Brief Report

Technical validation of a fully integrated NGS platform in real-world practice of Italian referral institution

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Technical Validation of a Fully Integrated NGS Platform in Real-World Practice of Italian Referral Institution

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Abstract: Aims: To date, precision medicine plays a pivotal role in the clinical administration of solid tumor patients. In this scenario, a rapidly increasing number of predictive biomarkers have been approved in diagnostic practice or are currently investigated in clinical trials. A pitfall in the molecular tests is the diagnostic routine sample available to analyze predictive biomarkers; scant tissue sample often represents the only diagnostical source of nucleic acids to assess molecular analysis. At the sight of these critical issues, Next Generation Sequencing (NGS) platforms emerged as referral testing strategy for molecular analysis of predictive biomarkers in routine practice but high-skilled personnel, extensive working-time drastically impact on the widespread diffusion of this technology in diagnostic setting. Here, we technically validate a fully integrated NGS platform on diagnostic routine tissue samples previously tested with NGS based diagnostic workflow by a referral institution. Methods: A retrospective series of n=64 samples (n=32 DNA, n=32 RNA samples), previously tested using a customized NGS assay (SiRe™ and SiRe fusion) were retrieved from internal archive of University of Naples Federico II. Each sample was tested by adopting Oncomine Precision Assay (OPA), able to detect 2769 molecular actionable alterations [hot spot mutations, copy number variations (CNV) and gene fusions on fully integrated NGS platform (Genexus, Thermofisher Scientifics. (26,27) Concordance rate between these technical approaches was carried out. *Results*: Genexus system successfully carried out molecular analysis in all instances. A concordance rate of 96.9% (31 out of 32) was observed between OPA and SiRe™ panel both for DNA and RNA based analysis. A negative predictive value of 100% and a positive predictive value of 96.9% (62 out of 64) was assessed. *Conclusions*: Fully automatized Genexus system combined with OPA (Thermofisher Scientifics) may be considered a technically valuable, saving time sequencing platform to test predictive biomarkers in diagnostic routine practice.

Keywords: NGS; predictive biomarkers; diagnostic samples

1. Introduction

In the last decades, personalized medicine lay the basis for a novel therapeutical option for solid tumor patients. (1,2) To date, target therapy is routinely available for the clinical administration of several solid tumor patients, including metastatic colorectal cancer (mCRC), melanoma (MM), non-small cell lung cancer (NSCLC), gastrointestinal stromal tumor (GIST), breast cancer (BC) patients. (3-9) Particularly, an increasing number of predictive biomarkers was approved in clinical practice to select lung cancer patients diagnosed with NSCLC type to the best therapeutical option. (8,9) In this evolving scenario, the minimal request in terms of predictive biomarkers to clinically administrate solid tumor patients has been regulated by international societies. (10-14) The most common diagnostic sample available to approach diagnosis and molecular tests in advanced tumor stage

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consists in a "scant sample" with low abundance of neoplastic cells to successfully carry out mandatory gene testing. (15-17) In this scenario, cytological specimens and small biopsies represent the most common biological source to accurately perform molecular analysis. In addition, cell block (CB), a hybrid preparation where the aspirated material is processed following standardized formalin fixation and paraffin embedding (FFPE), represents an alternative source of neoplastic cells affected by lowest quality and quantity of nucleic acids adopted in molecular tests. (18-19) Despite tissue specimens is considered "gold standard" for molecular testing, a not negligible percentage of patients does not access to molecular tests due to insufficient diagnostic material. (16-17) In this scenario, liquid biopsy becomes an integrating biological source to successfully perform molecular analysis when tissue is not available. Particularly, circulating tumor DNA (ctDNA) isolated from peripheral blood withdrawn consists in a reliable source to detect target molecular alterations. (21) At the sight of these aspects, single plex technology result inadequate to successfully analyze minimum gene panel established for each solid tumor. In this heterogenous landscape of biological sources, next generation sequencing (NGS) platforms play a crucial role in the molecular analysis of predictive biomarkers. (22-24) This technology allows to simultaneously analyze very low frequency clinically relevant biomarkers from very low amount of nucleic acids in a single run. (22,23) Remarkably, NGS systems are scalable decreasing reaction cost in accordance with the number of samples processed in each run. (24) On the other hand, adequate number of samples saving technical costs may be collected in more than 30 days for a not negligible number of small-medium institutions involved in molecular tests. This aspect drastically impacts on turnaround -time (TAT) resulting in a delay for the clinical administration of tumor patients. (24,25) In this scenario, Ion Torrent™ Genexus™ Integrated Sequencer (Genexus; Thermofisher Scientifics, Waltham Massachusetts) was designed to automatically carry out entire NGS workflow (from tissue and liquid biopsy derived nucleic acids extraction to data analysis) without other manual operations. (26-28) This technology allows to successfully carry out molecular analysis of a small batch of diagnostic specimens (1- 8) without impacting on Turn-around Time (TAT) of diagnostic workflow. We aimed to evaluate the concordance rate between Genexus system and Ion Torrent S5™ plus (Thermofisher Scientifics, Waltham Massacchusetts) on a retrospective series of extracted genomic DNA (gDNA) from solid tumor patients previously tested in our diagnostic routine.

2. Study design

A retrospective series of n=64 previously extracted DNA and RNA specimens from solid tumor patients [n=16 CRC, n=13 NSCLC, n=2 BC and n=1 MM and n=32 NSCLC cases for DNA and RNA related molecular analysis, respectively) was retrieved from internal archive of Predictive molecular pathology laboratory of University of Naples Federico II. Clinical pathological data were listed in Tables 1 and 2.

