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Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics

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Increasing use of fine needle aspiration for oncological diagnosis, while minimally invasive, poses a challenge for molecular testing by traditional sequencing platforms due to high sample requirements. The advent of affordable benchtop next-generation sequencing platforms such as the semiconductor-based Ion Personal Genome Machine (PGM) Sequencer has facilitated multi-gene mutational profiling using only nanograms of DNA. We describe successful next-generation sequencing-based testing of fine needle aspiration cytological specimens in a clinical laboratory setting. We selected 61 tumor specimens, obtained by fine needle aspiration, with known mutational status for clinically relevant genes; of these, 31 specimens yielded sufficient DNA for next-generation sequencing testing. Ten nanograms of DNA from each sample was tested for mutations in the hotspot regions of 46 cancer-related genes using a 318-chip on Ion PGM Sequencer. All tested samples underwent successful targeted sequencing of 46 genes. We showed 100% concordance of results between next-generation sequencing and conventional test platforms for all previously known point mutations that included *BRAF*, *EGFR*, *KRAS*, *MET*, *NRAS*, *PIK3CA*, *RET* and *TP53*, deletions of *EGFR* and wild-type calls. Furthermore, next-generation sequencing detected variants in 19 of the 31 (61%) patient samples that were not detected by traditional platforms, thus increasing the utility of mutation analysis; these variants involved the *APC*, *ATM*, *CDKN2A*, *CTNNB1*, *FGFR2*, *FLT3*, *KDR*, *KIT*, *KRAS*, *MLH1*, *NRAS*, *PIK3CA*, *SMAD4*, *STK11* and *TP53* genes. The results of this study show that next-generation sequencing-based mutational profiling can be performed on fine needle aspiration cytological smears and cell blocks. Next-generation sequencing can be performed with only nanograms of DNA and has better sensitivity than traditional sequencing platforms. Use of next-generation sequencing also enhances the power of fine needle aspiration by providing gene mutation results that can direct personalized cancer therapy.
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Personalized therapy by identification and targeting of tumor-specific molecular abnormalities is rapidly becoming an important component in the management of cancer patients. Consequently, algorithms for tumor diagnosis necessitate not only morphological and immunophenotypic assessment of tumors but also molecular mutational profiling. Among the solid tumors, mutational status is important to the clinical management of patients with thyroid carcinomas, non-small cell carcinomas of the lung, melanomas and colorectal carcinomas. For example, treatment of patients with non-small cell carcinoma of the lung depends on the histological type (adenocarcinoma *versus* squamous cell carcinoma) and the mutational status of the epidermal growth factor receptor (*EGFR*) and *KRAS* genes.[1] Thus, adequate tissue sampling is essential not only for pathological diagnosis but also for molecular testing that is required to guide therapeutic decisions.[1–3]

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In clinical practice, minimally invasive fine needle aspiration (FNA) is a helpful and convenient method for establishing the diagnosis of solid tumors. FNA procedures are included in the recommended guidelines for the diagnosis of thyroid carcinomas,[4,5] lung carcinomas[6,7] and sarcomas, such as Ewing Sarcoma/primitive neuroectodermal tumor.[8] Although the DNA yield obtained by FNA is adequate for morphological diagnosis, FNA material is not routinely processed for molecular analysis.[9] This is because mutational assessment of multiple genes by traditional sequencing platforms require a large quantity of DNA, which is often difficult to obtain by FNA. Fortunately, most FNA procedures routinely have either a concurrent biopsy or a follow-up surgical excision, which provide adequate tissue for molecular assessment.[10,11]

In some instances, however, cytological specimens may be the only material available for molecular testing. If the tumor is locally advanced or metastatic, surgical resection is not performed.[1,6] In some patients, tumor size, location or co-morbid conditions may preclude concurrent core needle or excisional biopsy.[1] Therefore, efficient strategies and novel technologies are needed to apply molecular testing to FNA samples. Studies have attempted to improve the yield of DNA from cytological samples by using Fast Technology for Analysis cards for high-quality DNA preservation.[9] Preparation of cell blocks using the tissue coagulum clot method has also increased the efficacy of tissue extraction from endobronchial and transbronchial FNA samples.[1] Additionally, use of COLD-PCR/direct sequencing for *EGFR* and *KRAS* detection in lung aspirates and locked nucleic acid-based PCR/sequencing assay in thyroid aspirates has improved the sensitivity of mutation detection.[10,12]

Massively parallel or next-generation sequencing (NGS) technology is increasingly being used for mutational analysis of tumors for both clinical and research applications. The advent of affordable bench top NGS platforms such as Ion Personal Genome Machine (PGM) has facilitated multi-gene mutational profiling using only nanograms (ng) of DNA.[13] PGM uses the semiconductor-based sequencing technology, which monitors the release of hydrogen ions during the incorporation of nucleotide. The resulting pH change detected by the ion-sensitive field-effect transistor is converted into sequence information by signal-processing software.[14] The need for extremely small amount of DNA makes this technology potentially applicable to FNA cytological specimens in a clinical laboratory. To our knowledge, no studies using NGS on cytology specimens have been reported to date.

The objective of this study was to evaluate the feasibility of applying NGS technology to the mutational analysis of routinely obtained FNA cytological specimens in a clinical molecular diagnostic laboratory.

Materials and methods

Case Selection/Patient Specimens

For this study, we selected FNA-obtained samples from 61 consecutive tumor specimens with known mutational status for clinically relevant genes between January 2010 and August 2012. The diagnoses of these samples were established using universally accepted criteria. Specimens included 33 smears and 28 formalin-fixed, paraffin-embedded cell blocks. Smears were stained with either Diff-Quik (methanol-fixed) or Papanicolaou (ethanol-fixed). The cell block sections were stained with hematoxylin and eosin. All slides were reviewed by pathologists to circle the tumor-rich areas and estimate the tumor percentage. Only cases in which tumor involvement was estimated to be $>20\%$ were included in this study.

DNA Extraction from FNA Smears and Cell Blocks

DNA was extracted directly from stained FNA smear(s). Coverslips were removed using xylene followed by hydration and air drying. Circled tumor-rich areas were scraped off the slides using a razor blade by manual microdissection. For cell blocks, DNA was extracted from a variable number of unstained sections, each 0.4 μm thick. Manual microdissection of tumor-rich areas was followed by deparaffinization. DNA was extracted using the Pico Pure DNA Extraction Kit (Arcturus, Mountain View, CA) followed by purification using the Agentcourt AMPureXP kit (Agentcourt Biosciences, Beverly, MA). DNA quantification was performed using Qubit DNA HS assay kit (Life Technologies, Carlsbad, CA). Only samples with a minimum DNA concentration of 0.8 ng/μl were selected for NGS.

