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Original article

Technical feasibility of a long read, fourth generation sequencing platform in diagnostic profiling of clinical routine samples: a proof-of-concept study

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Summary

Background. Next generation sequencing (NGS) impacted on clinical algorithm of solid tumor patients. A heterogeneous series of NGS platforms have been implemented in clinical practice but challenging handling procedures, high technical costs, and scant affordability on sequencing diagnostic routine specimens can leave behind some patients who could benefit from target drugs. Here, we sought to evaluate technical feasibility of Oxford Nanopore Technologies (ONT) sequencing accurate identification of tumor-associated molecular alterations, in a pilot series of real-world samples.

Methods. We developed a technical workflow adapting the SiRe® NGS panel, originally designed for Ion semiconductor sequencing, on MinION platform (Oxford nanopore technologies), a portable, cost effective long read sequencer. The SiRe® panel enables detection of 568 clinically actionable somatic mutations across six key genes (*EGFR, KRAS, NRAS, BRAF, cKIT, PDGFRα*) relevant to targeted therapies in several solid tumors. We implemented a multiplexed assay using pooled and barcoded samples, processed on a single MinION flow cell. Performance was benchmarked from a pilot series of nine FFPE samples against Ion Torrent sequencing data. A single liquid biopsy sample was also analyzed testing accuracy of MinION technology.

Results. The adapted ONT workflow demonstrated high concordance ratei in detecting clinically relevant molecular alterations on short-read fragments, achieving comparable accuracy with standardized second generation NGS platforms on tissue and liquid biopsy samples.

Conclusions. This proof of concept aimed to integrate ONT sequencing into molecular oncology workflows, providing practical, low-cost, and scalable alternative to conventional NGS platforms. The results support the potential of ONT technology to democratize access to precision oncology, particularly in laboratories with limited resources.

Keywords: NGS, tissue samples, bioinformatic pipelines, predictive biomarkers

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Introduction

In the era of precision medicine, accurate and saving time analysis of tumor-associated actionable alterations play a pivotal role in the clinical algorithms of advanced stage tumor patients. Lastly, a rapidly increasing

number of predictive biomarkers have been approved by international societies to optimize cancer care in advanced stages [1]. Standard Sanger sequencing techniques have been elected as milestone in the molecular profiling of tumor patients thanks to its ability to simultaneously detect actionable alterations in a single DNA fragment [2]. Technical limitations of Sanger sequencing platforms paved the way for the widespread diffusion of upgraded next generation sequencing (NGS) systems consistently detecting low frequency mutations in biological samples [3,4]. To date, massive parallel NGS tools emerged as cutting edge, scalable, highly sensitive technologies able to capture molecular fingerprints of scant diagnostic routine specimens [4]. In accordance with technical specifications, conventional NGS systems may be grouped in amplicon and hybridization-based systems, depending on the technical strategy to select molecular targets. NGS platforms are also classified in semiconductor or fluorescent signal detecting systems, demonstrating consistent heterogeneity in clinical routine practice [5,6]. It has been consolidated that these systems have significantly modified the testing paradigm comprehensively covering actionable alterations in several biological sources from clinical practice [4]. In particular, sequencing platforms cover target molecular biomarkers (spanning from 2 to 500 cancer-related genes) or wide genomic regions (whole exomes and whole genomes approaches), confirming scalability to tailorize clinical requests [7,8]. However, the lack of standardized preanalytical procedures significantly impacts on the successful rate of the most common short-read NGS platforms, leaving behind tumor patients who could benefit from targeted agents [9]. In addition, consistent technical costs challenging handling procedures and hard-to-use equipment are required to assess genomic profile in clinical settings. As a consequence, alternative sequencing strategies are investigated to solve these issues in real world samples [10].

In recent years, long-read the Oxford Nanopore Technologies (ONT) sequencing platform emerged as promising alternative to fill this gap. ONT system is an easy to use, rapid, accessible and saving cost strategy for sequencing clinically actionable biomarkers both in tissue and liquid biopsies thanks to simplified technical workflow. Historically, long-read sequencing platforms suffer from higher error rates (5.0-15.0%) compared to conventional sequencing strategies, dramatically affecting the widespread diffusion in clinical practice.[8,11].