Table 1. Clinical characteristics of archival cases and corresponding requests on DNA-based molecular alterations.

ID	Sex	Age	Sample Type	Tumour	N.C.	Clinical Request
DNA 1*	M	78	Resection	CRC	70.0%	*RAS, BRAF*
DNA 2*	M	78	Resection	CRC	70.0%	*RAS, BRAF*
DNA 3	M	89	Biopsy	CRC	50.0%	*RAS, BRAF*
DNA 4	F	68	Resection	NSCLC	70.0%	*EGFR, KRAS, BRAF*
DNA 5	M	73	Resection	CRC	50.0%	*RAS, BRAF*
DNA 6	M	53	Biopsy	NSCLC	30.0%	*EGFR, KRAS, BRAF*
DNA 7	M	66	Resection	CRC	40.0%	*RAS, BRAF*
DNA 8	F	78	Resection	CRC	40.0%	*RAS, BRAF*
DNA 9	F	67	Resection	NSCLC	60.0%	*EGFR, KRAS, BRAF*
DNA 10	F	51	Resection	CRC	30.0%	*RAS, BRAF*
DNA 11	M	50	Resection	CRC	80.0%	*c-KIT, PDGFRA*

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DNA 12	F	50	Biopsy	NSCLC	50.0%	*EGFR, KRAS, BRAF*
DNA 13	M	70	Biopsy	NSCLC	20.0%	*EGFR, KRAS, BRAF*
DNA 14	F	59	Resection	NSCLC	40.0%	*EGFR, KRAS, BRAF*
DNA 15	M	66	Biopsy	NSCLC	30.0%	*EGFR, KRAS, BRAF*
DNA 16	M	56	Resection	CRC	50.0%	*RAS, BRAF*
DNA 17	M	66	Resection	NSCLC	60.0%	*EGFR, KRAS, BRAF*
DNA 18	F	51	Biopsy	CRC	50.0%	*RAS, BRAF*
DNA 19	F	41	Biopsy	BC	30.0%	*PIK3CA*
DNA 20	F	82	Biopsy	CRC	30.0%	*RAS, BRAF*
DNA 21	M	67	Biopsy	CRC	50.0%	*RAS, BRAF*
DNA 22	M	82	Resection	NSCLC	80.0%	*EGFR, KRAS, BRAF*
DNA 23	M	74	Resection	NSCLC	70.0%	*EGFR, KRAS, BRAF*
DNA 24	M	74	Resection	CRC	40.0%	*RAS, BRAF*
DNA 25	F	44	Biopsy	CRC	40.0%	*RAS, BRAF*
DNA 26	F	69	Biopsy	NSCLC	60.0%	*EGFR, KRAS, BRAF*
DNA 27	M	54	Resection	CRC	30.0%	*RAS, BRAF*
DNA 28	F	74	Resection	MM	90.0%	*BRAF, NRAS*
DNA 29	F	63	Biopsy	NSCLC	40.0%	*EGFR, KRAS, BRAF*
DNA 30	M	56	Resection	NSCLC	50.0%	*EGFR, KRAS, BRAF*
DNA 31	F	52	Resection	CRC	60.0%	*RAS, BRAF*
DNA 32	F	45	Resection	BC	60.0%	*PIK3CA*

* Same patient, different lesions. Abbreviations: BC (Breast Cancer); BRAF (Murine Sarcoma Viral Oncogene Homolog B); c-KIT (KIT Proto-Oncogene); CRC (Colorectal Cancer); DNA (Deoxyribonucleic Acid); EGFR (Epidermal Growth Factor Receptor); F (Female); ID (Identifier); KRAS (Kirsten Rat Sarcoma Virus); M (Male); MM (Malignant Melanoma); N.C. (Neoplastic Cellularity); NSCLC (Non-Small-Cell Lung Cancer); PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha); RAS (Rat Sarcoma Virus).

Table 2. Clinical characteristics of archival cases and corresponding requests on RNA-based molecular alterations.

ID	Sex	Age	Sample Type	Tumour	N.C.	Clinical Request
RNA 1	M	56	Resection	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 2	F	58	Biopsy	NSCLC	70.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 3	M	77	Biopsy	NSCLC	25.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 4	M	79	Resection	NSCLC	70.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 5	M	79	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 6	M	59	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 7	F	70	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 8	M	62	Biopsy	NSCLC	25.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 9	M	61	Biopsy	NSCLC	40.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 10	M	66	Resection	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 11	M	68	Biopsy	NSCLC	40.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 12	M	64	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 13	F	65	Biopsy	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 14	M	58	Biopsy	NSCLC	20.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 15	F	79	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 16	M	52	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 17	M	67	Resection	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 18	M	87	Biopsy	NSCLC	40.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 19	M	25	Biopsy	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 20	F	60	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 21	M	60	Resection	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*

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RNA 22	F	36	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 23	M	66	Biopsy	NSCLC	60.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 24	F	47	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 25	M	67	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 26	F	64	Biopsy	NSCLC	10.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 27	M	54	Biopsy	NSCLC	40.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 28	F	37	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 29	M	79	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 30	F	71	Biopsy	NSCLC	30.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 31	M	68	Biopsy	NSCLC	50.0%	*ALK, ROS1, RET, MET, NTRK*
RNA 32	F	72	Biopsy	NSCLC	70.0%	*ALK, ROS1, RET, MET, NTRK*

Abbreviations: ALK (Anaplastic Lymphoma Kinase); F (Female); ID (Identifier); M (Male); MET (Tyrosine-Protein Kinase Met); N.C. (Neoplastic Cellularity); NSCLC (Non-Small-Cell Lung Cancer); NTRK (Neurotrophic Tyrosine Receptor Kinase); RET (RET Proto-Oncogene); RNA (Ribonucleic Acid); ROS1 (Proto-Oncogene Tyrosine-Protein Kinase ROS).

