extended the morphological characteristics of this rare entity. We stated that molecular confirmation is necessary for a definitive diagnosis and further studies are needed to determine the exact biological behavior.

The third paper is a review article focused on the main changes in the mesenchymal tumor chapter in the new 5th edition of WHO classification of skin tumors, primarily focusing on the new entities that were added [1]. These namely include *CRTC1::TRIM11*, *ACTIN::MITF*, and *MITF::CREM* rearranged tumors with melanocytic differentiation, *EWSR1::SMAD3*-rearranged fibroblastic tumors, superficial CD34-positive fibroblastic tumors, and *NTRK*-rearranged spindle cell neoplasms. Morphological features, immunohistochemical characteristics, molecular profile, and differential diagnosis were discussed in this article.

The second part focused on rare soft tissue tumors and included four articles.

The first article was focused on a rare soft tissue tumor with an indolent nature: inflammatory leiomyosarcoma (ILMS). We investigated the immunohistochemical profile and analyzed follow-up data to understand the long-term outcomes and, therefore, clinical behavior of this tumor. The molecular study results and immunohistochemical characteristics indicated that ILMS is not a subtype of leiomyosarcoma. All these findings for the first time suggested that ILMS originate from a more primitive myogenic lineage with smooth but mainly skeletal muscle differentiation. This has been confirmed in subsequent studies and eventually it has led to redefining of its current classification

from ILMS to a neoplasm with skeletal muscle immunophenotype in the upcoming WHO classification of soft tissue tumors.

In the second paper, we described a rare phenomenon of transformation of biphenotypic sinonasal sarcoma (BSNS) with *PAX3::MAML3* fusion into high-grade rhabdomyosarcoma (RMS). Therefore, we advocated for a careful clinical follow-up and thorough sampling of every BSNS case, as well as molecular profiling of sinonasal RMS of any type to prevent potential diagnostic errors.

In the third study, we investigated by then the largest case series of *EWSR1-PATZ1*-rearranged sarcomas (EPS), focusing on detailed clinicopathological analysis. We expanded the known morphological range and defined specific histological and immunohistochemical features of these tumors. Also, in some cases, we described more indolent clinical behavior than previously reported cases, suggesting that a subset of EPS may have a more favorable prognosis than previously thought, a finding confirmed in subsequent studies. Further investigation is needed to identify the potential morphological or molecular prognostic factors which can influence clinical outcomes.

In the fourth study, we focused on comprehensive clinicopathological, molecular, and methylation analysis of mesenchymal tumors with *NTRK* and other kinase gene aberrations, one of the largest series to date. A morphological spectrum defined by cellularity and degree of atypia was confirmed to correlate with chromosomal copy number changes as well as outcome. Several novel fusions were identified, including *PWWP2A::RET*, *NUMA1::RET*, *ITSN1::RAF1*, and *CAPZA2::MET* fusions. Additionally, the first cervical case with *BRAF* and *EGFR* mutations was discovered and published in this series. DNA sequencing revealed that secondary molecular alterations are rare in these mostly kinase fusion driven tumors. Most importantly, we have shown that the methylation profile of so called *NTRK*-rearranged spindle cell tumors largely overlaps with that of infantile fibrosarcoma, a currently separate entity in the WHO classification, thus supporting the notion that all these neoplasms are closely related and perhaps represent a morphological spectrum of one entity.

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GOALS AND HYPOTHESIS

The objectives of the dissertation "The Diagnostics of Rare Soft Tissue and Skin Tumors Using Histological, Immunohistochemical, and Molecular Biological Methods" were as follows:

1. To investigate rare soft tissue and skin tumors to identify specific clinical and morphological characteristics that could potentially enhance diagnostic accuracy.
2. To study genetic mutations and pathogenic molecular pathways involved in the tumorigenesis and progression of soft tissue and skin tumors to identify potential therapeutic targets and predictive markers.
3. To establish correlations between clinical presentation, histopathology, and molecular profiles to improve our understanding of the biological behavior of these rare entities.
4. To expand existing knowledge for soft tissue and skin tumors, facilitating research, data sharing, and collaborative efforts in understanding these diseases.
5. To extend and refine the current classification of soft tissue and skin tumors.