Optimized protocols can accelerate enhancement of accuracy rate. Indeed, advancements in ONT sequencing technology, such as V14 chemistry and R10.4.1 flow cells and base calling model (super- accuracy model), have substantially meliorated sequencing accuracy to 99.0%, in line with routinely available short reads sequencing technologies. Severely fragmented nucleic acids (cfDNA from liquid biopsies, or genomic DNA from formalin-fixed paraffin-embedded [FFPE] samples) need optimized workflow for consistent sequencing procedures on long-read platform.[12,13,14].

Here, we sought to technically validate the PromethION system (ONT) on a retrospective series of real-world samples previously tested for actionable biomarkers.

Study design

A pilot series of nine FFPE from non-small cell lung cancer (NSCLC) and a liquid biopsy sample from gastrointestinal stromal tumor (GIST) patients were retrieved from internal archive of Fondazione IRCCS Ospedale Casa Sollievo della Sofferenza Hospital to validate ONT sequencing technology on real world samples. Each case had been previously analyzed with custom NGS panel (SiRe® NGS DNA panel, Genedin) covering 568 clinically relevant hotspot mutations across seven genes (*EGFR*, *KRAS*, *NRAS*, *BRAF*, *cKIT*, and *PDGFR*α) in accordance with clinical requests[15]. An aliquot of SiRe® libraries were adapted for Nanopore sequencing system, as described in the methods below, and sequenced on MinION flow-cell. Written informed consent was collected and documented in accordance with 'The Italian Data Protection Authority'(http://www.garanteprivacy.it/web/guest/home/docweb/-/docwebdisplay/export/ 2485392). All information regarding human material was managed using anonymous numerical codes, and all samples were handled in compliance with the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/).

SiRe™ protocol

A series of five unstained slides (5 µm) and matched hematoxylin/eosin-stained slides from FFPE samples were collected. Highly trained pathologist visually inspected neoplastic cell fraction (> 20.0%) for nucleic acid's isolation. Genomic DNA (gDNA) was extracted using the QIamp DNA Micro kit (Qiagen) and quantified with the Qubit dsDNA HS and BR assay Kits (Life Technologies) in accordance with manufacturer instructions.

Regarding liquid biopsy, a total of 10 ml of peripheral blood was withdrawn. Plasma was separated centrifuging blood twice at 2300 rpm. following manufac-

turer procedures. Total circulating free DNA (cfDNA) was purified from plasma specimens by using the Helix Circulating Nucleic Acid Kit (IVD, Diatech Pharmacogenetics), according to manufacturers' instructions. Quality and quantity of gDNA were evaluated by Genomic ScreenTape whereas cfDNA was analyzed using the High Sensitivity D1000on TapeStation 2200 (Agilent Technologies). Briefly, cfDNA amount and fragmentation profile was automatically measured by proprietary TapeStation 4200 software. In detail, a median DNA concentration of 112.1 µl (from 10.1 to 308.0 ng/µl) and DNA integrity number (DIN) of 3.7 (from 2.6 to 5.1) were calculated using proprietary software. CfDNA sample highlighted 0.6 ng/µl; DIN was not assessed (supplementary Table I). Tissues and liquid biopsy sample were analyzed using SiRe® NGS DNA panel (Genedin) following a customized workflow. In brief, a series of 8 amplified libraries were diluted at 70 pM and pooled together into Ion Chef platform to sequence fragments on the GeneStudio S5 Plus Sequencer (Thermo Fisher Scientific). The raw data signal was processed using bioinformatic pipeline aligning sequences with human genome 19 reference (GRCh37/hg19), Data analysis was performed using the Coverage Analysis plug-in, the variant Caller and the ION Reporter software (https://ionreporter.thermofisher.com). In all cases, the reads' alignment was also visually inspected using the Golden Helix Genome Browser v.3.0.0 software (Bozeman, MT, USA). A threshold of at least ≥ 3500× coverage and an allelic frequency (AF) ≥ 0.5% was selected for mutation calling. Overall, non-synonymous insertions/deletions (indels) and mononucleotide variants (MNVs) in splicesite_5′ and splicesite_3′ regions that produce missense/nonsense mutations and frameshift deletions/frameshift insertions were considered. Mutations listed in ClinVar (https:// www. ncbi. nlm. nih. gov/ clinvar/) as pathogenic, likely pathogenic, drug response, conflicting and uncertain significance were annotated.