Each sample was previously tested by adopting a customized NGS assay (SiRe™ and SiRe fusion), that covers n=568 clinically relevant alterations in *BRAF, EGFR, KRAS, NRAS, PIK3CA, c-KIT, PDGFRA* and *ALK, ROS1, RET*, and *NTRK* gene fusions, as well as and MET exon 14 skipping alterations, routinely employed in molecular testing of solid tumor patients. (29) The Oncomine Precision Assay (OPA), able to detect 2769 molecular actionable alterations [hot spot mutations, copy number variations (CNV) and gene fusions, was combined with Genexus (Thermofisher Scientifics) platform to assess molecular profile of selected samples. (26,27) Concordance rate of OPA on Genexus system with SiRe™ on S5 plus platform was investigated. All information regarding human material will be managed using anonymous numerical codes, and all samples will be handled in compliance with the Helsinki Declaration (http://www.wma.net/ en/30publications/10policies/b3/).

3. Material and methods

3.1. Routine sample processing startegy

Nucleic acids were previously purified from n=4 representative slides of neoplastic area (>10%). Particularly, QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) was adopted following manufacturer instructions. DNA quantification was successfully carried out in all instances according to standardized procedures. Conversely, RNA volume was maximized for cDNA synthesis. Selected samples were routinely analyzed with SiRe™ and SiRe fusion panel on Ion S5™ plus (Thermofisher Scientifics) to assess mutational status in clinically relevant biomarkers for NSCLC patients. (29-31) Briefly, 15 µl of extracted DNA/cDNA was dispensed on Ion Chef system (Thermofisher Scientifics) for library preparation. A total of n= 8 samples were simultaneously processed following previously validated thermal condition. After pooling, templating procedure was carried out for n=16 libraries by using Ion 510™ & Ion 520™ & Ion 530™ Kit Chef (Thermofischer Scientifics) according to manufacturer instructions on 520 chip (Thermofisher Scientifics). Data were inspected by adopting designed bed files on proprietary Torrent Suite [v.5.0.2]. In details, variant inspection was performed with variant caller plug-in (v.5.0.2.1) able to filter variants with ⩾5X allele coverage and a quality score ⩾20, within an amplicon that covered at least 500X alleles.

3.2. Genexus analysis

A series of n=64 extracted gDNA and gRNA from solid tumor patients were retrospectively tested on Genexus (Thermofisher Scientifics) system. The platform enables entire NGS workflows (from library preparation to data interpretation) within 24 hours. OPA assay includes most clinically relevant actionable genes (*EGFR, BRAF, KRAS, ALK, ROS1*, NTRK, and *RET*) for NSCLC patients. (27,28) Briefly, samples were created on dedicated server and assigned to a new run. Genexus platform was loaded with OPA primers, strip solutions, strip reagents and supplies according to

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manufacturer instructions. A total of 10 ng was required by OPA assay on Genexus platform. Accordingly, each sample was diluted and immediately dispensed on 96-well plate, following manufacturer instructions. Finally, nucleic acids were sequenced on GX5TM chip that allows simultaneous processing of n=8 samples in a single line with OPA assay. Data analysis was performed on proprietary Genexus software. Particularly, detected alterations were annotated by adopting Oncomine Knowledgebase Reporter Software (Oncomine Reporter 5.0).

4. Results

4.1. Hot spot mutations

Overall, Genexus system successfully carried out molecular analysis in all DNA series. In details, a median number of total reads, mapped reads, mean read length, percent reads on target, mean depth, uniformity of amplicon coverage of 1134878.2 (ranging from 424900.0 to 1791041.0), 1074345.7 (ranging from 365139.0 to 1756414.0), 90.9 bp (ranging from 71 to 103 bp), 88.3% (ranging from 77.7 to 93.7%), 3602.9 (ranging from 994.00 to 6097.0) and 98.2% (ranging from 96.7 to 99.4%) were detected, respectively. (Table 3).

Table 3. Technical parameters from DNA-based analysis by using S5 plus and Genexus systems.

DNA Analysis Technical Parameters - S5 Plus (SiRe™ Panel) vs Genexus (OPA Panel)							
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**	**On Target Reads**	**Mean Depth**	**Uniformity**
DNA 1*	S5 Plus	254212	126	253622	94.6%	5712	100%
	Genexus	872831	76	736530	77.7%	2044	99.1%
DNA 2*	S5 Plus	215464	128	215047	92.6%	4740	100%
	Genexus	732691	84	663064	83.9%	2034	98.8%
DNA 3	S5 Plus	298541	135	297999	93.9%	6662	100%
	Genexus	1143038	91	1076855	88.8%	3528	98.1%
DNA 4	S5 Plus	524926	155	523086	92.3%	11489	100%
	Genexus	1419289	101	1393603	92.9%	5210	98.1%
DNA 5	S5 Plus	361148	137	360373	91.3%	7830	100%
	Genexus	1094620	98	1064051	91.5%	3810	98.6%
DNA 6	S5 Plus	314176	128	313706	99.2%	7406	100%
	Genexus	1090358	98	1049935	90.8%	3837	99,0%
DNA 7	S5 Plus	635201	142	634226	92.1%	13911	100%
	Genexus	1002231	92	946318	88.9%	3150	98.9%
DNA 8	S5 Plus	524182	131	523608	93.0%	11591	100%
	Genexus	1262760	95	1208543	90.9%	4176	98.9%
DNA 9	S5 Plus	942781	161	940605	94.6%	21192	100%
	Genexus	1791041	97	1756414	93,0%	6097	97.9%
	S5 Plus	393979	126	393371	89.5%	8381	100%