Fulfilling research aims to improve the understanding, diagnostic process, and management of rare soft tissues and skin tumors, which ultimately leads to better patient outcomes.

MATERIALS AND METHODS

The majority of cases were sourced from the Tumor Registry in Pilsen, with some additionally reviewed as part of collective consultation cases.

Clinical and follow-up information was obtained from medical records, patients, treating physicians, or referring pathologists.

In addition to morphological assessment, extensive panels of immunohistochemical markers were employed to ensure a comprehensive evaluation of expression profiles.

To further explore tumor biology, a range of molecular genetic techniques—including fluorescence in situ hybridization (FISH), reverse transcription polymerase chain reaction (RT-PCR), DNA and RNA-based next-generation sequencing (NGS), and methylation analysis—were utilized.

RESULTS

Rare skin tumors.

BAP1-inactivated melanocytoma is a histologically distinct lesion characterized by epithelioid cell morphology and genetically defined by the loss of *BAP1* gene function. It can be observed sporadically or as part of the *BAP1* tumor predisposition syndrome (BAP1-TPDS) [2].

In our study, we present a clinical, morphological, and molecular analysis of 50 BIMTs from 36 patients, with a female-to-male ratio of 2.3:1 (mean age of 30.4 years).

The primary aim of this study was to analyze the morphological features and to deepen the understanding of the mutational spectrum of this neoplasm.

Multiple lesions were found in approximately a quarter of cases. The most common tumor locations were the head and neck region and trunk, followed by the upper extremities and genital area. Clinically, all lesions appeared as flesh-colored exophytic nodules, sometimes with a pigmented rim. A history of malignancy was noted in 21 patients, including uveal and cutaneous melanoma, mesothelioma, renal, lung, breast, and thyroid cancer, as well as schwannoma of the cerebellopontine angle. Follow-up data (mean: 34.3 months) showed no recurrence or metastasis in most cases, except for two patients who experienced adverse outcomes, including nodal metastasis and local recurrence.

Most BIMTs presented as dermally-based neoplasms with dome-shaped or polypoid architecture. 90% were associated with remnants of a conventional nevus, while 10% were composed solely of *BAP1*-negative epithelioid melanocytes. Epithelioid cells typically exhibited dyscohesive or trabecular growth, often forming single-cell clusters at the lesion's edges.

BAP1-negative cells displayed significant pleomorphism. Multinucleated or giant epithelioid melanocytes were consistently present. Mitotic activity was generally low (2 per 1mm² with rare exceptions up to 5 per 1mm²).

Immunohistochemical findings showed nuclear loss of *BAP1* staining in epithelioid melanocytes. Remnants of conventional nevi retained *BAP1* nuclear expression in 90% cases.

A single-allele mutation of *BAP1* was detected in 16 out of 26 cases, while 4 neoplasms harbored biallelic mutations. During the study, several novel *BAP1* mutations were identified, including NM_004656.3: c.14 G > A p.(Trp5Ter), c.25 G > T p.(Glu9Ter), c.249_250delinsT p.(His84ThrfsTer3), c.390_412del p.(Ile131GlnfsTer4), c.700 G > C p.(Val234Leu), and c.856 A > T p.(Lys286Ter). Germline *BAP1* mutations were identified in four cases. Additionally, one patient with multiple neoplasms exhibited an identical *BAP1* mutation (c.1717delG) across different tumors, strongly suggesting a germline alteration. Among the 26 neoplasms analyzed, 24 harbored the

BRAFV600E mutation. Two cases with *RAF1* fusions, specifically *TRAK1::RAF1* and *IGIT2::RAF1*, had been previously reported [3]. Additional mutations were detected in nine BIMTs, including an isolated *MYC* gain, as well as *FGFR1* and *ERCC2* gains. One neoplasm exhibited gains in both *MYC* and *RREB1*. Tetraploid mononuclear cells were identified in five cases.