MinION protocol

For each sample, 200 fmol of the target library from the SiRe® NGS DNA panel (Genedin) was end-repaired and adapter ligated using the NEBNext Ultra II End Repair/dA-Tailing Kit (E7546S, NEB). Briefly, 1.75 µl of ULTRA II end prep reaction buffer and 0.75 µl of ULTRA II end prep enzyme mix was added to each sample. The mix was incubated at 20°C for 5 minutes followed by inactivation at 65°C for 5 minutes. Samples were purified using AMPure XP Beads (Beckman Coulter), washed twice with freshly prepared 80,0% absolute ethanol, and eluted in 10 µL of nuclease-free water. Native barcodes (NB01–NB24) from the Native Barcoding Kit 24 V14 (Oxford Nanopore Technologies, SQK-NBD114.24) were ligated to the end-prepped DNA using NEB Blunt/TA Ligase Master Mix (NEB, M0367). Barcoded samples were pooled together and purified using 0.4× AMPure XP Beads and eluted in 35.0 µL of nuclease-free water. Sequencing adapters (Native Adapter, NA) were ligated using the NEBNext® Quick Ligation Module (NEB, E6056). After incubating at room temperature, libraries were purified using AMPure XP Beads and washed twice using 125 µL of Short Fragment Buffer (SFB).

MinION R10.4.1 flow cells (FLO-MIN114) were checked before loading using MinKnow software to confirm a minimum of 800 active pores. Priming mix was prepared with 1170 µL of Flow Cell Flush (FCF), 30 µL Flow Cell Tether (FCT), and 5 µL bovine serum albumin (50 mg/mL, final concentration 0.2 mg/mL). Then, 800 µL of the prepared priming mix was loaded via the priming port, into the flow cell equilibrated for 5 minutes at room temperature before sequencing. DNA library was combined with 37.5 µL of sequencing buffer and 25.5 µL of well-resuspended library beads and finally loading into SpotON sample port dropwise following manufacturer recommendations. Flow cells were running throughout the day (24 hours). Data was verified using base-calling plugin with the Super Accurate (SUP) demultiplexing model. Genomic variants were annotated after aligning raw data (BAM files) with human GRCh38 reference genome using MiniMap2 [16]. Filtering chain was able to detect molecular alterations with variant allele fraction (VAF) ≥ 5% adopting the GATK HaplotypeCaller (germline alterations) and GATK Mutec2 (somatic variations) pipelines. Filtering mutations in GATK is a critical step for distinguishing true biological variants from technical artefacts, especially for low-frequency variants. We focused on: QualByDepth, which normalises confidence based on depth; FisherStrand indicating strand bias; and ReadPosRankSum, which assesses consistency of variant calling. Visual inspection of mutations was approached by Integrated Genome Viewer (IGV) after confirming technical quality checks.

Results

Overall, pilot series was successfully sequenced by MinION platform (Oxford nanopore technologies), system. Molecular analysis demonstrated consistent technical parameters achieving a mapped reads from 93.0% to 99.0% on target regions and an average medium depth ≥ 3000x.

The Phred-scale quality value (QV) calculated by

FastQC and MultiQC v was ≥20 in 92.0-96.0% of reads averaging 94.0% across all datasets (Fig. 1) In addition, a median of 62.0% of reads highlighted (QV) of 30 or higher. A perfect match between MinION workflow and conventional short-read sequencing pipeline was identified. In detail, 14 single-nucleotide variants (SNVs) across all 10 samples, were confirmed by ONT sequencing. In particular, six clinically relevant alterations were found adopting both ONT GATK Haplotype Variant Caller and the ONT GATK MuTect2 Variant Caller workflows. (Tabs. I-III)