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DNA 10	Genexus	989635	60	717385	64.9%	1459	98.9%
DNA 11	S5 Plus	451494	139	450779	94.4%	10127	100%
	Genexus	776893	78	679358	80.4%	1863	96.7%
DNA 12	S5 Plus	88915	129	88784	98.0%	2072	92.9%
	Genexus	1297992	91	1263558	92.7%	3996	93.9%
DNA 13	S5 Plus	296845	143	296434	96.2%	6790	100%
	Genexus	1196122	99	1174442	92.7%	4258	98.5%
DNA 14	S5 Plus	37206	133	37173	95.2%	842,7	97.6%
	Genexus	1125616	97	1093531	91.8%	3824	98.6%
DNA 15	S5 Plus	782397	150	780894	95.2%	17703	100%
	Genexus	1465786	92	1423741	91.9%	4574	95.3%
DNA 16	S5 Plus	378978	140	378373	93.3%	8402	100%
	Genexus	1084647	87	1012693	87.6%	3054	98.2%
DNA 17	S5 Plus	520304	135	519653	91.5%	11317	100%
	Genexus	1048030	98	1016324	91.4%	3617	98.8%
DNA 18	S5 Plus	49127	138	49055	95.3%	1113	97.6%
	Genexus	1294194	97	1256161	91.9%	4435	98.9%
DNA 19	S5 Plus	486407	147	485652	96.6%	11165	97.6%
	Genexus	1343529	97	1311776	92.3%	4658	99.4%
DNA 20	S5 Plus	346019	131	345464	97.4%	8010	97.6%
	Genexus	974476	71	759420	75.7%	2023	98.8%
DNA 21	S5 Plus	67488	130	67417	95.9%	1540	97.6%
	Genexus	1150249	90	1094010	90.3%	3519	98.8%
DNA 22	S5 Plus	52080	170	51956	90.4%	1119	100%
	Genexus	1494337	100	1470085	92.3%	5451	97.9%
DNA 23	S5 Plus	614960	141	613813	96.2%	14059	97.6%
	Genexus	1574234	91	1510266	91.2%	4865	97.7%
DNA 24	S5 Plus	188967	136	188623	98.1%	4407	97.6%
	Genexus	1093646	103	1071141	92.2%	4072	99.1%
DNA 25	S5 Plus	140163	145	139930	95.5%	3183	97.6%
	Genexus	949852	94	911448	90,0%	3064	99.4%
DNA 26	S5 Plus	40233	142	40180	96.7%	925,4	97.6%
	Genexus	1497022	99	1476425	93.7%	5365	98.3%

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DNA 27	S5 Plus	153378	133	153236	96.0%	3501	97.6%
	Genexus	1059772	95	1021186	90.2%	3498	98.7%
DNA 28	S5 Plus	155154	118	154695	96.5%	3553	92.8%
	Genexus	424900	75	365139	79.3%	994	97.4%
DNA 29	S5 Plus	358001	160	356995	95.2%	8095	100%
	Genexus	1165795	98	1134969	92.2%	4075	98.4%
DNA 30	S5 Plus	275579	149	274340	98.4%	6428	100%
	Genexus	1080846	92	1034348	90.3%	3392	98.4%
DNA 31	S5 Plus	259364	130	258623	92.6%	5702	100%
	Genexus	1109488	92	1054465	89.9%	3457	98.9%
DNA 32	S5 Plus	263420	126	262682	93.4%	5841	97.6%
	Genexus	710181	82	631880	82.5%	1893	96.7%

*Same patient with different lesions. Abbreviations: DNA (Deoxyribonucleic Acid); ID (Identifier).

Remarkably, n=29 out of 32 (90.6%) patients [n=16 CRC, n= 10 NSCLC, n=2 BC and n=1 MM) showed molecular alterations covered by OPA reference genes. Of note, 24 out of 29 (82.7%) cases highlighted clinically relevant molecular alterations referenced by SiRe™ panel. In particular, n=3 out 29 *EGFR* mutations [n=1 exon 19 c.2300_2308dup p.A767_V769dup; n=1 exon 21 c.2573T>G p.L858R and a concomitant EGFR exon 20 c.2369C>T p.T790M+ exon 21 c.2573T>G p.L858R; n=13 out of 29 *KRAS* molecular alterations [n=3 exon 2 c.35G>A p.G12D; n=2 exon 2 c.34G>T p.G12C; n=2 exon 2 c.35G>A p.G12V; n=1 exon 2 c.38G>A p.G13D; n=1 exon 3 c.182A>T p.Q61L; n=1 exon 3 c.181C>A p.Q61K; n=1 exon 4 c.436G>A p.A146T and n=2 concomitant KRAS exon 2 c.35G>A p.G12D+ c.38G>A p.G13D; *KRAS* exon 2 c.38G>A p.G13D+ c.38_39delinsAA p.G13E]; n=3 out of 29 *BRAF* mutations [n=2 exon 15 c.1799T>A p.V600E and n=1 exon 15 c.1801A>G p.K601E]; n=4 out of 29 *PIK3CA* hot spot mutations [n=2 exon 9 c.1633G>A p.E545K and n=2 exon 20 c.3140A>G p.H1047R]; n=3 out 29 *NRAS* mutations [n=2 exon 3 c.181C>A p.Q61K and n=1 exon 3 c.182A>G p.Q61R]; n=1 out of 29 c-KIT molecular alterations [exon 11 c.1727T>C p.L576P] were detected. (Table 4).

