THERAPEUTIC ADVANCES in
Medical Oncology

Brief Report

Enhancing precision in sarcoma diagnosis: nCounter fusion panel implementation in a middle-income country

Ther Adv Med Oncol

2025, Vol. 17: 1–8

DOI: 10.1177/17588359251318159

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Abstract

Background: Sarcoma diagnosis is challenging due to numerous subtypes with similar histopathological features and the high cost of fusion detection methods, particularly in middle-income countries.

Objectives: To implement a cost-effective custom-based nCounter approach previously validated for fusion analysis of suspected sarcoma in Brazil.

Design and methods: RNA isolated from 56 routine sarcomas, which were formalin-fixed and paraffin-embedded, was analyzed using a custom nCounter assay that detects 174 common sarcoma gene fusions. The results were compared to fluorescence in situ hybridization (FISH)/next-generation sequencing (NGS) and clinicopathological data.

Results: The nCounter assay was conclusive in 98.2% of cases, identifying 25 gene fusions with 82.5% accuracy, 76.6% sensitivity, and 100% specificity compared to FISH/NGS.

Conclusion: Although it does not identify all sarcoma fusions, especially for rare subtypes, the present nCounter assay is a rapid, affordable, and accurate tool for sarcoma diagnosis in resource-limited settings.

Keywords: biomarkers, low- and middle-income countries, molecular diagnostics, nCounter, sarcoma

Received: 11 October 2024; revised manuscript accepted: 20 January 2025.

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Introduction

Sarcomas are the most common mesenchymal human tumors, can occur in children and adults, and have been divided into two large subgroups: bone sarcomas and soft tissue sarcomas.[1] Both subgroups comprise a variety of histological subtypes and often present considerable diagnostic difficulties due to their rarity and overlapping histologic and immunohistochemical features.[2–4] To overcome these challenges, their biological characterization has enabled deciphering a constantly increasing number of subtypes at the molecular level, associated mainly with unique gene fusions.[2,5]

The 2020 WHO Classification of Tumors of Soft Tissue and Bone introduces new tumor categories, updates prognostic information for various sarcomas, and includes recent genetic alterations.[5] This revision improves the distinction between rare and common tumor subtypes, aiding accurate diagnosis.[6] Moreover, integrating clinical, pathological, and molecular data, the classification enhances tumor biology understanding, leading to better diagnostic accuracy, customized treatments, and improved patient outcomes, leading to personalized medicine.[6–8]

Nevertheless, molecular testing has yet to become routine in many middle-income countries due to technological and financial issues.[9] The most common current molecular approaches, such as next-generation sequencing

(NGS)[10] or fluorescence in situ hybridization (FISH), which require a high-quality tumor specimen, are time-consuming, demanding highly trained technicians, and costly. The nCounter platform (NanoString) demands minimal technical and bioinformatics expertise, is reliable even in low-quality biospecimens, is semi-automatized, has relatively low cost, and has a rapid turnaround time.[7,11]

Previous studies have validated a pan-sarcoma nCounter custom-based approach in North America, Europe, and Asia populations.[12] Herein, we aimed to implement a routine molecular assessment of sarcomas in a Brazilian public oncological hospital and to compare it with other molecular approaches.

Methods

Case selection

The present study retrospectively analyzed formalin-fixed and paraffin-embedded (FFPE) tissue from 56 suspected sarcomas (28 from biopsy and 28 from surgical specimens) admitted to Barretos Cancer Hospital, between 2014 and 2022, in Barretos, Brazil. The patients were selected based on a positive FISH assay ($n=30$) and/or a positive NGS assay ($n=5$) for both assays (Supplemental Figure 1). Data on patient demography, tumor characteristics, and molecular tests were collected from medical records. The study was approved by the Research Ethics Committee of Barretos Cancer Hospital (number 6.000.083 CAAE:68028423.3.0000.5437). The reporting of this study conforms to the STARD 2015 statement[13] (Supplemental Table 1).

The patient's clinical pathological features are summarized in Table 1.

The most common primary tumor locations were head and neck (19.6%), trunk (19.6%), and lower extremity (19.6%). Ewing's sarcoma was the most frequent histological subtype (25%; Table 1). Metastases were detected in 41.1% of patients (Table 1).