Notably, SNVs showing a variant allele frequency (VAF) ranging from 0.5% to 5.0% were consistently identified by the GATK MuTect2 pipeline and confirmed visually inspecting BAM files on Genomics Viewer (IGV) tool. Notably, a visual inspection of the raw data using the IGV tool confirmed the detection of low-frequency genomic alterations, ranging from

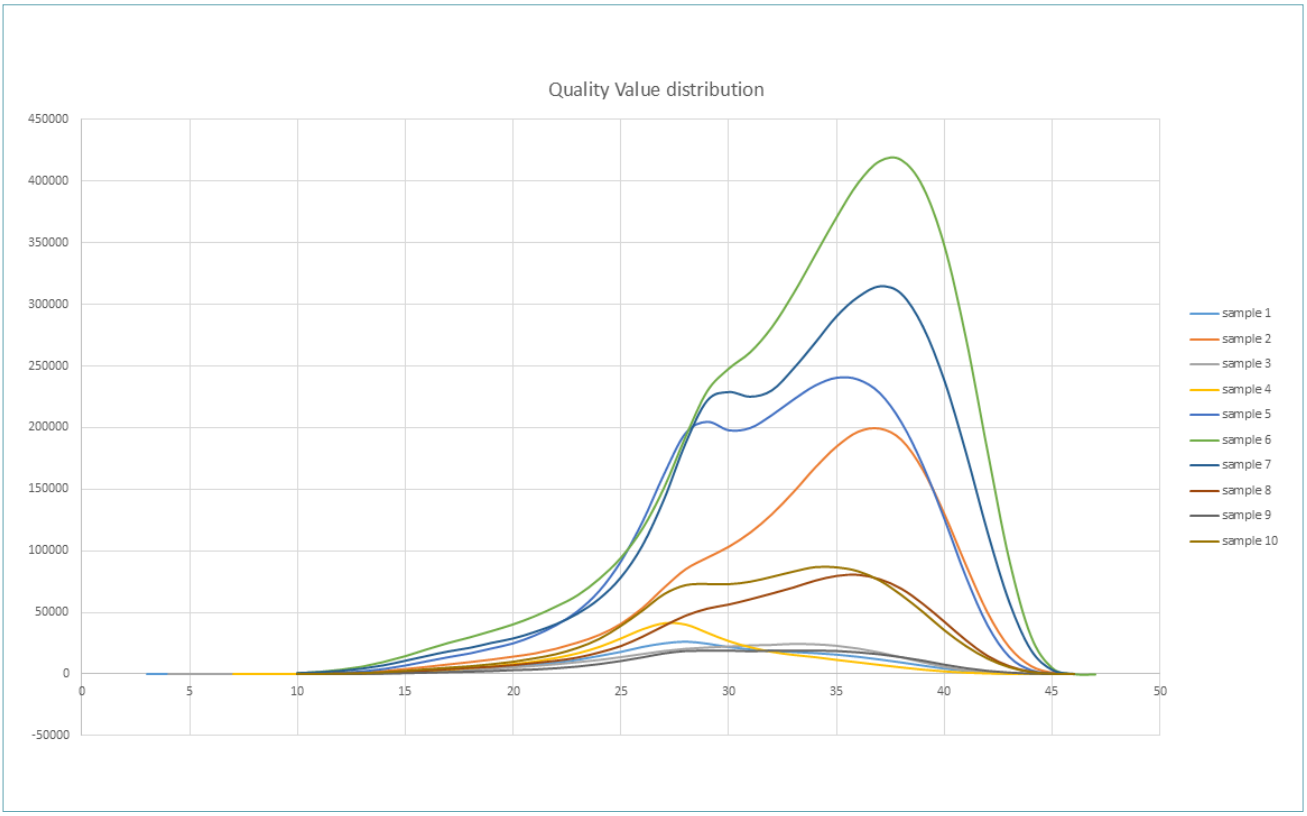

Figure 1. Quality value distribution across samples.

Table I. List of clinically relevant molecular alterations detected by S5 (Thermofisher) and MinION (Nanopore) systems in a pilot series of diagnostic routine samples.