Table 4. Comparison of DNA-related molecular alterations between S5 plus and Genexus platforms.

ID	S5Plus (SiRe™ Panel)	Genexus (OPA Panel)
DNA 1*	**KRAS** p.G12C 27.6% **PIK3CA** p.H1047R 35.0%	**KRAS** p.G12C 32.9% **PIK3CA** p.H1047R 33.2%
DNA 2*	**KRAS** p.G12C 37.2% **PIK3CA** p.H1047R 42.2%	**KRAS** p.G12C 32.7% **PIK3CA** p.H1047R 36.4%
DNA 3	**KRAS** p.G12D 20.7%	**KRAS** p.G12D 18.9%
DNA 4	**EGFR** p.L858R 27.7%	**EGFR** p.L858R 18.9%
DNA 5	**KRAS** p.G12V 34.5%	**KRAS** p.G12V 33.0%
DNA 6	**WT**	**WT**
DNA 7	**KRAS** p.G12D 57.2%	**KRAS** p.G12D 60.8%
DNA 8	**KRAS** p.Q61K 16.8%	**KRAS** p.Q61K 19.3%
DNA 9	**WT**	**WT**
DNA 10	**KRAS** p.G12D 50.6%	**KRAS** p.G12D 55.3%
DNA 11	**c-KIT** p.L576P 68.0%	**c-KIT** p.L576P 63.8%
DNA 12	**EGFR** p.A767_V769dup 67.2%	**EGFR** p.A767_V769dup 72.8%
DNA 13	**WT**	**WT**

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DNA 14	**WT**	**WT**
DNA 15	**BRAF** p.K601E 16.3%	**BRAF** p.K601E 16.1%
DNA 16	**KRAS** p.G12D 9.3% **KRAS** p.G13D 14.1%	**KRAS** p.G12D 8.2% **KRAS** p.G13D 12.1%
DNA 17	**KRAS** p.Q61L 32.7%	**KRAS** p.Q61L 36.3%
DNA 18	**NRAS** p.Q61K 19.3%	**NRAS** p.Q61K 18.2%
DNA 19	**PIK3CA** E545K 0.8%**	**PIK3CA** E545K 7.2%
DNA 20	**BRAF** p.V600E 30.5%	**BRAF** p.V600E 30.0%
DNA 21	**NRAS** p.Q61K 46.7%	**NRAS** p.Q61K 36.2%
DNA 22	**KRAS** p.G13D 47.4%*** **KRAS** p.G13E 47.9%***	**KRAS** p.G13D 41.9%*** **KRAS** p.G13E 42.0%***
DNA 23	**WT**	**WT**
DNA 24	**KRAS** p.A146T 30.80%	**KRAS** p.A146T 26.4%
DNA 25	**WT**	**WT**
DNA 26	**BRAF** p.V600E 27.3%	**BRAF** p.V600E 30.3%
DNA 27	**KRAS** p.G13D 14.9%	**KRAS** p.G13D 12.2%
DNA 28	**NRAS** p.Q61R 34.3%	**NRAS** p.Q61R 28.2%
DNA 29	**EGFR** p.L858R 9.7% **EGFR** p.T790M 9.5%	**EGFR** p.L858R 9.3% **EGFR** p.T790M 11.0%
DNA 30	**WT**	**WT**
DNA 31	**KRAS** p.G12V 51.2% **PIK3CA** p.E545K 32.2%	**KRAS** p.G12V 59.2% **PIK3CA** p.E545K 31.0%
DNA 32	**WT**	**WT**

* Different lesion of same patient. ** Below 5%; *** Concominant SNV. Abbreviations: BRAF (Murine Sarcoma Viral Oncogene Homolog B); c-KIT (KIT Proto-Oncogene); DNA (Deoxyribonucleic Acid); EGFR (Epidermal Growth Factor Receptor); ID (Identifier); KRAS (Kirsten Rat Sarcoma Virus); PIK3CA (Phosphatidylinositol-4,5-Bisphosphate 3-Kinase, Catalytic Subunit Alpha); RAS (Rat Sarcoma Virus); WT (Wild-Type).

Molecular profile detected by OPA on Genexus platform matched with Sire panel on S5 plus system in 31 out of 32 patients (96.9%). Remarkably, positive results previously identified adopting SiRe panel were confirmed in 23 out of 24 (95.8%) patients. Particularly, ID#19 showed exon 9 *PIK3CA* p.E545K hot spot mutation not observed by using S5 system with standardized clinical cut-off. (Figure 1)