Our study expanded the morphological spectrum of BIMTs and identifies novel *BAP1* mutations. While BIMTs likely evolve from conventional melanocytic nevi, the presence of cases without detectable *BAP1* alterations and the occasional presence of tetraploidy suggests more complex pathological mechanisms. Despite their atypical features, BIMTs generally follow an indolent course, though long-term follow-up is needed. The only metastatic case involved a child with a nevoid-like lesion displaying bland cytology, resembling previously reported melanomas with *BAP1* inactivation [4, 5]. Further studies are necessary to clarify the definition of BIMTs and assess additional morphological and molecular features that can help identify adverse outcomes.

Cutaneous tumours with melanocytic differentiation of non-melanocytic origin represent a growing category of neoplasms with the distinct molecular profiles. Recently, it has been broadened by newly identified fusion-associated tumors, namely *ACTIN::MITF*, and *MITF::CREM* tumors.

In this study, we report a case of rare *MITF::CREM* rearranged tumor, occurring in the temporal region of a 1-year-old girl. Including the two previously described cases, a total of three neoplasms with the this fusion have been identified to date of publication [6, 7].

Morphologically, the lesion was an ill-defined, polypoid, dermal-based tumor infiltrating between the underlying collagen bundles, with no junctional component present. The tumor was composed of cellular solid sheets or small nests of epithelioid to spindle-shaped cells, primarily with eosinophilic cytoplasm and, less frequently, clear cytoplasm. The nuclei were round to ovoid, exhibiting moderate to high-grade atypia with prominent nucleoli. There were 11 mitoses per 10 HPFs, with occasional atypical mitotic figures. Necrosis, as well as vascular or perineural invasion, was absent. A sparse perivascular lymphohistiocytic infiltrate with scattered plasma cells was present at the tumor periphery. The overlying epidermis showed focal ulceration with reactive changes.

Immunohistochemically, the tumor exhibited strong and diffuse expression of S100 protein, SOX10, and MITF, while HMB45, tyrosinase, and Melan-A were negative. The Ki-67 index was high (up to 40%).

Extensive RNA sequencing identified an *MITF* (exon 6)::*CREM* (exon 6) gene fusion.

No evidence of disease was noted after 9 months of the initial diagnosis.

In conclusion, we reported an additional case of an *MITF::CREM*-rearranged tumor, further contributing to the characterization of the clinicopathological features of these rare neoplasms. Due

to the extreme rarity of *MITF::CREM*-rearranged tumors, molecular confirmation is strongly recommended for a definitive diagnosis. Also, further studies are necessary to precisely determine their biological potential and clarify their nosologic status.

The review article on updates in cutaneous mesenchymal tumors in the 5th edition of the WHO Classification of Skin Tumors, with an emphasis on newly recognized fusion-associated neoplasms, included *CRTC1::TRIM11*-, *ACTIN::MITF*-, and *MITF::CREM*-rearranged tumors with melanocytic differentiation; *EWSR1::SMAD3*-rearranged fibroblastic tumors; superficial CD34-positive fibroblastic tumors; *NTRK*-rearranged spindle cell neoplasms (*NTRK-SCN*); as well as other relevant updates introduced in the mesenchymal tumor chapter of the 5th edition. Rare soft tissue tumors.

Rare soft tissue tumors

ILMS is an extremely rare and under-recognized soft tissue neoplasm which was characterized by smooth muscle differentiation and a prominent intrinsic inflammatory component. ILMS was thought to have favorable prognosis but follow-up data were limited due to the lack of long-term observations [8]. It was shown to have specific near-haploid chromosomal changes, sometimes accompanied by a hyperdiploid karyotype. Importantly, one study revealed a distinct myogenic gene overexpression, particularly *MYOD1*, *MYOG*, and *PAX 7* but did not verify these findings on immunohistochemical level [8]

Therefore, the main aim of our study was to improve the understanding of clinicopathological features of these neoplasms with a special focus on long-term follow-up as well as to characterize the immunohistochemical profile with a special emphasis on the expression of skeletal muscle markers.