Next-Generation Sequencing

Library preparation and emulsion PCR. An amplicon library was generated from 10 ng of DNA from each sample using the Ion Ampliseq Cancer Panel (Life Technologies). Formalin-fixed, paraffin-embedded cell pellets of the H2122 cell line diluted in the HL60 cell line were used as control. The 46 genes in the panel for detection of 'hotspot' mutations included: *AKT1*, *BRAF*, *FGFR1*, *GNAS*, *IDH1*, *FGFR2*, *KRAS*, *NRAS*, *PIK3CA*, *MET*, *RET*, *EGFR*, *JAK2*, *MPL*, *PDGFRA*, *PTEN*, *TP53*, *FGFR3*, *FLT3*, *KIT*, *ERBB2*, *ABL1*, *HNF1A*, *HRAS*, *ATM*, *RB1*, *CDH1*, *SMAD4*, *STK11*, *ALK*, *SRC*, *SMARCB1*, *VHL*, *MLH1*, *CTNNB1*, *KDR*, *FBXW7*, *APC*, *CSF1R*, *NPM1*, *SMO*, *ERBB4*, *CDKN2A*, *NOTCH1*, *JAK3*, and *PTPN11*. Primers for PCR amplification included the 190-primer pair pool (provided by the vendor) with an additional primer pair that was custom added to cover the 'hotspot' location on codon 17 of *AKT1*. Following PCR amplification of target sequences, barcodes were ligated to the amplicons

using the Ion Xpress Barcode Adaptors Kit (Life Technologies). Library quantification was then performed using the Bioanalyzer High Sensitivity DNA Chip (Agilent Technologies, Santa Clara, CA). The library was diluted in nuclease-free water to obtain a final concentration of 16 pM. Emulsion PCR was performed manually using the Ion Xpress Template Kit (Life Technologies) followed by manual breaking of the emulsion to isolate the ion spheres (ISPs). The quality of the DNA following PCR was measured using the Qubit IonSphere Quality control kit (Life Technologies). Selective ISPs with DNA were isolated and sequenced on a Ion 316 Chip (4 samples/chip) or a Ion 318 Chip (8 samples/chip) using the vendor-provided sequencing kit (Life Technologies).

Successful sequencing of a sample required at least 300 000 reads with a quality score of AQ20 (1 misaligned base per 100 bases). For a wild-type call, a minimum coverage of 250× was required. As tumor specimens were admixed with normal tissue, a minimum coverage of 500× with at least 10% frequency was used as cutoff for a variant to be considered true. All variants detected by Ion PGM with at least 10% frequency were selected for confirmation by alternate platforms.

Data Analysis

Sequence alignment and base calling was performed by Torrent Suite software V2.0.1 (Life Technologies) using Human Genome Build 19 (Hg19) as the reference. Torrent Variant Caller software V1.0 (Life Technologies) was used for the detection of variants whereas the Integrative Genomics Viewer (IGV) was used to visualize variants.[15] The IGV provides a visual picture of the sequence alignment with the reference, thus facilitating detection of false variants as a result of possible sequencing errors or strand bias. Oncoseek software, developed by one of us (MJR), was used to integrate the data generated by Torrent variant caller for annotation and visualization in IGV.[16] Further, it was used to generate refined data by filtering errors generated due to the presence of homopolymers. Other custom-designed tools in Oncoseek include ready annotation of the results using standard nomenclature and side-by-side comparison between specimens. During interpretation of NGS data, the results of the traditional mutation analysis were completely blinded.

Confirmation of Mutations by Traditional Sequencing Platforms: Sanger Sequencing, Pyrosequencing and Sequenom Massarray System

At least one of the three conventional platforms was used to confirm the results obtained by the Ion Ampliseq Cancer Panel in our clinical laboratory. The results derived by using the Ion Ampliseq Cancer Panel were confirmed by at least one of the three conventional platforms used in our clinical laboratory. For Sanger sequencing, PCR amplification of genomic regions of interest was performed using 10 ng of DNA template and M13-tagged primers; M13 forward, 5′-TGTAAAACGACGGCCAGT-3′; or M13 reverse, 5′-CAGGAAACAGCTATGACC-3′. Primers were designed and optimized to ensure similar PCR conditions for all the genes. PCR amplification was followed by purification using AMPure magnetic beads (Agentcourt). Sanger sequencing was performed on a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA) using 5 μl of PCR product (1:5 dilution in water) and analyzed using SeqScape v2.5 and/or v2.7 software (Applied Biosystems). For pyrosequencing, PCR amplification was confirmed by electrophoresis on an agarose gel. In all, 15 μl of the PCR product along with the appropriate sequencing primer underwent pyrosequencing in PSQ96 HS System (Biotage AB, Uppsala, Sweden). The program of nucleotide dispensation order was customized to each of the genes to detect all possible mutations. The Sequenom multiplex assay was used to assess the mutational status of hotspot regions in 11 genes: *AKT1*, *BRAF*, *GNAS*, *GNAQ*, *IDH1*, *IDH2*, *KRAS*, *MET*, *NRAS*, *PIK3CA*, and *RET*. PCR amplification was performed using 10 ng of DNA in each of the nine wells and designed primers from Integrated DNA Technologies (Coralville, IA), designed by using Sequenom's MassARRAY Designer software. Dephosphorylation of unincorporated nucleotides was performed using shrimp alkaline phosphatase. Single base primer extension using mass modified di-deoxy nucleotides (iPLEX Gold kit) was followed by product analysis using MALDI-TOF mass spectrometry (Sequenom).

Results

Case Selection

Sixty-one consecutive cytological tumor specimens obtained by FNA and in which mutation analysis of selected genes had been requested as a part of clinical care were selected for the study group. Fifty-five cases ($n=55$) had been tested by one of the conventional testing platforms and six cases were primarily received for NGS testing. The minimum DNA concentration required for successful NGS analysis using the Ion PGM platform is 10 ng. The minimum amount was based purely on validation studies performed on surgical biopsy specimens (data not shown). The above required DNA concentration was obtained in 31 of the 61 (51%) samples derived from 15 cell blocks and 16 aspirate smears. These samples were selected for NGS-based testing. In the remaining cases, 17 of the 33 (52%) FNA smears and 13 of the 28 (46%) cell blocks yielded suboptimal DNA and were not tested by NGS. However, all of these 'failed' cases were successfully analyzed by one of the conventional testing platforms for mutations in ≥1 genes.

Table 1 Clinico-pathological data and specimen characteristics of the cytological cases that qualified for testing by Ion Torrent's next-generation sequencing

Case no.	*Site*	*Diagnosis*	*Tumor %*	*Stain type*	*Slides used*	*DNA yield (ng/ul)*
Smears						
1	Thyroid	Papillary thyroid carcinoma	70	Pap	2	2.00
2	LN	Papillary thyroid carcinoma[a]	90	Pap	1	3.14
3	Thyroid	High grade carcinoma	95	Pap	1	2.07
4	LN	Adenocarcinoma, lung[a]	70	Pap	1	21.00
5	LN	Adenocarcinoma, lung[a]	60	DQ	1	12.70
6	LN	Adenocarcinoma, lung[a]	60	DQ	1	3.83
7	Lung	Adenocarcinoma, lung	80	Pap	1	4.78
8	LN	Adenocarcinoma, lung[a]	60	Pap	2	1.32
9	Pleural fluid	Adenocarcinoma, lung[a]	25	Pap	1	2.70
10	LN	Adenocarcinoma, lung[a]	80	Pap	1	0.30
11	LN	Non-small cell carcinoma, lung[a]	>20	Pap	2	7.71
12	LN	Adenosquamous carcinoma, lung[a]	50	Pap	1	7.63
13	Liver	Adenocarcinoma, colorectal[a]	90	Pap	1	1.17
14	LN	Melanoma[a]	>20	Pap	2	0.91
15	LN	Melanoma[a]	100	DQ	1	8.64
16	LN	Pheochromocytoma[a]	70	Pap	1	2.90
Blocks						
17	LN	Medullary thyroid carcinoma[a]	50	NA	4	1.71
18	LN	Adenocarcinoma, lung[a]	35	NA	4	1.93
19	Lung	Adenocarcinoma, lung	60	NA	6	1.28
20	Lung	Adenocarcinoma, lung	50	NA	1	13.80
21	Lung	Adenocarcinoma, lung	75	NA	5	4.85
22	Pleural fluid	Adenocarcinoma, lung[a]	70	NA	2	0.28
23	Lung	Adenocarcinoma, lung	30	NA	7	1.40
24	Pleural fluid	Adenocarcinoma, lung[a]	80	NA	4	3.03
25	Pleural fluid	Adenocarcinoma, lung[a]	80	NA	3	1.48
26	Lung	Adenocarcinoma, lung	60	NA	7	14.50
27	LN	Squamous cell carcinoma, lung[a]	85	NA	3	1.43
28	Bone	Adenocarcinoma, prostate[a]	50	NA	4	1.32
29	LN	Melanoma[a]	95	NA	10	1.79
30	LN	Melanoma[a]	25	NA	4	2.94
31	Soft tissue[b]	Alveolar soft part sarcoma	80	NA	4	0.85