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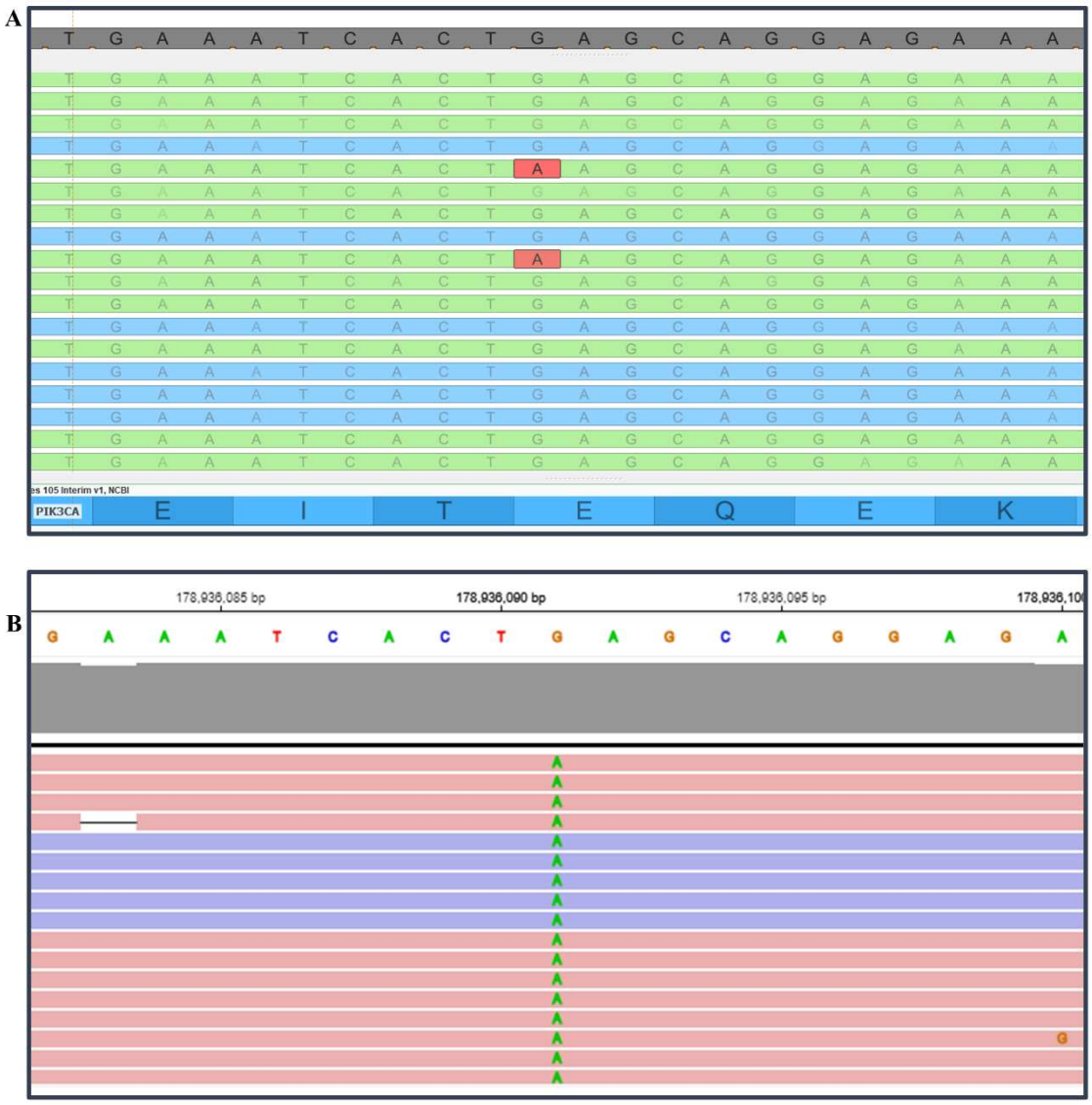

Figure 1. PIK3CA p.E545K hotspot mutations manually inspected with Golden Helix Genome Browser v.2.0.7 (Bozeman, MT, USA) (A) and automatically annotated on on proprietary Genexus software (B).

No significant variations in accordance with histological groups, mutation type and mutant allele fraction levels between Genexus and previously tested samples on S5 platform were identified. In addition, OPA assay also identified n= 16 out of 32 (50.0%) DNA based molecular alterations in other genes not covered by SiRe panel. As regards, 12 out of 16, 1 out of 16 and 1 out of 16 highlighted *TP53*, *CTNNB1* and *MTOR* hotspot molecular altercations, respectively. Moreover, a concomitant *TP53* (exon 7 p.G279E plus exon 5 p.V197M) and *TP53* (exon 4 p.R175H) in association with *CTNNB1* (exon 3 p.S45F) hotspot mutations were identified in ID#2 and ID#16 cases. (Table 5).

Table 5. Expanded list of molecular alterations covered by OPA on Genexus platform.

ID	Other Mutations (OPA Panel)
DNA 1*	**MTOR** p.R2217W 4.5%
DNA 2*	**TP53** p.G279E 4.8% **TP53** p.V197M 4.0%
DNA 7	**TP53** p.H179Y 75.8%
DNA 9	**TP53** p.R273H 35.0%

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DNA 12	**TP53** p.V197M 77.7%
DNA 14	**TP53** p.R273H 10.0%
DNA 16	**CTNNB1** p.S45F 41.1% **TP53** p.R175H 13.2%
DNA 18	**TP53** p.Y220C 19.7%
DNA 19	**TP53** p.L194F 9.9%
DNA 20	**TP53** p.P151S 54.7%
DNA 21	**TP53** p.K132R 51.4%
DNA 23	**TP53** p.C238S 25.3%
DNA 27	**CTNNB1** p.S45F 21.8%
DNA 30	**TP53** p.H179Y 24.6%
DNA 31	**TP53** p.Y220C 56.1%
DNA 32	**TP53** p.E285K 4.8%

*Same patient, different lesion. Abbreviations: CTNNB1 (Catenin Beta 1); DNA (Deoxyribonucleic Acid); ID (Identifier); MTOR (Mammalian Target Of Rapamycin); TP53 (Tumor Protein P53).

4.2. Fusions rearrangements

Regarding RNA samples, Genexus platform successfully analyzed all retrieved cases. Briefly, a median number of total reads, mapped reads and mean read length of 1721491.0 (ranging from 1471817.00 to 2462555.00), 158230.4 (ranging from 37387.0 to 1029745.00), 98.8 bp (ranging from 91 to 104 bp) were identified, respectively. (Table 6).