Case number	SiRe - IonTorrent	SiRe - MinION
#ID1	*KRAS* p.Gly12Asp (c.35G > A) 6.5%	*KRAS* p.Gly12Asp (c.35G > A) 5.9%
#ID2	*KRAS* p.Gly12Ala (c.35G > C) 22.5%	*KRAS* p.Gly12Ala (c.35G > C) 27.8%
#ID3	WT	WT
#ID4	WT	WT
#ID5	WT	WT
#ID6	*KRAS* p.Gly12Val (c.35G > T) 20.9%	*KRAS* p.Gly12Val (c.35G > T) 19.8%
#ID7	*KRAS* p.Gly12Val (c.35G > T) 45.2%	*KRAS* p.Gly12Val (c.35G > T) 45.4%
#ID8	*KRAS* p.Gly12Asp (c.35G > A) 10.5%	*KRAS* p.Gly12Asp (c.35G > A) 11.1%
#ID9	WT	WT
#ID10	*KIT* (p.Leu576Pro; c.1727T > C) 0.5%	*KIT* (p.Leu576Pro; c.1727T > C) 0.4%

Table II. Coverage analysis for clinically relevant mutation performed with Ion Seq Torrent variant caller (Thermofisher), ONT Gatk Haplotype,ONT Gatk Mutec2 tools and ONT data IGV bam visual inspection. In detail, target coverage and variant coverage were reported.

Case number	Variant detected	Analysis software	Target Coverage	Variant Coverage
#ID1	KRAS p.Gly12Asp	Ion Seq Torrent Variant caller	1998	130
		ONT Gatk Haplotype Variant Caller	nd	Nd
		ONT Gatk Mutec2 Variant Caller	2280	140
		ONT data IGV Bam Visual Ispection	1026	65
#ID2	KRAS p.Gly12Ala	Ion Seq Torrent Variant caller	1992	448
		ONT Gatk Haplotype Variant Caller	16075	3668
		ONT Gatk Mutec2 Variant Caller	16717	4328
		ONT data IGV Bam Visual Ispection	6537	1710
#ID6	KRAS p.Gly12Val	Ion Seq Torrent Variant caller	1997	417
		ONT Gatk Haplotype Variant Caller	29698	5747
		ONT Gatk Mutec2 Variant Caller	29133	5738
		ONT data IGV Bam Visual Ispection	31292	7082
#ID7	KRAS p.Gly12Val	Ion Seq Torrent Variant caller	1997	903
		ONT Gatk Haplotype Variant Caller	23896	10313
		ONT Gatk Mutec2 Variant Caller	23547	10209
		ONT data IGV Bam Visual Ispection	27144	12664
#ID8	KRAS p.Gly12Asp	Ion Seq Torrent Variant caller	1993	210
		ONT Gatk Haplotype Variant Caller	643	63
		ONT Gatk Mutec2 Variant Caller	712	60
		ONT data IGV Bam Visual Ispection	1602	135
#ID10	KIT p.Leu576Pro	Ion Seq Torrent Variant caller	2000	9
		ONT Gatk Haplotype Variant Caller	nd	nd
		ONT Gatk Mutec2 Variant Caller	nd	nd
		ONT data IGV Bam Visual Ispection	3842	17

Table III. Comparative analysis of variants across genes referenced by SiRe™ panel adopting ONT Gatk Haplotype and ONT Gatk Mutec2 tools.

Case number	Ion Seq Torrent	ONT Gatk Haplotype	ONT Gatk Mutec2
#ID2	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 71.5%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 71.6%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 72.0%
#ID3	*PIK3CA* (p.?; c.352+40) MAF: 50.0% *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 49.9%	*PIK3CA* (p.?; c.352+40) MAF: 49.5% *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 48.0%	*PIK3CA* (p.?; c.352+40) MAF: 49.5% *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 47.6%
#ID4	*KIT* (p.Leu862 = ; c.2586G > C) MAF: 52.3%	*KIT* (p.Leu862 = ; c.2586G > C) MAF: 47.8%	*KIT* (p.Leu862 = ; c.2586G > C) MAF: 47.0%
#ID7	*PIK3CA* (p.Glu545Lys; c.1633G > A) MAF: 2.6% *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 48.6%	*PIK3CA* (p.Glu545Lys; c.1633G > A) MAF: 0.2%* *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 48.5%	*PIK3CA* (p.Glu545Lys; c.1633G > A) MAF: 4.6% *EGFR* (p.Gln787 = ; c.2361G > A) MAF: 48.9%
#ID8	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 51.6%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 52.0%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 52.0%
#ID9	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 49.0%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 55.0%	*EGFR* (p.Gln787 = ; c.2361G > A) MAF: 55.0%
	*After visual inspection		

0.1% to 5.0% MAF, identified by the SiRe™ MinION pipeline.