RNA isolation and nCounter assay

Four 10-µm-thick FFPE tissue sections, each containing a minimum of 60% tumor cells, were subjected to RNA isolation using the RNeasy FFPE Kit (Qiagen, Hilden, Germany) as reported.[14] Total RNA quantification was performed using a Nanodrop spectrophotometer (ThermoFisher, Waltham, MA, USA).

For probe hybridization and nCounter analysis, 200 ng of tumor RNA was hybridized overnight (18 h) with previously validated 172 sarcoma probes and 4 reference genes (*ACTB*, *GAPDH*, *SDHA*, and *UBC*) from the previous studies.[11,12] Hybridization was conducted in a thermocycler at 67°C with a heated lid set to 72°C. The RNA-probe complexes were subsequently loaded onto a nCounter cartridge and analyzed on a nCounter SPRINT platform following the manufacturer's standard operating procedures (NanoString nCounter Technologies, Seattle, WA, USA).

Data analysis (nCounter assay)

The Reporter Code Count files containing the probe set's raw data were analyzed using nSolver Software v.4.0 (NanoString). The geometric mean (GM) of the raw counts of the four reference genes was calculated, and samples with GM <500 were excluded from the analysis.[11] Data normalization using internal control probes corrected differences in hybridization efficiency. The probe median was established as a fixed value after the initial determination and was used consistently for all subsequent samples. The probe ratio was computed by dividing the normalized value by the corresponding probe median (probe ratio = normalized count/probe median). The sample median represented the median of the probe ratios calculated for each sample. Subsequently, a sample ratio (SR) was determined by dividing the probe ratio of each sample by the sample median of that respective sample (SR = probe ratio/sample median). An SR $\geqslant 5.0$ indicated a positive result for the corresponding gene fusion, while an SR <5.0 was interpreted as negative.[12]

Fluorescence in situ hybridization

The presence of gene fusions was evaluated by FISH using 5-µm thick FFPE sections, using the specific probes from ZytoVision (ZytoLight SPEC Dual Color Break Apart Probes—SS18, EWSR1, DDIT3, TFE3, FUS, CIC; ZytoLight SPEC Dual Color Single Fusion—FOXO1/PAX3, FOXO1/PAX7) using standard FISH protocol (ZytoVision, Bremerhaven, Germany),

Table 1. Patient's clinical pathological features.

Characteristics	Parameters	*N* = 56
Sex	Female	26 (46%)
	Male	30 (54%)
Age at diagnosis, years	⩾18	44 (79%)
	⩽18	12 (21%)
Primary tumor localization	Head/neck	11 (20%)
	Trunk	11 (20%)
	Lower extremities	11 (20%)
	Retroperitoneum	9 (15%)
	Abdominal	5 (9%)
	Upper extremities	5 (9%)
	Others	4 (7%)
Histology	Ewing sarcoma	14 (25%)
	UPS	9 (16.1%)
	Synovial sarcoma	8 (14.3%)
	Alveolar rhabdomyosarcoma	3 (5.4%)
	Extrarenal rhabdoid tumor	3 (5.4%)
	Undifferentiated sarcoma	3 (5.4%)
	Desmoplastic small round cell tumor	2 (3.6)
	Malignant peripheral nerve sheath tumor	2 (3.6%)
	NTRK rearranged spindle cell neoplasm	1 (1.8%)
	Myxoid liposarcoma	1 (1.8%)
	Undifferentiated embryonal sarcoma	1 (1.8%)
	Myxofibrosarcoma	1 (1.8%)
	High-grade spindle cell sarcoma	1 (1.8%)
	Epithelioid malignant neoplasm	1 (1.8%)
	Desmoplastic fibroma	1 (1.8%)
	Gliosarcoma	1 (1.8%)
	Embryonal rhabdomyosarcoma	1 (1.8%)
	High-grade poorly differentiated sarcoma rich in giant cells	1 (1.8%)
	Osteosarcoma	1 (1.8%)

(Continued)

Table 1. (Continued)