Abbreviations: LN, lymph node; NA, not applicable.
DNA extracted from these cases met the minimum required concentration as measured by Qubit.
[a]Metastatic from a primary tumor.
[b]Pelvic site.

Specimen Characteristics

The clinical and pathological information of the 31 patient specimens assessed by NGS in this study are described in Table 1. The tumors included 16 cases of primary or metastatic adenocarcinomas of the lung, 4 cases of metastatic melanomas, 4 cases of thyroid carcinomas (2 papillary, 1 medullary and 1 high-grade) 2 colonic adenocarcinomas and 1 case each of squamous cell carcinoma of the lung, non-small cell carcinoma of the lung, adenosquamous carcinoma of the lung, alveolar soft part sarcoma and pheochromocytoma.

Estimation of tumor burden based on tumor percentage and the number of slides used for DNA extraction of the 31 cases with adequate DNA are shown in Table 1. As a prerequisite, only cases with a tumor percentage >20% were selected for performing NGS. The median tumor percentages in the smears and cell blocks were 70% (range, 25–100, $n=14$) and 60% (range, 25–95, $n=15$), respectively. The median number of slides used to extract DNA was 1 for smears (range, 1–2) and 4 for cell blocks (range, 1–10). The median DNA concentration for smears and cell blocks were 3.02 ng/μl (range, 0.295–21) and 1.71 ng/μl (range, 0.276–14.5), respectively. The selected smear samples included both Papanicolaou (ethanol-fixed) and Diff-Quik (methanol-fixed)-stained FNA smears. The process of extraction and yield of DNA was not affected by the type of fixation or staining. Images of the slides used for DNA extraction from these 31 cases are shown in Figure 1.

In comparison, we analyzed the specimen characteristics of 30 cases with suboptimal DNA for NGS testing. The median tumor percentages in the smears and cell blocks were 80% (range, 30–100) and 65% (range, 40–90), respectively. The median number of slides used to extract DNA was 1 for smears (range, 1–4) and 3.5 for cell blocks (range, 1–8). The median DNA concentration for the smear and cell block samples were 0.276 ng/μl (range, 0.001–0.673) and 0.001 ng/μl (range, 0.00–0.613), respectively. Based on this, there were no significant differences between the samples that yielded DNA adequate for NGS testing *versus* the samples that did not yield

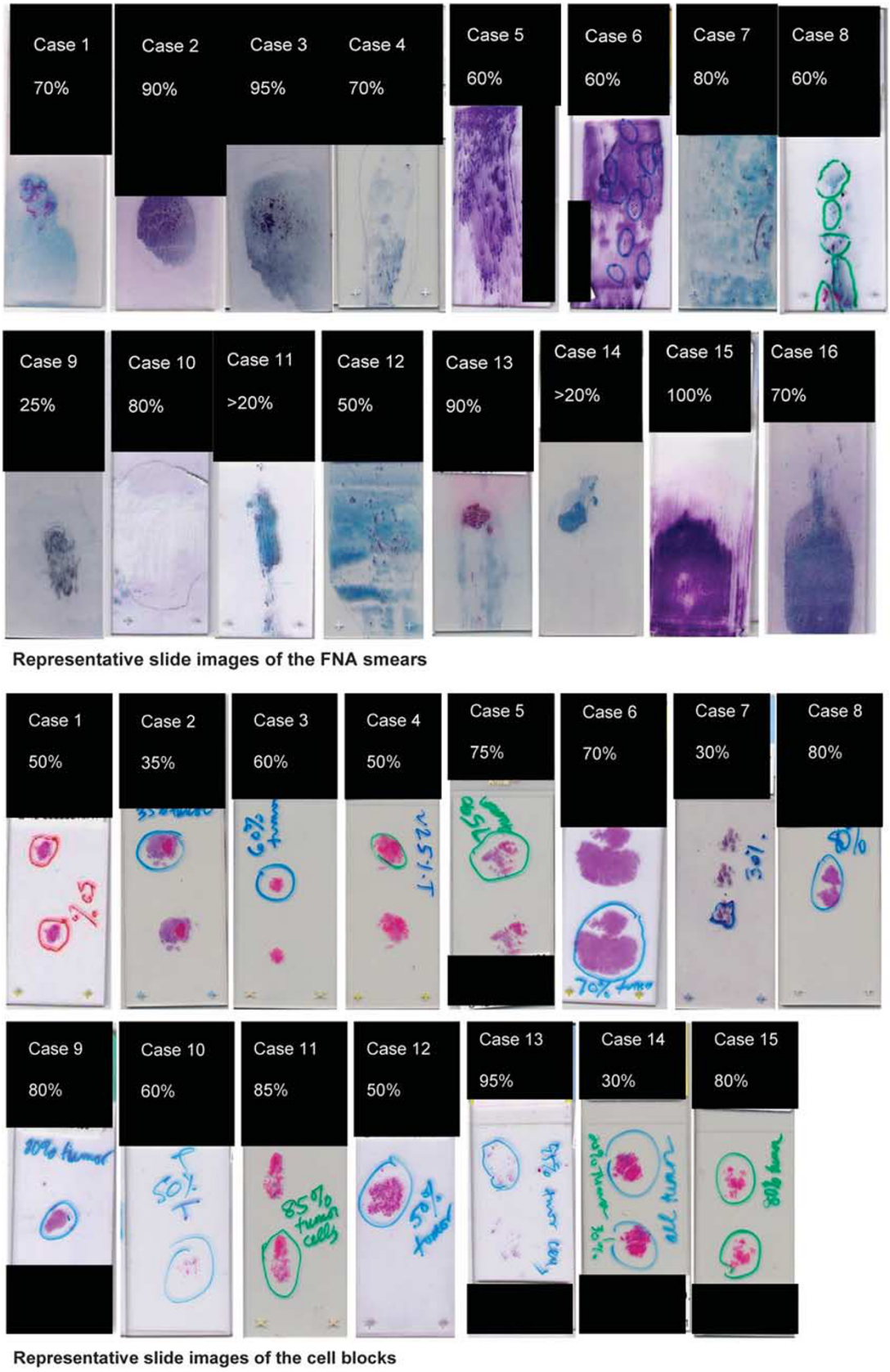

Figure 1 Representative image of the FNA smears and cell blocks used for the study. The amount of available material was variable, but these cases had sufficient DNA for analysis.