Discussion

Herein, we designed a proof of analytical equivalence study evaluating technical feasibility of a rapid, ver-

satile and ready to use long read next generation sequencer (Oxford Nanopore) in a diagnostic setting. These platforms enable real-time long reads sequencing on native DNA or RNA strands, without any chemical or enzymatic modifications. In particular, this system is built on proprietary device (flow cell), containing nanoscale pores (nanopores) that separate two sequencing chambers under constant ionic current (electrical current). When a single-strand DNA or RNA passes the nanopore electrical current is modified accordingly.[11,12].

The rapidly evolving scenario of Nanopore technologies led to the development of several technical upgrades (including R9 and R10 flow cells) significantly enhancing efficiency and accuracy of sequencing strategies. [11,12,13,14]. confirming that QV30 signal is aligned with technical performance of diagnostically diffuse NGS methods (semiconductor and fluorescent labelled signal). [5,6,7]. Additionally, nanopore technologies are saving costs, ready to use systems not requiring substantial investments in terms of equipment and highly skilled personnel supporting clinical applications in resource-limited areas [12].

Furthermore, ONT technology has been largely optimized for long-read sequencing. Conversely, short fragments derived from liquid biopsy or FFPE samples remain challenging. [17,18,19].

A pilot series of 10 diagnostic samples (n = 9 FFPE and n = 1 liquid biopsy) were investigated to evaluate the technical performance of nanopore sequencing in clinical settings, which demands both rapid turnaround times and flexible reporting schemes. We overviewed sequencing yield to inspect technical parameters supporting analytical procedure. ONT output dataset showed quality values comparable with the gold standard next-generation sequencing technologies demonstrating consistent technical results.

It has been ascertained that sequencing yield primarily depends on three factors: the number of active nanopores, timing of DNA translocation across nanopore, and the running time [20, 21].

If DNA translocations and running time can be set up by working personnel, the number of active pores is directly influenced by the flow cell's storage time. As recommended, optimal shelf life is 90 days storing the flow cell at 2-8°C. Despite lower pore activity yielding a reduced number of reads, technical parameters enabled secondary data analysis in all samples. Of note, technical management of the flow cell can significantly impact on the widespread diffusion of this technology in clinical scenario. Preanalytical quality checks (storage conditions and shelf life) from manufacturers guidelines are crucial, validating molecular results. If handling procedures are not aligned with technical requirements, lower analytical performance can be assessed. [22]. In particular, the analytical performance of two distinct flow cells was compared to optimize the diagnostic analytical workflow and the cost-benefit ratio, particularly for small libraries of 7 kb. Three barcoded pool libraries (samples 1, 3, and 4) were loaded after five months expiring time. In this case, 200 active pores at starting check were identified.

In contrast, the other seven barcoded libraries (samples 2, 5, 6, 7, 8, 9, and 10) were loaded onto a newer flow cell. This flow cell was used within two months of its arrival, well within the manufacturer's recommended timeframe, and had approximately 1,500 active pores at the time of the run.