In our study, we collected 9 cases. The clinical features included 5 male and 4 female patients aged 25–54 years, with an average age of 37.8 years. Tumors were predominantly located in the somatic soft tissues, particularly the lower limbs and back, with two exceptions occurring in the lung and omentum. Most somatic soft tissue tumors presented as intramuscular masses. Tumor sizes ranged from 2.6 to 9 cm. Treatment modalities included surgical resection in most of the cases, with additional chemotherapy in case of the omental tumor. Follow-up data (average 10.6 years) showed no recurrence or metastasis in somatic soft tissue cases, while the omental tumor spread locally but did not metastasize; the patient remained disease-free 7 years post-treatment.

Tumor architecture revealed an expansively growing, well-circumscribed neoplasm surrounded by a fibrous capsule, occasionally accompanied by lymphoid aggregates at the margin. Growth patterns varied from fascicular and storiform to haphazard, with variable nuclear pleomorphism and hyperchromatism. Tumors typically presented as low- to intermediate-grade sarcomas with rare mitotic activity (0-1 per 10 HPF, except in one case with 2 per 10 HPF).

Cytomorphologically, tumors predominantly consisted of spindle-shaped cells, with epithelioid to rhabdoid cells present in all cases. Deeply eosinophilic cytoplasm was characteristic, without cross-striated intracytoplasmic inclusions.

The intrinsic inflammatory component was present in all cases. Prominent collections of xanthoma cells were seen in 6 out of 9 cases, with small foamy cell aggregates observed in the remaining cases. Additional features included focal tumor necrosis in 3 cases, hemosiderin deposition in 6 cases, and the presence of calcospherites and cholesterol crystals in 2 cases each.

The tumor cells consistently expressed desmin and/or smooth muscle actin (SMA). In all desmin-positive cases, a subset of cells with histiocyte-like foamy cytoplasm showed strong desmin expression but was negative for CD163. H-caldesmon and muscle-specific actin demonstrated variable immunoreactivity. MyoD1, PAX-7, and myogenin also showed variable positivity, ranging from focal to diffuse expression. Histiocytic markers, such as CD163, exhibited diffuse and strong positivity in all tested cases, highlighting histiocytic aggregates and spindled histiocytes interspersed among neoplastic cells.

The Archer FusionPlex sarcoma kit was used for testing, but no rearrangement was found in the 4 cases that were analyzed. Four additional cases could not be assessed due to poor quality of the archival tissue. Two cases were examined using the IlluminaTS170 assay, with one case showing mutations in the *NFI* gene (c.1738dupT; p.Tyr580LeufsTer8) and the *ERBB4* gene (c.2087A>C; p.Glu696Ala).

In conclusion, this study has greatly expanded the understanding of the immunohistochemical profile and clinical behavior of ILMS, confirming generally indolent nature of this tumor. It also provided strong evidence that ILMS is not a subtype of leiomyosarcoma. The results of gene expression profiling and the common co-expression of both smooth and skeletal muscle markers suggest that it originates from a more primitive myogenic cell with a predominant skeletal muscle differentiation. Based on the findings initially observed in our study and confirmed in subsequent reports, the upcoming WHO classification will include this neoplasm in the chapter on skeletal muscle tumors under the name inflammatory rhabdomyoblastic tumor.

BSNS is a low-grade sarcoma with neural and myogenic differentiation [9]. Most cases show co-expression of SMA and S100 protein, with a subset also demonstrating morphological or immunohistochemical features indicative of skeletal muscle differentiation [9, 10, 11, 12].

In our study, we present a case of a 67-year-old male with a sinonasal tumor displaying areas of typical BSNS that abruptly transitioned into high-grade rhabdomyosarcoma.