Table 6. Technical parameters from RNA-based analysis by using S5 plus and Genexus systems.

RNA analysis Technical Parameters - S5 Plus (SiRe Fusion Panel) vs Genexus (OPA Panel)				
ID	**Platform**	**Total Reads**	**Mean Read Length**	**Mapped Reads**
RNA 1	S5 Plus	503832	92	489474
	Genexus	2355408	99	170105
RNA 2	S5 Plus	829380	124	823978
	Genexus	1748261	99	140327
RNA 3	S5 Plus	641591	89	348169
	Genexus	2462555	104	54529
RNA 4	S5 Plus	254394	93	242076
	Genexus	1667488	100	37387
RNA 5	S5 Plus	234803	67	176276
	Genexus	1755508	91	111713
RNA 6	S5 Plus	357284	89	319350
	Genexus	1542252	101	72995
RNA 7	S5 Plus	1070656	111	1067615
	Genexus	1571469	100	150711
RNA 8	S5 Plus	535701	103	526127
	Genexus	1737696	96	1029745
RNA 9	S5 Plus	494550	87	421901
	Genexus	1634624	103	72104
RNA 10	S5 Plus	161964	100	153003
	Genexus	1815512	96	51505
RNA 11	S5 Plus	190170	98	187044
	Genexus	1597727	98	386493
RNA 12	S5 Plus	677654	91	513093
	Genexus	1554237	101	171919

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RNA 13	S5 Plus	765186	129	753177
	Genexus	1777747	100	178846
RNA 14	S5 Plus	222717	103	217972
	Genexus	1503566	102	48005
RNA 15	S5 Plus	490208	125	483482
	Genexus	1523971	99	61024
RNA 16	S5 Plus	20405	91	17060
	Genexus	1878041	97	42572
RNA 17	S5 Plus	367743	117	346142
	Genexus	1769313	97	80920
RNA 18	S5 Plus	191027	99	189336
	Genexus	1513615	97	365130
RNA 19	S5 Plus	240954	126	239481
	Genexus	1744270	100	133226
RNA 20	S5 Plus	203214	86	195547
	Genexus	1284559	94	173554
RNA 21	S5 Plus	195912	91	185689
	Genexus	1940917	96	60947
RNA 22	S5 Plus	464854	119	462638
	Genexus	1715374	98	294552
RNA 23	S5 Plus	258734	93	251939
	Genexus	1644449	99	141394
RNA 24	S5 Plus	287598	104	284682
	Genexus	1573653	103	68184
RNA 25	S5 Plus	297871	114	294124
	Genexus	1587686	99	111160
RNA 26	S5 Plus	428858	118	426903
	Genexus	1682103	100	185977
RNA 27	S5 Plus	173120	98	171187
	Genexus	1471817	98	252247
RNA 28	S5 Plus	187176	145	185591
	Genexus	1903859	98	126388
RNA 29	S5 Plus	311784	84	262726
	Genexus	1839064	102	45998
RNA 30	S5 Plus	416422	93	393110
	Genexus	1727113	101	57972
RNA 31	S5 Plus	240891	112	239186
	Genexus	1598494	99	133522
RNA 32	S5 Plus	156106	63	97917
	Genexus	1965363	93	52222

Abbreviations: ID (Identifier); RNA (Ribonucleic Acid).

Of note, 10 out of 32 (31.2%) patients highlighted aberrant transcripts by using Genexus platform. Among them, 5 out of 10 and 2 out of 10 patients showed *ALK* and *RET* rearrangements, respectively. Moreover, three patients were positive for *ROS1*, *NTRK* aberrant transcripts and MET Δ 14 skipping mutation, respectively. (Table 7) Interestingly, rearranged genes were identified by OPA on Genexus platform in 9 out of 10 (90.0%) retrieved cases showing a concordance rate of 96.9% (31 out of 32 cases) with SiRe panel on S5 system. Particularly, ID#1 was positive for *NTRK3-KANK1* fusion transcript not previously detected with SiRe panel on S5 platform. No significant variations

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were observed in accordance with histological groups, rearranged genes, fusion partners, and mapped reads levels between Genexus and previously tested samples on S5 platform.

Table 7. Comparison of RNA-related molecular alterations between S5 plus and Genexus platforms.

ID	S5Plus (SiRe Fusion Panel)	Genexus (OPA Panel)
RNA 1	**No Fusion**	**NTRK3** (ex14) - KANK1 (ex3) 1571 reads *
RNA 2	**No Fusion**	**No Fusion**
RNA 3	**No Fusion**	**No Fusion**
RNA 4	**No Fusion**	**No Fusion**
RNA 5	**No Fusion**	**No Fusion**
RNA 6	**No Fusion**	**No Fusion**
RNA 7	**ALK** (ex20) - EML4 (ex6) 601 reads	**ALK** (ex20) - EML4 (ex6) 353 reads
RNA 8	**No Fusion**	**No Fusion**
RNA 9	**No Fusion**	**No Fusion**
RNA 10	**No Fusion**	**No Fusion**
RNA 11	**No Fusion**	**No Fusion**
RNA 12	**No Fusion**	**No Fusion**
RNA 13	**ALK** (ex20) - unknown partner 149 reads	**ALK** (ex20) - DCTN1 (ex26) 2268 reads
RNA 14	**No Fusion**	**No Fusion**
RNA 15	**No Fusion**	**No Fusion**
RNA 16	**No Fusion**	**No Fusion**
RNA 17	**No Fusion**	**No Fusion**
RNA 18	**No Fusion**	**No Fusion**
RNA 19	**ROS1** (ex34) **-** CD74 (ex6) 2208 reads	**ROS1** (ex34) - CD74 (ex6) 1992 reads
RNA 20	**ALK** (ex20) - EML4 (ex6) 43 reads	**ALK** (ex20) - EML4 (ex6) 1040 reads
RNA 21	**No Fusion**	**No Fusion**
RNA 22	**ALK** (ex20) - EML4 (ex13) 11335 reads	**ALK** (ex20) - EML4 (ex13) 7212 reads
RNA 23	**No Fusion**	**No Fusion**
RNA 24	**RET** (ex12) - KIF5B (ex15) 4063 reads	**RET** (ex12) - KIF5B (ex15) 2417 reads
RNA 25	**No Fusion**	**MET** (ex13) - MET (ex15) 9638 reads
RNA 26	**No Fusion**	**No Fusion**
RNA 27	**No Fusion**	**No Fusion**
RNA 28	**ALK** (ex20) - EML4 (ex20) 6293 reads	**ALK** (ex20) - EML4 (ex20) 1140 reads
RNA 29	**No Fusion**	**No Fusion**
RNA 30	**No Fusion**	**No Fusion**
RNA 31	**No Fusion**	**No Fusion**
RNA 32	**RET** (ex12) - CCDC6 (ex1) 494 reads	**RET** (ex12) - CCDC6 (ex1) 172 reads