Characteristics	Parameters	*N* = 56
	Poorly differentiated malignant neoplasm consisting of small, round cells BCOR mutated	1 (1.8%)
Metastasis	Yes	23 (41%)
	No	33 (59%)
Disease stage at diagnosis	Ia	7 (13%)
	Ib	2 (3.6%)
	IIa	4 (7.1%)
	IIIa	8 (14%)
	IIIb	8 (14%)
	IVa	8 (14%)
	IVb	10 (18%)
	Missing	9 (16%)
Histologic grade	G2	1 (1%)
	G3	34 (61%)
	Missing	21 (38%)

UPS, undifferentiated pleomorphic sarcoma.

and evaluated on a Nikon Eclipse 50i Microscope with 100× magnification using DAPI, FITC, and Texas red filters.

Next-generation sequencing

Archer FusionPlex Solid tumor Panel (ArcherDX, Inc., Boulder, CO, USA) was used to detect 53 gene fusions as previously reported.[14]

Results

Molecular profile of sarcomas

The nCounter evaluation was conclusive in 55 cases (Figure 1(a)). We observed 25 gene fusions, namely 9 *SS18::SSX1*, 8 *EWSR1::FLI1*, 3 *PAX3::FOXO1*, 2 *EWSR1::WT1*, 1 *EWSR1::NR4A3*, 1 *HEY1::NCOA2*, and 1 *CDH11::USP6* (Figure 1(b)).

The FISH analysis was performed in 40 cases and NGS in 17 patients (Figure 1(c)). Among the 30 positive FISH cases, 2 (6.7%) exhibited a nCounter negative result since no probes were available in the custom nCounter panel (*EWSR1::FEV* fusion transcript in an Ewing Sarcoma, and a *NUMA::NTRK1* fusion transcript in a NTRK-rearranged spindle cell neoplasm). NGS also validated these two fusions. In five (16.7%) cases, the FISH showed the presence of fusion at *CIC* (Ewing sarcoma), two *EWSR1* (Ewing sarcoma), *FUS* (myxofibrosarcoma), and *DDIT3* (myxoid liposarcoma), yet the nCounter results were negative despite the presence of the target probe. Interestingly, in one case (Ewing sarcoma), both nCounter and NGS were negative, in contrast to an *EWSR1* FISH break-apart result. In the other four cases, the NGS was not performed ($n = 3$), or it did not contain the target (*CIC*).

Overall, the comparative methodological analysis showed an accuracy of 82.5%, a sensitivity of 76.6%, and a specificity of 100% for the custom-based nCounter.

