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ORIGINAL ARTICLE

# Profiling of potential driver mutations in sarcomas by targeted next generation sequencing

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Comprehensive genetic profiling by massively parallel sequencing, commonly known as next generation sequencing (NGS), is becoming the foundation of personalized oncology. For sarcomas very few targeted treatments are currently in routine use. In clinical practice the preoperative diagnostic workup of soft tissue tumours largely relies on core needle biopsies. Although mostly sufficient for histopathological diagnosis, only very limited amounts of formalin fixated paraffin embedded tissue are often available for predictive mutation analysis. Targeted NGS may thus open up new possibilities for comprehensive characterization of scarce biopsies. We therefore set out to search for driver mutations by NGS in a cohort of 55 clinically and morphologically well characterized sarcomas using low input of DNA from formalin fixated paraffin embedded tissues. The aim was to investigate if there are any recurrent or targetable aberrations in cancer driver genes in addition to known chromosome translocations in different types of sarcomas. We employed a panel covering 207 mutation hotspots in 50 cancer-associated genes to analyse DNA from nine gastrointestinal stromal tumours, 14 synovial sarcomas, seven myxoid liposarcomas, 22 Ewing sarcomas and three Ewing-like small round cell tumours at a large sequencing depth to detect also mutations that are subclonal or occur at low allele frequencies. We found nine mutations in eight different potential driver genes, some of which are potentially actionable by currently existing targeted therapies. Even though no recurrent mutations in driver genes were found in the different sarcoma groups, we show that targeted NGS-based sequencing is clearly feasible in a diagnostic setting with very limited amounts of paraffin embedded tissue and may provide novel insights into mesenchymal cell signalling and potentially druggable targets. Interestingly, we also identify five non-synonymous sequence variants in 4 established cancer driver genes in DNA from normal tissue from sarcoma patients that may possibly predispose or contribute to neoplastic development.

**Keywords** Sarcoma, mutation, FFPE, NGS


## Introduction

Sarcomas represent only 1% of all malignant tumours and form a heterogeneous group with widely variable characteristics in terms of incidence, growth rate, malignant potential and prognosis (1). Currently more than 150 different benign and malignant tumours of soft tissue and bone are recognized (2013 WHO-classification). Over the last two decades, improvements in molecular techniques have provided important general insights into the biology of neoplasia. This is particularly obvious in the context of sarcomas, where nearly 100 gene fusions of pathogenetic importance have been described (2). Even though a large number of translocations in solid tumours are believed to be “passengers”, i.e. non-oncogenic aberrations caused by the inherent genomic instability of tumour cells, some of these rearrangements code for oncogenic fusion-gene “drivers” (3) that confer a proliferative advantage to the neoplastic cell. Sarcomas can be cytogenetically subdivided into those with simple karyotypes with balanced or unbalanced translocations and those with complex karyotypes or even chaotic chromosomal alterations (4). Examples of the former category are t(11;22) in Ewing sarcoma, t(X;18) in synovial sarcoma and t(12;16) in myxoid liposarcoma that are all likely primary genetic events essential for tumour formation. In other

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sarcomas specific point mutations are pathogenetically crucial, for instance in GIST where the *KIT* or *PDGFRA* genes are mutated in approximately 85% of the cases (5). For many of the solid tumour types that have so far been comprehensively studied, single-base substitutions outnumber chromosomal translocations by at least one order of magnitude (3). It is thus possible that sarcomas with strong fusion-gene drivers may also harbour other driver gene mutations.

The massive parallel sequencing that is the foundation of so called next generation sequencing (NGS) has opened up new avenues to comprehensively study cancer related genes (6) and is now widely used in clinical diagnostic laboratories. Also focused gene panels (16-50 genes) for NGS platforms cover a substantial portion of known driver mutations that are clinically relevant to guide treatment decisions. Little is currently known about the clinical feasibility of this strategy in the clinical management of sarcomas, where only very limited amounts of neoplastic tissue are often available from core needle biopsy specimens to obtain predictive genetic information that might potentially guide neoadjuvant therapy. We therefore set out to characterize the occurrence of driver gene mutations in sarcomas using a medium-sized gene panel requiring only a small input of DNA (10 ng) from formalin fixated paraffin embedded (FFPE) specimens. This makes it possible to reach a large depth of sequencing to detect also rare subclonal mutant alleles that might avoid detection with a lower number of sequencing reads that are usually obtained by whole-genome or exome sequencing. In this work we have analysed DNA and RNA from a total of 55 previously clinically well characterized malignant soft tissue and bone tumours, mostly specimens from primary untreated tumours, with different biological behaviours and characteristics. On one hand gastrointestinal stromal tumour (GIST) represents a soft tissue tumour with in most cases a strong oncogenic driver due to point mutations or insertions/deletions (indels) in *KIT* or *PDGFRA*. On the other hand, myxoid liposarcoma, synovial sarcoma and Ewing sarcoma represent sarcomas driven by characteristic fusion genes (*FUS-DDIT3*, *SS18-SSX1/2* and *EWSR1* translocations). We find that low input amounts of DNA extracted from FFPE material to interrogate a panel of tumour-associated driver genes can readily be used in a clinical setting. In the present work we are furthermore able to identify and verify a potentially targetable driver mutation in some of these tumours that may have an impact on individually tailored treatment. We also show that some sarcoma patients harbour non-synonymous germline sequence variants in genetic loci that have been reported in the COSMIC database of somatic mutations in cancer.