*Not covered from SiRe Fusion Panel. Abbreviations: ALK (Anaplastic Lymphoma Kinase); CCDC6 (Coiled-Coil Domain-Containing Protein 6); CD74 (HLA Class II Histocompatibility Antigen Gamma Chain); DCTN1 (Dynactin Subunit 1); EML4 (Echinoderm Microtubule-Associated Protein-Like 4); EX (Exon); ID (Identifier); KANK1 (KN Motif And Ankyrin Repeat Domains 1); KIF5B (Kinesin Family Member 5B); MET (Tyrosine-Protein Kinase Met); NTRK (Neurotrophic Tyrosine Receptor Kinase); RET (RET Proto-Oncogene); RNA (Ribonucleic Acid); ROS1 (Proto-Oncogene Tyrosine-Protein Kinase ROS).

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5. Discussion

In the era of personalized medicine, the rapidly increasing number of predictive biomarkers yet approved in clinical practice have revolutionized the treatment strategy for solid tumor patients. (1-2,32) Although the widespread diffusion of single-gene testing platforms in the vast majority of laboratories involved in molecular tests, low multiplexing biomarker's analysis discouraging their implementation as pivotal diagnostic platform in clinical practice (23-24). As regards, NGS techniques allows to simultaneously cover clinically relevant molecular alterations from a plethora of diagnostic routine specimens saving technical costs and maintaining adequate TAT (33). Moreover, NGS platforms may also benefit of automatized technical procedures that allows accurate and reproducible analysis spending low bench-working time (33). Genexus system consists in a scalable, versatile and fully automatized sequencer able to carry out each technical procedure without manual operations (34). This system is built to integrate analytical procedures (nucleic acids extraction, libraries preparation, template generation, sequencing) with data analysis by adopting pre-customized pipeline analysis. Here, we have validated Genexus system in our diagnostic routine by comparing its analytical performance on a retrospective series of clinical cases previously analyzed with a custom NGS panel on S5 system. As expected, all diagnostic specimens (n=64) were successfully analyzed by using this fully automatized system. Overall, a concordance rate of 96.9% (62 out of 64) was reached by adopting Sire panel on S5 system as reference standard. Interestingly, molecular analysis unmatched with previously archived data in only two cases (DNA-ID#19 and RNA-ID#1). Of note, DNA-ID#19 sample derived from a BC patient resulted positive for *PIK3CA* exon 9 p.E545K hotspot alteration on Genexus system with a mutant allele fraction (MAF) of 7.2%. Following manufacturer clinical cut-off (MAF ≥5%), previous analysis did not show any clinically relevant molecular alteration. By approaching visual inspection of raw data, the same alteration at 0.9% was detected. Similarly, RNA-ID#1 showed *NTRK3* (ex14) - *KANK1* (ex3) aberrant transcript not previously detected with the standard reference approach. In this case *NTRK3* was not covered by reference range of SiRe fusion panel.

In a not negligible percentage of cases, synchronous lesions may be observed in CRC patients. In this scenario, NGS may be considered an affordable technical strategy to comprehensively evaluate molecular assessment of CRC patients where heterogeneous specimens are clinically available (28). DNA-ID#11 and DNA-ID#2 represent synchronous lesions of a CRC elected to molecular test. Interestingly, both S5 and Genexus systems revealed *KRAS* exon 2 p.G12C and *PIK3CA* exon 20 p.H1047R hot spot mutations demonstrating a common origin of these lesions. Moreover, NGS systems overcome technical issues from the analysis of "complex" molecular alteration. DNA-ID#22 case confirmed two concomitant *KRAS* exon 2 hotspot mutations p.G13D+p.G13E on Genexus platform previously detected by reference technology. Although this study provides encouraging results for the implementation of Genexus system in clinical routine setting of solid tumor patients, some limitations may be identified. Firstly, this technical report aims to compare analytical parameters of two NGS-based technologies on a series of diagnostic routine specimens without any clinical considerations. Secondly, this retrospective study is based on the analysis of a small group of cases retrieved from internal archive of University of Naples Federico II. All these crucial points warrant further analysis, but this preliminary data may suggest that fully automatized Genexus system integrated with commercially available OPA (Thermofisher Scientifics) represent a technically affordable, saving time sequencing platform enable to analyze clinically relevant molecular alterations in diagnostic routine specimens.

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