Patient presented with a soft tissue mass involving the right ethmoid, maxillary, and frontal sinuses with extraconal orbital invasion. Endoscopic examination revealed a $4.4 \times 3.4 \times 2.2$ cm mass that blocked the nasal airway and centered around the middle turbinate. Initial biopsy confirmed a high-grade sarcoma with myogenic differentiation, diagnosed as embryonal RMS without evidence of metastatic disease. The patient began neoadjuvant chemotherapy, but after three cycles, local progression was observed.

The resection specimen revealed a proliferation of uniform spindle cells with moderate to high cellularity, occasional fascicular arrangement, minimal atypia, and no mitotic activity. Acanthotic projections of the surface mucosa were surrounded by tumor cells. No rhabdoid differentiation was observed in these areas. However, in some sections, abrupt transitions to a high-grade sarcoma with rhabdoid features were observed, accompanied by extensive mitotic activity and areas of necrosis.

Immunohistochemical features of conventional BSNS areas were characterized by patchy SMA expression and diffuse S100 protein positivity. PAX7 showed strong diffuse staining, while MyoD1 displayed patchy expression. Desmin and myogenin were negative. In contrast, high-grade areas showed complete negativity for S100 protein and SMA, with only patchy PAX7 expression. Desmin, MyoD1, and myogenin exhibited diffuse positivity.

Molecular analysis using the Archer FusionPlex assay revealed a *PAX3*(exon7)::*MAML3*(exon2) fusion, confirmed by FISH using *MAML3* (4q31.1) and *PAX3* (2q36.1) breakapart probes (both from SureFISH, Agilent). FISH analysis confirmed rearrangement in both conventional and high-grade components.

Despite undergoing radiotherapy and chemotherapy, the patient died from the disease 15 months after the diagnosis.

Our case illustrates that a very small subset of BSNS may progress into high-grade RMS, in cases where the low-grade area is not sampled, such cases could be mistaken for embryonal or pleomorphic RMS due to overlapping morphology and immunophenotype of the high-grade areas. In such cases (as was the case in the initial biopsy of our case), distinguishing between the two entities becomes impossible without molecular investigation.

Since the clinical behavior and response to treatment of embryonal and pleomorphic RMS may differ from RMS arising from BSNS, we believe it is reasonable to perform molecular studies in suspicious cases. This can be done using a *PAX3* FISH probe or, preferably, with an adequate RNA-sequencing panel.

In conclusion, we presented a unique case of BSNS with transformation into high-grade RMS. This case contributes to our understanding of this novel phenomenon. Although the risk of high-grade

transformation in BSNS appears low, it has significant clinical and diagnostic implications. We advocate for careful follow-up of patients with BSNS, thorough sampling of every case, and molecular profiling of sinonasal RMS of any type.

The first case of EPS was described 20 years ago [13]. However, only after the introduction of advanced molecular testing methods, such as NGS, the identification and publication of additional cases has increased, broadening our understanding of this rare entity [14, 15, 16, 17, 18]. Before the wide application of genomic alterations testing, this tumor was classified under the umbrella of Ewing-like sarcomas as were round cell sarcomas with *CIC* and *BCOR* gene fusions [19]. While the latter two were reclassified as distinct entities in the latest WHO classification of soft tissue and bone tumors, EPS remained within the category of round cell sarcomas with *EWSR1*-non-ETS fusions which includes provisional entities with clinicopathological features that are still being defined [2]. However, in the upcoming 6th edition of WHO classification, EPS will be included as a separate entity.

In our study we presented nine cases of EPS. There were four males and five females, with ages ranging from 10 to 81 years (average age: 49 years). The tumors were located in the abdominal wall soft tissues, the thorax, and the back of the neck.

Morphological spectrum was broad and included a mix of spindled, ovoid, round, and epithelioid cells, with considerable variation in both architectural and stromal features. Based on the predominant cell shape and nuclear grade, cases in our observation were divided into three subgroups: low-grade spindle/round cell tumors, intermediate/high-grade tumors with predominantly round or ovoid cell morphology, and high-grade spindle/round cell sarcomas. We found this approach useful for differential diagnosis, while clinical significance of this subclassification remains uncertain.