Table 2 Performance metrics and detected variants of selected fine needle aspirate smear samples in our study

Case no.	*Total reads*	*Filtered Q20 reads (% total)*	*Variants*	*Failed amplicons/ genes (<250× coverage)*
1	523092	429525 (82)	**NM_004333.4(*BRAF*):c.1799T>A p.V600E** NM_004119.2(*FLT3*):c.2039C>T p.A680V[a]	*TP53*
2	744563	610704 (82)	**NM_004333.4(*BRAF*):c.1799 T>A p.V600E** NM_002253.2(*KDR*):c.1444 T>C p.C482R[a]	*CDKN2A*
3	680436	559115 (82)	NM_000546.5(*TP53*):c.638G>T p.R213L[a]	*KIT*
4	563731	460325 (82)	**NM_033360.2(*KRAS*):c.34G>T p.G12C** NM_000546.5(*TP53*):c.475G>C p.A159P[a]	
5	622091	418175 (67)	**NM_033360.2(*KRAS*):c.37G>T p.G13C**	
6	711955	549189 (77)	NM_000141.4(FGFR2):c.1647T>A p.N549K[a] NM_002253.2(KDR):c.1444T>C p.C482R[a] NM_000222.2(KIT):c.1621A>C p.M541L[a] NM_000546.5(TP53):c.273G>A p.W91*[a]	None
7	810521	658770 (81)	NM_002253.2(*KDR*):c.3433G>C p.G1145R[a]	
8	555218	451324 (81)	***EGFR* exon 19 15bp deletion** NM_002253.2(*KDR*):c.1444T>C p.C482R[a] NM_006218.2(*PIK3CA*):c.1030G>A p.V344M[a]	*FLT3; NOTCH1*
9	580104	495402 (85)	NM_000455.4(*STK11*):c.1062C>G p.F354L[a]	
10	976974	749289 (77)	NM_033360.2(*KRAS*):c.183A>T p.Q61H[a] NM_000546.5(*TP53*):c.892G>T p.E298*[a]	*FLT3*
11	553205	456865 (83)	***EGFR*: p.E745_A750Del15bp exon 19**	*CDKN2A*, *RET*
12	1154270	908330 (79)	**No variants detected**	None
13	588443	480470 (82)	*APC* p.E1306_E1309Del8bp Exon 16[a] NM_000222.2(*KIT*):c.1621A>C p.M541L[a] **NM_033360.2(*KRAS*):c.35G>T p.G12V** **NM_006218.2(*PIK3CA*):c.3062A>Gp.Y1021C** NM_000546.5(*TP53*):c.817C>T p.R273C[a]	*TP53*
14	1484662	1167524 (79)	**NM_002524.4(*NRAS*): c.182A>G p.Q61R** NM_000546.5(*TP53*): c.637C>T p.R213*[a]	
15	830528	649191 (78)	NM_000077.4(*CDKN2A*):c.181G>T p.E61*[a] NM_002524.4(*NRAS*):c.181C>A p.Q61K[a] NM_000546.5(*TP53*):c.833C>T p.P278L[a]	
16	863550	658317 (76)	No variants detected	*CDKN2A*

Variants detected by both conventional and next-generation sequencing (NGS) methods are in bold.
[a]Additional variants detected by NGS method for which conventional testing was not performed.

adequate DNA. We are unable to draw any conclusions about the minimum amount of FNA sample required.

Performance of NGS Testing Using Cytology Specimens

All 31 samples with adequate DNA underwent successful targeted sequencing of the 46 genes. The median number of filtered Q20 alignment reads for smears was 554 152 (range, 167 391–1 019 698) and for cell blocks was 475 299 (range, 418 175–1 167 524). All but one smear had an AQ20 read well over the minimum level of 300 000 for analysis. The median filtered AQ20 alignment read for formalin-fixed, paraffin-embedded biopsy or resection specimens that were tested on the same runs was 451 324. Thus, both cytological and surgical specimens showed comparable performance metrics. Importantly, neither the fixatives (methanol, ethanol or formalin) nor the stains (Diff-Quik, Papanicolaou stains) seemed to interfere with NGS-based analysis. No significant differences were noted in the depth of coverage achieved between the cytology specimens and biopsy/resection specimens from different patients analyzed on the same NGS run (data not shown). The summary of performance metrics of the specimens including failed amplicons are listed in Tables 2 and 3 and illustrated in Figure 2.

Mutation Results

The 740 hotspot mutations in 46 genes included multiple targets of oncogenesis, prognosis or therapy. All mutations detected by conventional sequencing platform were also detected by NGS. There was a 100% concordance between the results of conventional sequencing platforms and NGS. These results are summarized in Tables 2 and 3.

Mutational analysis of genes relevant to the tumor type was performed on every case by one of the conventional platforms. Among the 16 FNA smears, conventional sequencing platforms detected seven single-nucleotide variants (SNVs), including *BRAF* V600E; *KRAS* G12C, G12V and G13C; *PIK3CA* Y 1021C; and *NRAS* Q61R as well as two indels in the *EGFR* gene (15 bp deletions in exon 19). Among the cell blocks, conventional sequencing platforms detected seven SNVs, including *RET* M918T; *EGFR* L858Rand A750P; *MET* N375S; *TP53* R248W; and *BRAF* V600E as well as four indels in the *EGFR* gene (15 bp deletions in exon 19, 9 bp deletions in exon

Table 3 Performance metrics and detected variants of selected cell blocks prepared from fine needle aspirate samples in our study

Case no.	*Total reads*	*Filtered Q20 reads (% total)*	*Variants*	*Failed amplicons/ genes (<250× coverage)*
17	611521	456261 (75)	**NM_020975.4(*RET*):c.2753 T>C p.M918T**	
18	552230	424284 (77)	**NM_005228.3(*EGFR*):c.2573 T>G p.L858R** NM_000249.3(*MLH1*):c.1151 T>A p.V384D[a]	
19	681281	475299 (70)	***EGFR* p.K745_A750Del15bp Exon 19**	*CDKN2A*
20	615842	167391 (27)	**NM_005228.3(*EGFR*):c.2573T>G p.L858R** NM_000222.2(*KIT*):c.1621A>C p.M541L[a] NM_005359.5(*SMAD4*):c.1414C>A p.P472T[a]	*SMO*, *KIT*, *RET*, *RB1*, *APC*, *MPL*, *TP53*, *CDKN2A*
21	444366	355738 (80)	NM_000051.3(*ATM*):c.9007A>C p.N3003H[a] NM_000051.3(*ATM*):c.2572T>C p.F858L[a] NM_001904.3(*CTNNB1*):c.110C>T p.S37F[a]	*APC; KIT; RB1; TP53*
22	592282	473115 (80)	**NM_005228.3(*EGFR*):c.2239_2248delinsC p.L747fs*1** NM_005228.3(*EGFR*):c.2248G>C p.A750P[a] NM_002834.3(*PTPN11*):c.205_211delinsAGAA p.E69fs*2[a]	*FLT3; RET; KIT*
23	581392	469639 (81)	No variants detected	*NOTCH1; STK11*
24	732506	599459 (82)	**EGFR 9 bp deletion NM_005228.3(*EGFR*):c.2248G>C p.A750P** NM_002834.3(*PTPN11*):c.205_211delinsAGAAp.E69fs*2[a]	*FLT3*
25	1281471	1019698 (80)	**EGFR 9 bp deletion** NM_000546.5(*TP53*):c.711G>A p.M237I[a]	*FLT3*
26	724982	562360 (78)	NM_033360.2(*KRAS*):c.35G>T p.G12V[a]	*FLT3*
27	612845	487329 (80)	**No variants detected**	*APC*
28	681281	525820 (77)	NM_001904.3(*CTNNB1*):c.98C>A p.S33Y[a] **NM_001127500.1(*MET*):c.1124A>G p.N375S NM_000546.5(*TP53*):c.742C>T p.R248W**	*PTEN*, *RET*
29	457378	373344 (82)	NM_002524.4 *NRAS*: c.182A>T p.Q61L[a]	
30	765725	606727 (79)	**NM_004333.4(*BRAF*):c.1799 T>A p.V600E** NM_000222.2(*KIT*):c.1621A>C p.M541L[a]	
31	677538	546910 (81)	NM_000455.4(*STK11*):c.149T>G p.L50R[a] NM_000546.5(*TP53*):c.742C>G p.R248G[a]	None

Variants detected by both conventional and next-generation sequencing (NGS) methods are in bold.
[a]Additional variants detected by NGS method for which conventional testing was not performed.