Both flow cells were run for two 24-hour periods. Sequencing data was then verified before and after base-calling with the SUP model and subsequent demultiplexing. Consistently, the flow cell exhibiting lower pore activity yielded fewer number of reads Nonetheless, full coverage for all targeted library regions was successfully obtained across all samples without any significant correlation with flow cell type. To date, the lack of optimized bioinformatic pipelines, meliorating high false positive rate, poses barriers for the clinical applications of MinION platform (Oxford nanopore technologies), in current practice [23]. FastQC and MultiQC tools were adopted to measure technical accuracy of ONT reads based on the Phred-scale quality value (QV).[24] Of note, we highlighted consistent technical parameters (QV > 20 in 94.0% of reads, QV > 30 in 62.0% of reads) parameters, in line with conventional short reads sequencing strategy (Fig. 1). To evaluate accuracy and identify genetic variants, ONT sequencing reads were aligned to the human GRCh38 reference genome using MiniMap2 [25] Following the alignments, BAM files were used for genotyping and identifying molecular alterations by the Sire™ panel using two complementary variant caller tools [26]. The HaplotypeCaller has a benchmarked sensitivity for VAF greater than 0.05 [27]. In contrast, the Mutect2 tool was specifically developed to detect somatic mutations, below 0.05 of VAF [28]. All single nucleotide variants reported in Ion sequencing results were accurately identified showing a significant correlation between VAF of standard procedure and nanopore technology.

The ONT sequencer offers a rapid and cost-effective method for identifying genetic variants, achieving genotype accuracy of target sequences in line with second-generation sequencing. In particular, long-read sequencing platforms can benefit from shorter turnaround time than conventional NGS strategies. In brief, a rapid workflow (7-24 hours) for 30X genome analysis spanning from sample preparation and data sequencing has been certified. Conversely, short-

read sequencing platforms require 2-3 working days before analyzing genomic data [19]. However, improvements are needed to address current criticisms, such as enhancing the flow cell's shelf life and increasing the reproducibility of coverage among target regions. Furthermore, ONT sequencing has promising applications for integrative analysis of various molecular alterations, including point mutations, copy number variations, simple and complex structural variants, as well as epigenetic markers and ctDNA fragmentation signatures. By simultaneously analyzing these factors with a single technology, we can gain a more comprehensive understanding of individual cancer cases. Despite these advances, several limitations affect this study. Firstly, the sample set is inadequate to statistically prove robust technical performance of ONT sequencer compared with standard second-generation sequencing platforms. Moreover, the small size of the NGS panel (targeting 6 actionable genes) needs further investigations exploring technical performance of ONT sequencing platform on comprehensive reference range. secondly, no clinical data are available to support technical accuracy of ONT technology predicting clinical response to target drugs; third, different reference range was selected between ONT technology and short-read sequencing approach potentially impacting on concordance rate due to post-analytical gaps. heterogeneous biological samples (tissue, liquid biopsy) require further investigations to validate ONT sequencing strategy in clinical routine.

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Conflict of interests statement

Francesco Pepe has received personal fees as speaker bureau from Menarini, Roche, Themrofisher Scientific, Jansen, Boehringer Ingelheim for work performed outside of the current study. Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen and Bayer, unrelated to the current work. Umberto Malapelle has received personal fees (as consultant and/or speaker bureau) from Boehringer Ingelheim, Roche, MSD, Amgen, Thermo Fisher Scientific, Eli Lilly, Diaceutics, GSK, Merck and AstraZeneca, Janssen, Diatech, Novartis and Hedera unrelated to the current work.

Patient consent for publication

Not applicable.

Ethical consideration

IRB approval is not required.

Author Contributions

Conceptualization, Domenico Vitale, Lorenzo Colarossi, Francesco Pepe, Giancarlo Troncone and Umberto Malapelle.; methodology, all the authors; software, Domenico Vitale, Lorenzo Colarossi, validation, all the authors; formal analysis, all the authors; data curation, Domenico Vitale, Lorenzo Colarossi, Francesco Pepe, Gianluca Russo and Umberto Malapelle.; writing—original draft preparation, Domenico Vitale, Francesco Pepe; writing—review and editing, Lorenzo Memeo, Lucia Anna Muscarella, Giancarlo Troncone and Umberto Malapelle.; visualization all the authors; supervision, Giancarlo Troncone, and Umberto Malapelle.; project administration, Giancarlo Troncone and Umberto Malapelle All authors have read and agreed to the published version of the manuscript.

Data availability statement

Data are available on request to the corresponding author. All data relevant to the study are included in the article or uploaded as supplementary information All data that are publicly available and used in the writing of this article in the text and the reference list.

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