The most consistent immunohistochemical markers in EPS were S100-protein, GFAP, and skeletal muscle markers (MyoD1, PAX-7, and desmin). A key finding of this study was the frequent PAX-7 expression, observed in almost all analyzed cases. Myogenin was the least sensitive skeletal muscle marker, appearing in only a small subset of cases.

A novel and diagnostically useful observation was the discreet perinuclear staining pattern of broad-spectrum cytokeratins (AE1/3), while cytoplasmic AE1/3 staining was noted in only one case.

Other findings included strong, diffuse nuclear expression of OLIG2 in some cases, weak expression of synaptophysin and CD56, weak cytoplasmic positivity for Pan-TRK in one case, and diffuse positivity for H-caldesmon in another. H3K27me2 expression varied, with some cases showing partial or complete loss. SOX-10 was strongly expressed at the tumor periphery in one case. In a few cases nuclear MDM2 positivity was observed. Additionally, focal expression of CD34 and smooth muscle actin was noted in some cases.

The *EWSR1* and *PATZ1* genes are both located on chromosome 22, approximately 2Mb apart. Due to this close genomic proximity, visualization and interpretation of break-apart FISH signals can be challenging [2]. This was also evident in our study, as all four tested cases showed negative *EWSR1* break-apart FISH results.

CDKN2A deletions were identified in two cases, with one exhibiting heterozygous and the other homozygous loss. Neither case showed *MDM2* gene amplification.

NGS findings detected an intrachromosomal rearrangement on chromosome 22, involving *EWSR1* exon 8 and *PATZ1* exon 1 in all 9 cases. Comprehensive DNA analysis in one case revealed a pathogenic *TP53* (p.C275Y) mutation, which might also have a potential prognostic relevance, and a low TMB (<5 mutations/Mb). The last finding was also observed in one more case (2 mutations/Mb). Additionally, *CDKN2A/B* gene loss was identified in one case.

Previous reports suggested very aggressive clinical behavior in most cases of EPS [14]. However, our data suggest that certain morphological features may be associated with a more favorable prognosis, including low-grade morphology, well-circumscribed tumors, partial encapsulation, and low mitotic activity. On the other hand, one case with intermediate-grade morphology, higher mitotic activity, and extensive pleural involvement remained disease-free five years after tumor resection and adjuvant chemotherapy as well. The role of chemotherapy remains unclear, as neither clinical nor histological responses were documented.

In conclusion, we presented the largest clinicopathological analysis of EPS until that point, highlighting its broad morphological spectrum alongside consistent microscopic features. These tumors exhibit a characteristic immunophenotype, marked by the co-expression of neural (S100-protein, GFAP) and skeletal muscle (desmin, MyoD1, Pax-7) markers in most cases. These findings, along with previous gene expression studies, support EPS as a distinct clinicopathologic entity. The favorable outcomes observed in some cases suggest that a subset of EPS may have a more indolent clinical course than previously thought which was confirmed in subsequent studies [20]. Further studies integrating clinical, morphological, and molecular data is necessary to determine potential prognostic factors and clarify the role of chemotherapy in treatment.

Kinase alterations are increasingly identified as key oncogenic drivers in a diverse group of mesenchymal tumors, including dermatofibrosarcoma protuberans (DFSP), inflammatory myofibroblastic tumor (IMT), and infantile fibrosarcoma (IFS) [21]. This work focuses on activating neurotrophic receptor kinase (NTRK) fusions in various entities, including IFS with canonical *ETV6::NTRK3* fusion [22, 23], *NTRK*-rearranged spindle cell neoplasms (*NTRK*-SCN)—an emerging entity recently included in the WHO classification [19, 24], and other tumors with alternative kinase gene alterations such as *NTRK1/2/3*, *BRAF*, *RAF1*, *RET*, *MET*, etc [21, 25, 26, 27,

28]. All these tumors are characterized by overlapping pathogenesis, as well as histological, immunohistochemical, and clinical features. The primary aim of this study is to enhance the understanding of *NTRK*-driven tumorigenesis and its diagnostic and clinical implications.