19 and exon 20). All of these alterations were accurately detected by NGS platform. An example, each of SNV and indel, is shown in Figure 3 and Figure 4 respectively.

In 12 of the 16 (75%) FNA smears studied by NGS, in addition to the alterations detected by conventional sequencing platforms, an additional 19 SNVs and 1 indel were identified. The additional variants included *FLT3* A680V, *KDR* C482R, *TP53* R213L, *TP53* A159P, *TP53* W91*, *TP53* E298*, *TP53* Arg213*, *TP53* P278L, *FGFR2* N549K, *KDR* G1145R, *PIK3CA* V344M, *STK11* F354L, *KRAS* Q61H, *KRAS* G12V, *KIT* M541L, *CDKN2A* E61* and *NRAS* Q61K. In a single case, an 8-bp deletion in exon 16 of the *APC* gene (p.E1306_E1309Del8bp) was also detected. Similarly, in 11 of the 15 (73%) cell blocks studied by NGS, in addition to the alterations detected by conventional sequencing platforms, an additional 16 SNVs and 2 indels were detected. These additional variants included *MLH1* V384D, *SMAD4* P472T, *KIT* M541L, *CTNNB1* S37F, *ATM* N3003H, *ATM* F858L, *EGFR* A750P, *TP53* M237I, *KRAS* G12V, *CTNNB1* S33Y, *NRAS* G61L and *STK11* L50R. An indel in *PTPN11* was detected in two cases (NM_002834.3(*PTPN11*):c.205_211delinsAGAA p.E69fs*2).

We tested six lung adenocarcinoma specimens for indels in *EGFR* gene. All six cases showed deletion (variable from 9 bp to 15 bp) of *EGFR* in exons 19 and 20. Following NGS testing, all cases showed the presence of deletion accurately. In 2 of the 6 cases, deletions were not called by the variant caller. However, deletions were visualized on IgV viewer.

Discussion

We have presented data demonstrating that NGS-based testing for gene mutational profiling can be performed reliably using nanogram amounts of DNA obtained from FNA cytology smears and cell blocks. The results of this study thereby enhance the utility of FNA that can be used for routine diagnosis as well as mutation profiling to guide personalized cancer therapy.

Minimally invasive FNA is a commonly used procedure in the workup and diagnosis of solid tumors. FNA is a standard pre-operative diagnostic screening technique, for example, for the workup of patients with thyroid nodules.[4,5] In fact, the Bethesda standardized system of reporting thyroid lesions is specifically designed for FNA samples.[17–19] Cytological diagnostic procedures have been included in lung cancer staging guidelines based on the similar diagnostic efficacy compared with surgical excisional biopsies.[6,7]

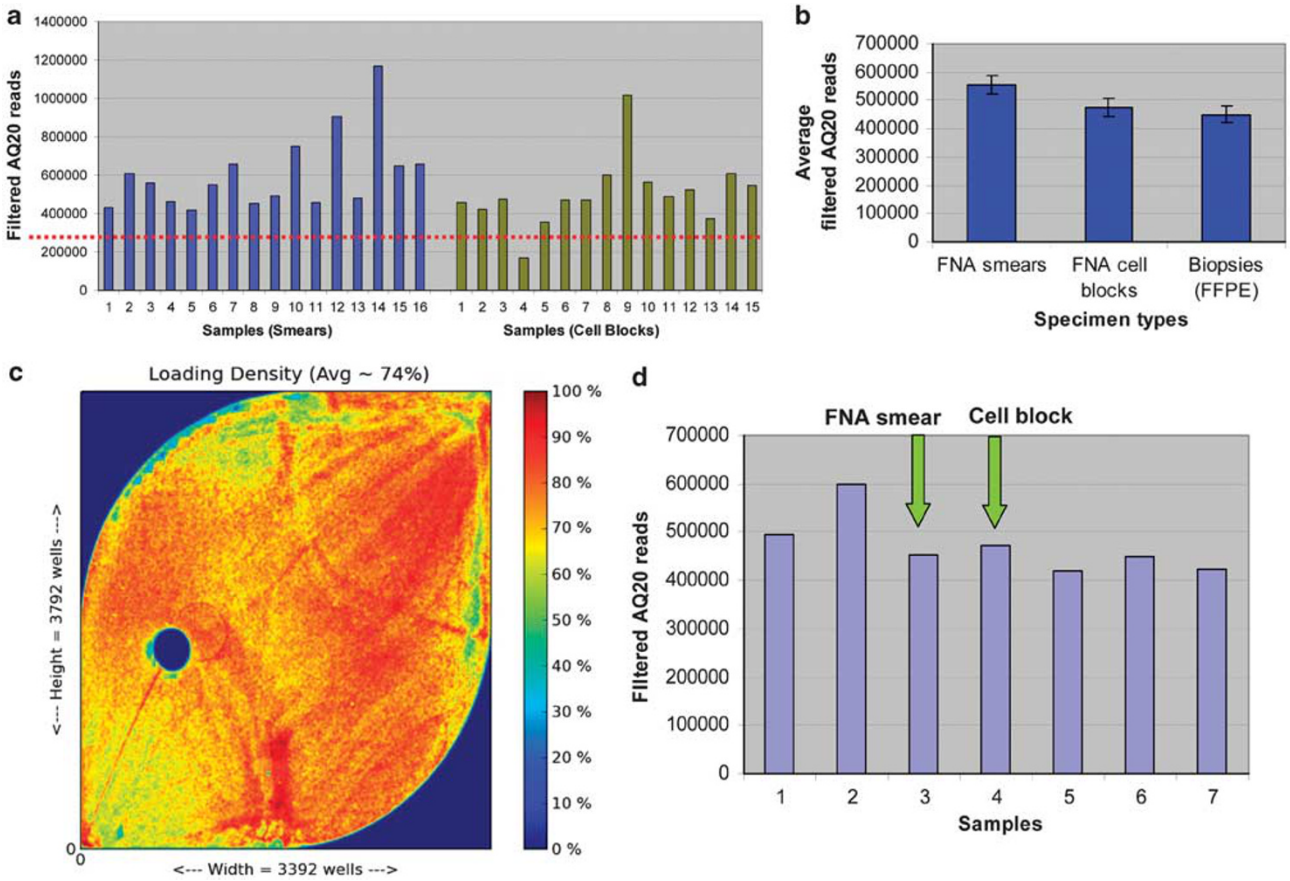

Figure 2 (**a**) Filtered AQ20 reads of the fine needle aspirate smears (blue, left) and cell blocks (green, right) used in our study. All samples (except case 20, cell block) demonstrated an AQ20 read of over the minimum set at 300 000. (**b**) Comparable average filtered AQ20 reads from fine needle aspirate (FNA) smears, cell blocks and FFPE material retrieved from excisional biopsies. (**c**) Representative heat map of wells, ISP, of a representative sample run that included five excisional biopsies (FFPE material), one FNA smear and one cell block; (**d**) Filtered AQ20 reads from the same run that included five excisional biopsies (FFPE material), one FNA smear (sample 3) and one cell block (sample 4).