## Materials and methods

### Routine diagnostic and genetic analysis of tumours

Samples from 55 patients with soft tissue tumours (9 GIST, 14 synovial sarcomas, 7 myxoid liposarcomas, 22 Ewing sarcomas and 3 Ewing-like small round cell tumours) were used in the study (Table 1). All samples were examined by at least two soft tissue pathologists, and the diagnosis was supported by morphological and immunohistochemical analyses in combination with identification of mutations or chromosomal translocations characteristic for each entity by Sanger sequencing, Fluorescence In Situ Hybridization (FISH) and/or RT-PCR according to routine diagnostic practice.

Mutations in *KIT* (exons 9, 11 and 13) or *PDGFRA* (exons 12 and 18) were analysed by Sanger sequencing. FISH was used on interphase nuclei to detect rearrangements of *EWSR1*, *DDIT3* and *SS18* genes using Vysis Break Apart probes (Abbott Laboratories, IL, USA). FISH was performed according to standard protocols.

For demonstration of fusion transcripts by RT-PCR (*SS18-SSX1*, *SS18-SSX2*, *FUS-DDIT3*, *EWSR1-FLI1* type 1 and 2, *EWSR1-ERG*), RNA was isolated from FFPE sections using RNeasy FFPE kit (Qiagen Gmbh, Hilden, Germany), following the manufacturer's instructions. RNA concentration was measured using NanoDrop 2000 (ThermoFisher Scientific, Waltham, MA, USA). 300 ng of RNA was used to detect the fusion transcripts within house designed primers (available on request) and the OneStep RT-PCR (Qiagen) kit according to the manufacturer's instruction.

### Preparation of sample library and next generation sequencing (NGS)

DNA was isolated from FFPE sections using QIAamp DNA FFPE tissue kit (Qiagen), following the manufacturer's instructions. DNA concentration was determined using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA). 10 ng of DNA was used to prepare barcoded libraries with the Ion AmpliSeq™ Library kit 2.0 (Life Technologies). The Cancer Hotspot Panel v2 (Life Technologies) covering 207-targeted regions in 50 cancer related genes was used (https://tools.lifetechnologies.com/content/sfs/brochures/Ion-AmpliSeq-Cancer-Hotspot-Panel-Flyer.pdf). Template preparation and enrichment were performed with the Ion OneTouch™ 2 system (LifeTechnologies). Eight barcoded samples were pooled per Ion 316™ chip and sequenced on the Ion PGM™ System (LifeTechnologies). All steps were performed according to the manufacturer's instruction.

### Data processing

Alignment to the hg19 human reference genome and variant calling was performed by the Torrent Suite Software v4.2.1.0 (LifeTechnologies). To identify hotspot variants/compelling variants the Ingenuity® Variant Analysis™ (Qiagen) was used.

Selection criteria for Call Quality and Read Depth were, respectively, 100 and 250. Previously database identified SNPs were filtered out. Alignment was visually inspected with the Integrative Genomics Viewer (IGV) modified by IonTorrent v4.2.003 (Broad Institute, Cambridge, MA, USA). The identified variants from Ingenuity® Variant Analysis™ were verified in VariantCaller in the Torrent Suite Software v4.2.1.0. Coverage. Quality parameters and tumour allele frequency presented in Tables 2 and 3 are from the VariantCaller.

### Validation of NGS-results

All called mutations were validated by allele specific PCR or direct Sanger sequencing. The same DNA as extracted for NGS was analysed. *BRAF* p.V600E and *NRAS* p.A146T mutations were confirmed with the B-Raf Codon 600 Mutation