This study presents a comprehensive clinicopathological and molecular analysis of 22 cases of IFS and other kinase gene-altered spindle cell neoplasms (male to female ratio 1:1). Paediatric and adult patients were included. We proposed a morphological subdivision based on cellularity and degree of atypia. Low-grade (LG) group characteristics included bland spindle cells in abundant stroma, low cellularity, minimal atypia and very few mitoses (≤ 2 per 10 HPF). Tumors often exhibited lipofibromatosis-like neural tumor pattern (LNT) and a prominent stromal and perivascular hyalinization (SPH). Three intermediate grade (IG) cases exhibited greater polymorphism, increased hyperchromasia, and occasional tumor cell overlapping. Cells were arranged in a disorganized manner or short fascicles. These cases resembled LG-MPNST or displayed an SPH or myopericytomatous pattern. Mitotic activity was higher than in the LG group (≤ 6 per 10 HPF). Necrosis was absent. The high grade (HG) group was characterized by a scarce stroma and a densely packed, hypercellular proliferation of cells with notable nucleus overlapping. Marked cytological atypia and pleomorphism were observed. In some cases, LG components were also present. The tumour cells exhibited various growth patterns, including a herringbone, poorly defined fascicles, or solid sheets. Mitotic activity was generally high, reaching up to 56 mitoses per 10 HPF. Small foci of necrosis were commonly observed.

Immunohistochemical analysis revealed focal positivity for CD34 and S100 protein in approximately half of the cases. All 12 cases with *NTRK* fusions exhibited positivity for pan-Trk.

RNA sequencing identified *NTRK1/3* fusions in 12 cases, including 4 with IFS morphology and *ETV6::NTRK3* fusion. The tumor with the *EML4::NTRK3* fusion showed LG-SPH areas transitioning into IFS. The *STRN::NTRK3* fusion tumor exhibited a highly myxoid LG-SPH pattern, while the case with the *RBPMS::NTRK3* fusion displayed the same LG-SPH pattern without myxoid changes. *NTRK1*-fused tumors spanned the entire morphological spectrum and were present in all three morphological subgroups. *RET* fusions were also identified across the entire morphological spectrum. A novel *PWWP2A::RET* fusion was found in a tumor with an IFS pattern, while a newly identified *NUMA1::RET* fusion was detected in a case with epithelioid/rhabdoid morphology. *MET* fusions displayed distinct morphological features, including a unique LG triphasic morphology combined with HG-IFS areas in a tumor with the *TFG::MET* fusion. The case with the *CAPZA2::MET* fusion exhibited an IG myopericytomatous appearance. Both *RAF1* and *BRAF* fusion cases demonstrated IFS morphology. One case with an LG-SPH pattern harbored a pathogenic *BRAF* deletion-insertion (p.V600K) and an *EGFR* p.S768_D770dup mutation. DNA sequencing also uncovered additional likely pathogenic mutations in *PALB2* and *ROS1*, with the *ROS1* mutation

confirmed as germline in origin. MYC duplication was detected in one case and confirmed by the methylation profiling CNV plot. Additionally, a high TMB was observed in two cases, while MSI remained low in all tested cases.

Methylation profiling revealed that one case with a *PWWP2A::RET* fusion matched the DKFZ sarcoma methylation class of infantile fibrosarcoma with a high calibrated score. Some cases showed lower-confidence matches to MPNST-like, DFSP, and rhabdomyosarcoma-like methylation classes. Unsupervised clustering of methylation profiles using t-SNE demonstrated that the eight kinase-rearranged cases clustered with the IFS methylation class. One case, which contained a significant proportion of admixed inflammatory cells, clustered with IMT. Comparative analysis of genome-wide CNVs showed that HG tumors had significantly higher genomic complexity than LG/IG subgroups. HG tumors frequently exhibited gains in *CCND1*, *MDM2/CDK4*, and *CDK6*, as well as *CDKN2A/B* losses, whereas LG tumors generally had fewer alterations, though *CDKN2A/B* loss was also observed in two cases.