Performing mutation analysis on FNA specimens is advantageous for several reasons. First, in situations where the diagnosis is not obvious, mutation data can be used to improve diagnostic accuracy, thus averting unnecessary surgery and associated complications.[20–22] For example, in thyroid lesions, the indeterminate categories under Bethesda standardized reporting, constitutes almost 30% of the cases.[17–20,23] Detection of *BRAF* mutation in such cases strongly favors a diagnosis of papillary thyroid carcinoma.[12,24–27] Second, the presence of mutation can help stratify patients into less aggressive or more aggressive groups.[12,25,28,29] Third, a cytological specimen may be the sole diagnostic material available for a patient's tumor. For example, only a third of the lung carcinomas are resectable at the time of initial presentation.[12] If the lung mass is large, locally advanced or metastatic, or is located in an inaccessible site, excisional biopsy or resection cannot be performed.[1,6] We also believe that FNA samples may be more representative of the tumor due to sampling of multiple areas of tumor by this technique compared with the unidirectional core needle biopsy. Thus, there is a critical need to develop and use efficient strategies and novel technologies for mutation testing of FNA samples.[6] Further, due to potentially clinically actionable implications carried with the presence of these mutations, proper validation studies are essential for the laboratory test development. This was acknowledged at a symposium on molecular diagnosis on tissues and cells, held in 2011.[30]

Several studies have assessed the suitability of cytological specimens, using fresh or fixed smears or cell blocks, for the detection of somatic mutations in several genes. The genes tested have included *EGFR* *KRAS*, *BRAF* and *PIK3CA* in the lung specimens and *BRAF*, *RET*, *RAS*, *TRK* and *PPR*γ in the thyroid nodules.[6,7,10,23,30–38] Due to the low analytical sensitivity of direct sequencing, ultrasensitive techniques such as high resolution melting, real-time PCR and pyrosequencing have been utilized to improve detection sensitivity.[7] However, assessment of multiple genes in cytological specimens is sometimes not possible, because traditional platforms require large amounts of DNA. NGS technology can

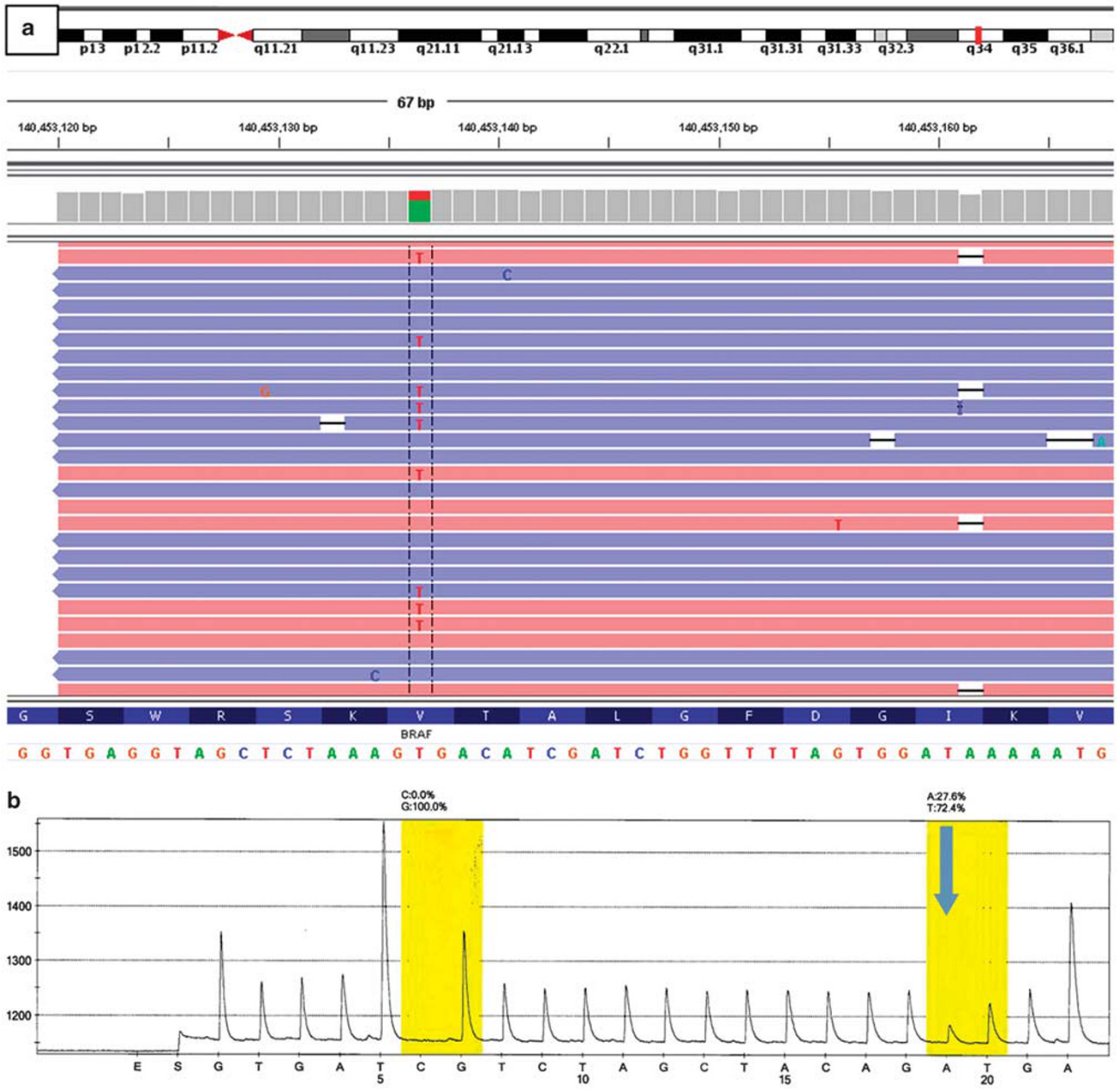

Figure 3 (**a**) Illustration of a mutation detected in FNA smear by the Ion Torrent next-generation sequencing platform as visualized in IGV (a case of papillary thyroid carcinoma, NM_004333.4(*BRAF*):c.1799 T>A p.V600E); (**b**) Same mutation identified by pyrosequencing.

be potentially used to overcome this problem. In our study, we used an Ion PGM NGS platform to detect variants in 46 genes in different types of cytological specimens. The amount of DNA required was markedly less compared with conventional platforms, and it allowed the assessment of multiple genes simultaneously using the same small quantity of DNA. The rationale for selection of the Ion PGM compared with other current NGS platforms such as Illumina Miseq (Illumina Inc., San Diego, CA) was mainly the differences in the DNA input. The DNA requirements for a 46-gene AmpliSeq panel on Ion PGM was markedly less (10 ng) compared with a 48-gene TruSeq panel on Illumina Miseq (250 ng). As most of our solid tumor specimens in our laboratory are FNA smears, FFPE cell blocks and core needle biopsies, we were unable to obtain a yield of 250 ng of DNA. Even by lowering the input to 10 ng for NGS testing by Ion PGM, only 31 of the 61 cytological samples had a sufficient DNA concentration by Qubit assessment for the 46 gene mutation panel analysis.