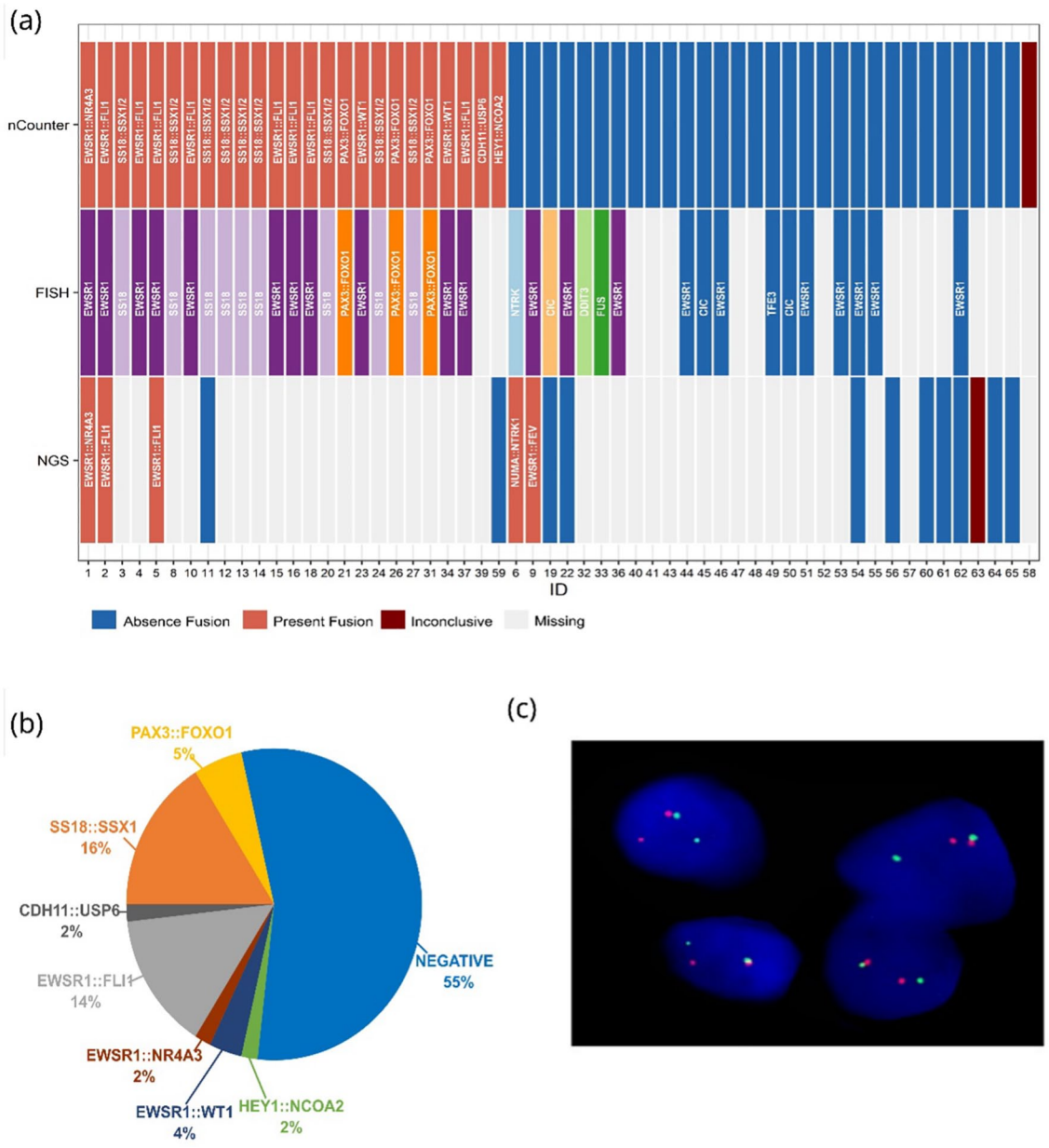

Figure 1. nCounter panel sarcoma, FISH, and NGS assay results. (a) Heatmap with the 56 samples of nCounter Platform, FISH, and NGS results. (b) Frequency of the gene fusions detect by nCounter. (c) FISH of break-apart positive *EWSR1* gene of a round small cell tumor, classified as Ewing Sarcoma (ID 9).
FISH, fluorescence in situ hybridization; NGS, next-generation sequencing.

Discussion

In this study, we successfully implemented a previously nCounter-based panel to detect sarcoma gene fusion in a Brazilian public Hospital. Our findings demonstrate this molecular diagnostic tool's feasibility and clinical utility in a resource-limited context.

Traditionally, sarcoma diagnosis has primarily depended on the histological evaluation of tissue samples, supported by clinical and imaging data. Although the classification of sarcoma subtypes continues to rely largely on histological characteristics, molecular testing, including immunohistochemistry to identify fusion gene products or overexpressed oncogenes, serves as a valuable adjunct in many cases.[15] When integrated with traditional methods, these molecular tools have the potential to improve diagnostic accuracy and enhance clinical practice. For optimal outcomes,

it is advisable that histologic diagnoses of sarcoma be conducted in specialized reference centers by experienced pathologists to ensure diagnostic precision and reliability.[2,16,17] However, the large number of existing histological types and subtypes demands an even more significant number of immunohistochemical markers, which directly implies higher costs and a greater quantity of tumor tissue available. Considering that most diagnoses are made through biopsy to guarantee a quick and accurate result to guide therapeutic management, especially neoadjuvant treatment, the availability of tumor tissue is limited, and it can be quickly depleted in large histochemistry panels. In addition, a growing number of genetically defined sarcomas are present in the latest edition of the WHO Classification of Soft Tissue and Bone Tumor.[1,5] Thus, a diagnostic technique, such as nCounter, capable of simultaneously evaluating a large number of fusions in a quick and accurate manner, adds value to the daily practice of diagnosing sarcomas while saving resources.[11,12]