Treatment and follow-up data were available in most of the cases with a follow-up duration up to 130 months. Recurrence and metastasis rates were 19% and 6%, respectively. The only fatal case exhibited HG-IFS morphology and metastasized within 30 months. Surgery, with or without chemotherapy, was the primary treatment. Of these, five patients remained disease-free, one experienced recurrence, and one was alive with disease. Six patients received targeted therapy (entrectinib, larotrectinib, or selpercatinib) in combination with surgery and/or chemotherapy. Most showed significant tumor regression or complete response.

The distinction between IFS and NTRK-SCN is generally clear due to patients' age differences. Nevertheless, in older pediatric patients where age criteria cannot be straightforwardly applied the differentiation can be challenging due to otherwise overlapping diagnostic criteria, especially in the subset of cases with alternative kinase fusions and high-grade IFS morphology.

In this study, we deepened the understanding of clinicopathological features of these neoplasms in one of the largest studies of this tumor type to date. A morphological spectrum defined by cellularity and degree of atypia was confirmed to correlate with chromosomal copy number changes as well as with outcome. Several novel fusions were identified, including *PWWP2A::RET*, *NUMA1::RET*, *ITSN1::RAF1*, and *CAPZA2::MET* fusions. Additionally, the first cervical case with *BRAF* and *EGFR* mutations was discovered and published in this series. DNA sequencing revealed that secondary molecular alterations are rare in these mostly kinase fusion driven tumors. Most importantly, we have shown that the methylation profile of so called NTRK-rearranged spindle cell tumors largely overlaps with that of infantile fibrosarcoma, a currently separate entity in the WHO classification, thus supporting the notion that all these neoplasms are closely related and perhaps represent a morphological spectrum of one entity.

CONCLUSIONS

The Ph.D. thesis is the culmination of postgraduate research in pathology by Dr. Antonina Kalmykova, MD.

The objectives were thoroughly explored and documented in publications.

This six-year study focused on the histological, immunohistochemical, and molecular characteristics of rare soft tissue and skin tumors. The research resulted in two first-author papers and five co-authored publications.

All findings presented in the doctoral thesis were published in peer-reviewed American and European journals with impact factors.

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LIST OF OWN PUBLICATIONS

1. Michal M, Rubin BP, Kazakov DV, Michalová K, Šteiner P, Grossmann P, Hájková V, Martínek P, Švajdler M, Agaimy A, Hadravský L, **Kalmykova AV**, Konishi E, Heidenreich F, Michal M. Inflammatory leiomyosarcoma shows frequent co-expression of smooth and skeletal muscle markers supporting a primitive myogenic phenotype: a report of 9 cases with a proposal for reclassification as low-grade inflammatory myogenic tumor. *Virchows Arch.* 2020 Aug;477(2):219-230. doi: 10.1007/s00428-020-02774-z. Epub 2020 Feb 20. PMID: 32078043.
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PRESENTATION

1. POSTER - 109th ANNUAL MEETING USCAP, Los Angeles, California, USA, 2020. PHF1-TFE3-rearranged Ossifying Fibromyxoid Tumors (OFMT): Are They Overrepresented Among Malignant OFMT?
2. ORAL PRESENTATION - 25th Joint Meeting of the ISDP, May 2022. A rare vascular tumor that mimics well-differentiated angiosarcoma.
3. ABSTRACT - 111th ANNUAL MEETING USCAP, Los Angeles, California, USA, 2022. Molecular Analysis of Myxoinflammatory Fibroblastic Sarcoma (MIFS) with High-Grade (HG) Progression Reveals a Novel BRAF-MKLN1 Gene Fusion and Frequent BRAF and VGLL3 Gene Amplifications
4. POSTER - EADV Congress 2025, Athens, Greece, April 3-5, 2025
Clinical and pathologic findings of Atypical Spitz tumor with ALK rearrangement: case report.