All samples that had sufficient DNA were successfully sequenced by NGS. Thus, adequate DNA concentration appears to be the only prerequisite for successful sequencing. To enhance the DNA yield, in all of our cases DNA extraction was performed following selection of tumor-rich areas.

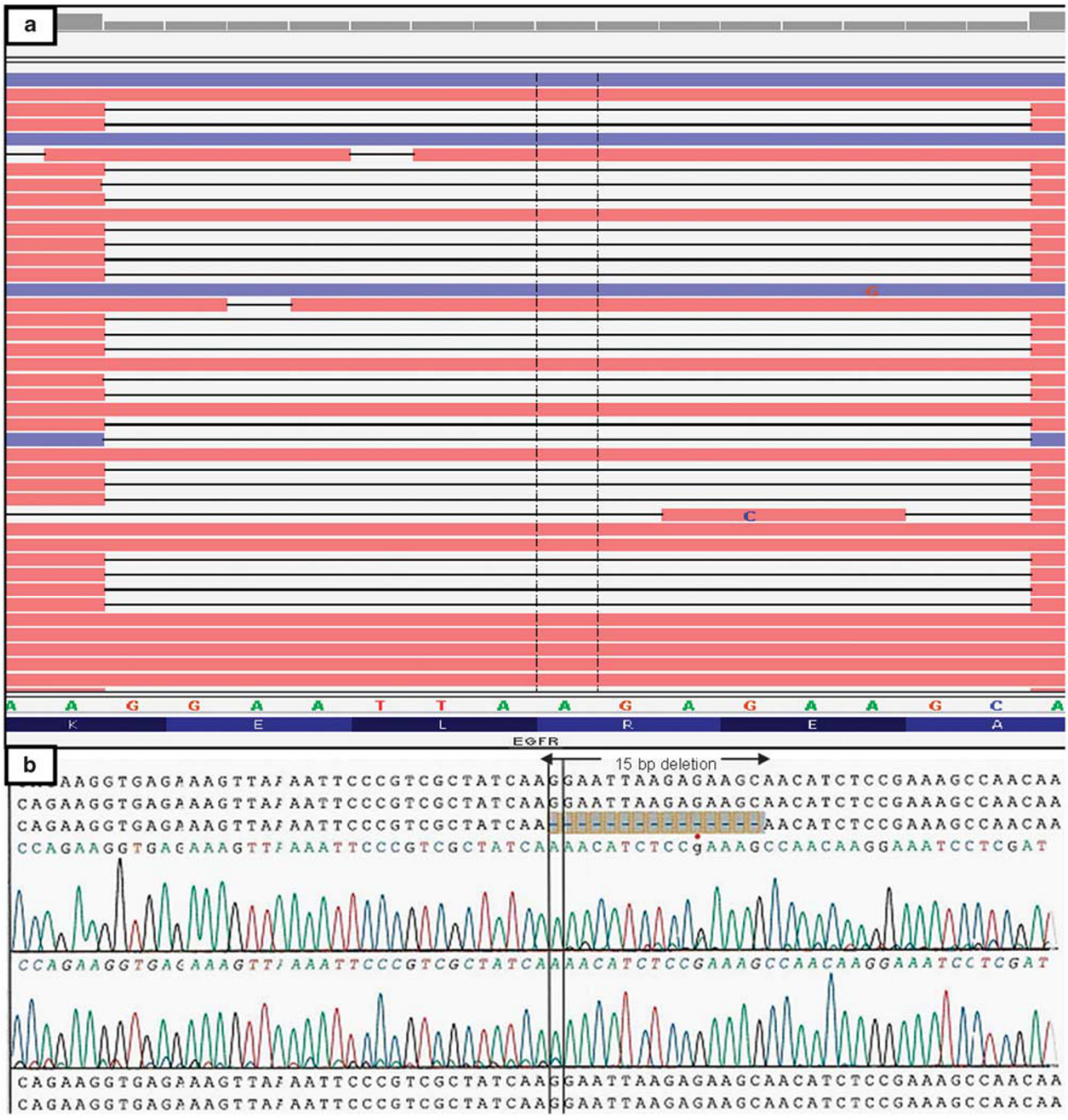

Figure 4 (**a**) Illustration of a 15-bp deletion in *EGFR* gene detected by Ion Torrent next-generation sequencing platform as visualized in IGV (a case of lung adenocarcinoma). (**b**) Same mutation identified by Sanger sequencing.

We also preselected cases with >20% tumor only, where DNA was extracted by manual scraping. In our lab, we routinely perform laser capture microdissection (LCM) on samples with <20% tumor. The NGS-based testing is not yet validated on the LCM samples.

There was no difference in the median tumor percentages between the groups of 31 cytological specimens that yielded sufficient DNA compared with 30 cases that yielded with insufficient DNA. One potential explanation is that we did not measure the tumor surface area on the slides for each of the cases. Second, for our NGS testing, we require at least 10 ng of DNA in 12 μl of nuclease-free water. Majority of our study group included left-over DNA samples from clinical specimens that had undergone either retrospective or concurrent testing by $\geq$1 of the conventional testing platforms. Thereby, following DNA extraction, DNA was eluted in sufficient volume of nuclease-free water for utilization for several clinical tests. Thus, for certain samples, in spite of availability of sufficient total