Our study showed a high rate of conclusive evaluation using the nCounter platform, with 98.2% (55/56) of cases yielding definitive results and detecting 25 (83.3%) gene fusions among the 30 FISH-positive cases. This performance is comparable to that observed in the study by Chang et al.,[12] where the NanoString assay confirmed fusion gene expression in 63 cases (89%) previously tested by FISH and RT-PCR. Similarly to Song et al.,[11] no false-positive results were observed in our study, reinforcing the high specificity of the method in all three studies (100%). Concerning false-negative results, our assay showed seven false-negative results (12.5%), two of which were undetected due to a limitation of the test; as it lacks a probe for fusion detection, whereas Song et al. reported eight cases (7.6%), and Chang et al. reported four cases (1.8%).

Song et al.[11] reported a higher sensitivity (85%) than ours (76.6%), which may be attributed to the diversity of histological subtypes included in their study. In addition, the discordance observed in our study in one case (ID22) between FISH and the molecular methods (nCounter and NGS) highlights the importance of integrating different diagnostic techniques to minimize false negatives.

The significant advantages of the NanoString platform are its ability to test for multiple gene fusions in a single assay (174 probes), the minimal manual sample preparation, since several steps are fully automated, it is quick (2 days), and the reliable results even in low amount and poor-quality specimens, such as small FFPE biopsies. In addition, the sarcoma nCounter assay costs around a single FISH assay. Therefore, compared with FISH labor time and expertise in interpretation, multiple probes needed, and a much higher percentage of inconclusive results due to pre-analytical issues, NGS costs, and bioinformatics expertise required, the present sarcoma nCounter is a feasible approach. Nevertheless, it also shows some limitations, such as the current probes' design, which is specific to the breakpoint locations, making it unsuitable for detecting genes with multiple possible gene partners or high variability at the exon junctions. Moreover, despite the relatively low cost of the assay, the equipment is expensive, making it a barrier to initial implementation.

Conclusion

The present custom sarcoma nCounter panel allows accurate detection of a broad range of gene fusions in a single assay, which is cost-effective for precise molecular classification of soft tissue tumors in middle-income countries.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Barretos Cancer Hospital (protocol CAAE: 78910824.6.0000.5437).

Consent for publication

Patient consent was waived due to the study's retrospective nature.

Author contributions

Flávia Escremim de Paula: Conceptualization; Data curation; Formal analysis; Methodology; Project administration; Writing – original draft; Writing – review & editing.

Murilo Bonatelli: Data curation; Formal analysis; Methodology; Writing – review & editing.

Monise Tadin dos Reis: Data curation; Formal analysis; Methodology; Writing – review & editing.

Karla Emília de Sá Rodrigues: Data curation; Formal analysis; Investigation; Methodology; Writing – review & editing.

Léon C. van Kempen: Conceptualization; Data curation; Methodology; Resources; Writing – review & editing.

Gustavo Ramos Teixeira: Data curation; Formal analysis; Investigation; Methodology; Writing – review & editing.

Rui Manuel Reis: Conceptualization; Funding acquisition; Project administration; Supervision; Writing – original draft; Writing – review & editing.

Acknowledgements
None.

Funding
The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This study was supported by Brazilian Ministry of Health (MoH), National Council for Scientific and Technological Development—CNPq, Brazil, and Brazilian National Program of Genomics and Precision Health—Genomas Brasil (MS-SECTICS-Decit/CNPq n° 16/2023—INOVACRIANÇA—Inovação terapêutica em crianças com tumores sólidos: uma jornada de precisão); Barretos Cancer Hospital, Barretos, São Paulo, Brazil; Public Ministry of Labor Campinas (Research, Prevention, and Education of Occupational Cancer); Rui Manuel Reis is a recipient of a CNPq Productivity (Brazil).

Competing interests
The authors declare that there is no conflict of interest.

Availability of data and materials

Data and materials will be available upon reasonable request to Dr Rui Manuel Reis.

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Supplemental material

Supplemental material for this article is available online.

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