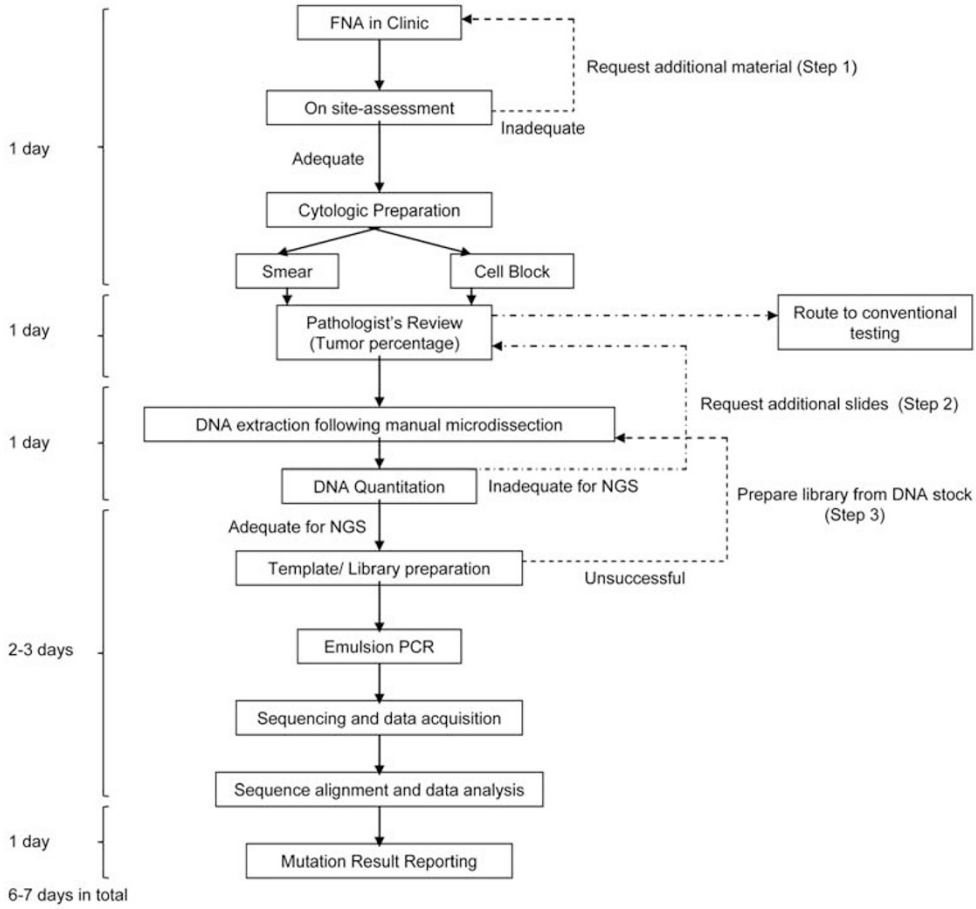

Figure 5 Illustration of optimized workflow of cytological specimens for next-generation sequencing technique in our clinical laboratory.

DNA, the concentration of DNA precluded NGS testing. If these cytological specimens had been extracted solely for NGS purposes, concentration may have been modified to achieve a total of 10 ng DNA to accommodate NGS-based testing.

Our study included stained smears as well as unstained cell block sections. Both types of specimens underwent successful sequencing by NGS, and neither of them showed better performance compared with each other. The success we had analyzing archival, stained cytological smears in this study is particularly convenient. Of the 16 FNA smears, we had included 3 methanol-fixed Diff-Quik-stained smears in addition to the 13 ethanol-fixed Papanicolaou-stained smears. The type of cytological stain and fixative did not appear to influence the performance of this test. This experience is in contrast to earlier studies in which DNA extracted from May-Grunwald–Giemsa-stained cytological smears seemed to be of superior quality compared with DNA extracted from Papanicolaou-stained slides.[38] With the cell blocks, similar to the surgical biopsy specimens, we did not observe any sequence artifacts associated with formalin fixation, such as transitional mutations.[39,40] Further, DNA from all the samples extracted from cell blocks underwent successful PCR amplification for sequencing.

In this study, there was 100% concordance between the mutations detected by conventional sequencing platforms and NGS. However, additional variants were identified by NGS in approximately 60% of the cases, with far less DNA required for analysis. The advantages of NGS testing seem clear. One can argue that the clinical significance of some of these additional gene variants is unclear, but long-term follow-up will likely lead to a better understanding. For example, due to broader amplicon coverage, the p.Met541Leu variant in exon 10 of *KIT* gene was frequently identified by NGS, whereas Sanger sequencing in our laboratory has been designed only for exons 9, 11, 13 and 17. The pMet541Leu variant has been described as a germline polymorphism in normal individuals.[41] However, there have been conflicting results in another study that showed significantly increased frequency of this variant in patients with chronic myelogenous leukemia compared with healthy volunteers.[42] Thus, in the long run, NGS results may help determine the clinical significance of variants that currently are poorly understood.

A large number of samples of lung adenocarcinomas were included in our study. FNA of lung masses is a common diagnostic modality at our institution. Critical to the mutational analysis of lung tumors is

the detection of point mutations as well as deletions and insertions in the *EGFR* gene. Detection of indels in NGS data is known to be difficult. In this study, all six cytological specimens from lung adenocarcinomas with known deletions in *EGFR* were identified. In two cases, deletions were not called by the Torrent Variant Caller. However, deletions could be visualized on the mapped reads in IGV, and in such cases, alternative computational approaches and indel detection softwares may be of help.[43] The number of base pairs deleted also correlated with the Sanger data. Thus, lung cytological specimens are suitable for mutation detection by NGS.

We only used fixed cytological specimens for NGS in this study. Fresh specimens contain unaltered DNA, free from fixation and processing, and theoretically will yield better results. However, it is not possible to accurately quantify tumor percentage or separate tumor cells from contaminating non-tumor cells using fresh samples. Therefore, possible non-representation of tumor makes it impossible to predict the outcome of mutational results using fresh tissue. Ulivi *et al*[7] showed that fixed stained cytological specimens are more reliable for molecular analysis than fresh specimens. In their study, 2 of the 36 cases in which DNA was obtained from fresh cytological specimens showed false-negative mutation results for *EGFR* and *KRAS* testing. The false-negative results were attributed to lack of accurate cancer cell selection and quantification.

A limitation of our study includes the absence of a parallel evaluation of matched cytological and concurrent surgical specimens from the same patient. There were only three cases with concurrent (within 3 months of FNA) cytological and excisional biopsy or surgical resection specimens that were sent to the laboratory for mutational assessment. Two of these samples analyzed by conventional platforms did not show any mutations in the tested genes, which was consistent with FNA findings. One case did not yield any PCR product. All three cases had limited amount of DNA left over for NGS analysis. Our study also looked at the most frequent tumors that undergo cytological evaluation. Extending the study to other types of malignancies that undergo FNA would further validate the methods used.

We used our experience to propose an optimized workflow for cytology specimens for NGS testing in our laboratory (Figure 5). The most challenging part was that only about 50% of the cases qualified for testing by NGS. We were unable to determine any particular characteristic of the specimens that yielded the desired amount of DNA. As our study is retrospective, we presume that pre-dilution of DNA for other testing may have been a confounding factor. For this reason, we suggest that on-site adequacy assessment of FNA specimens should be made for both morphological analysis and molecular studies. Further, as smears were as good as cell blocks for NGS testing, additional FNA passes performed specifically for molecular testing may contribute to greater success (Figure 5, step 1). If extracted DNA is of inadequate quality (Figure 5, step 2), requesting additional smear slides to enhance the DNA concentration may be attempted, before routing to a conventional testing platform. In any case, one of the critical steps included tumor enrichment by pathologist's review of the slides. Manual microdissection of tumor-rich areas significantly enhanced DNA yield in cytological specimens where tumor cells are closely intermixed with contaminating non-malignant cells. Following DNA extraction, if the library preparation is unsuccessful, the process can be repeated from the DNA stock (Figure 5, step 3). Due to high false-positive rate and generation of clinically actionable results by this test, we used the following criteria to establish the validity of any variant call. Minimum criteria included uniformly high coverage with a minimum of 300 000 AQ20 alignment reads, mutation frequency of 5–10%, with presence of SNVs in both forward and reverse strands. All variant calls were confirmed by an alternate platform unless the SNV had been well established in our patient cohort based on validation studies on other samples (data not shown). With optimization, NGS testing seems very promising in a clinical laboratory setting on cytology specimens. After streamlining the appropriate steps in the workflow, the turnaround time for reporting molecular profiling results from the time of FNA approaches approximately 1 week for timely patient care.

In summary, this is the first study to demonstrate the feasibility of using NGS-based methods to perform gene mutation analysis in routine cytological specimens obtained by FNA, including stained smears and cell blocks. The implications of this study are broad. Mutational profiling of multiple genes is possible using an extremely low quantity of DNA (nanograms), which can be extracted from specimens obtained by FNA, thereby enhancing the utility of the FNA approach. We believe that NGS-based gene mutation analysis is a valuable addition to the workup of FNA specimens obtained from patients with various tumors.

Disclosure/conflict of interest

The authors declare no conflict of interest.

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