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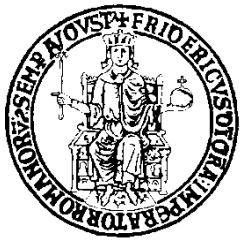

DIPARTIMENTO DI SANITA' PUBBLICA

DOTTORATO DI RICERCA IN SANITA' PUBBLICA E MEDICINA PREVENTIVA - XXIX CICLO

"Integration of Next-generation sequencing technologies in pathological diagnostics"

Relatore:
Ch.mo Prof. Giancarlo Troncone

Coordinatore:
Ch.mo Prof. Stefania Montagnani

Candidata:
Dott. Elena Vigliar

Anno Accademico 2015 – 2016

Contents

Chapter 1 - Introduction 3

1.1 Illumina technology 4

1.2 Ion Torrent technology 4

1.3 Data analysis 6

Chapter 2 - Next-generation sequencing analysis of colon carcinomas 7

2.1 - Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients 8

2.1.1 Materials and methods 8

2.1.2 Results 10

2.1.3 Discussion 12

2.2 - Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of 653 routine cases 14

2.2.1 Materials and methods 14

2.2.2 Results 14

2.2.3 Discussion 17

Chapter 3 - Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective 20

3.1 - Can the Ion AmpliSeq Cancer Hotspot Panel v2 Be Used for Next-Generation Sequencing of Thyroid FNA Samples? 26

3.2.1 Materials and methods 27
3.2.2 Results 28
3.2.3 Discussion 32

3.2 - Next-generation sequencing and cytopathology in basic science 35
3.2.1 Cytological samples of patient-derived xenografts
3.2.2 NGS 35
36

Bibliography 39

Chapter 1

Introduction

Today, with the better understanding of the molecular events involved in malignancy and the mechanisms of pharmacotherapy, larger gene panels are more helpful than single biomarker detection. After the completion of the first human genome sequence in 2004[1], the growing need to sequence a large number of individual genomes in a fast, low-cost and accurate way has directed a shift from traditional Sanger sequencing methods towards new high-throughput genomic technologies.[2] In 2005, the development of next generation sequencing (NGS) methods has represented one of the more significant technical advances in molecular biology.[3] NGS, also known as massive parallel sequencing because of the ability to allow the parallel analysis of a very large number of DNA molecules, is beginning to show its full potential for diagnostic and therapeutic applications. Until recently, NGS platforms were envisioned for large-scale applications, focused on whole genome sequencing, with protocols, consumable costs and a turnaround time (TAT) unsuitable for the needs of small diagnostic laboratories. The development of miniaturised technology by benchtop NGS sequencers decreased sequencing costs, moving NGS from a few large sequencing core centers to a much larger number of individual laboratories.[4] Currently, most pathology departments acquired an NGS benchtop sequencer,[5] thus NGS is adopted for routine molecular diagnostics, including cytological samples. NGS may increase both analytical sensitivity and breadth of examined genomic regions, enabling the simultaneous detection of multiple mutations in multiple genes by the parallel sequencing of millions of different DNA fragments.[6] tremendously increases the throughput, making it feasible to sequence entire human

genomes in days.[7] Each nucleotide is read several times, ensuring a high degree of sensitivity.

To understand the current and future application of NGS in the field of pathology, modern pathologists need to understand its basic principles. Regardless of the specific features of any single platform type, the NGS workflow follows four sequential phases, as follows: (1) the generation of a short fragment DNA library; (2) single fragment clonal amplification; (3) massive parallel sequencing and (4) sequencing data analysis.

1.1 Illumina technology

The Illumina platforms perform clonal amplification and massive parallel sequencing on instrument without manual intervention. The DNA fragments clonally amplified, as seen before on a flow cell, are bound by complementary primers and extended in subsequent cycles of sequencing by synthesis reactions employing reversible dye terminators. All four nucleotides are provided in each cycle but not in a specific order because each nucleotide carries an identifying fluorescent label; thus, Illumina performs well in homopolymeric regions and the error rate, predominantly substitution errors, is only 0.5%.17 A fundamental advantage of the Illumina system is that it is very efficient in mapping the reads to the corresponding regions in the reference genome; this is thanks to the so-called paired-end sequencing, namely producing sequence data from both ends of each library fragment.[8] The Illumina benchtop sequencer Miseq has a run time as short as 8 h from the preparation of DNA for variant detection moreover, it can run numerous samples from different patients simultaneously by tagging the samples with a barcode so that they can be identified.[8]

1.2 Ion Torrent technology

Ion Torrent sequencing exploits emulsion PCR, to establish clonal templated beads from isolated single DNA library molecules, also generating a read for each single well.[9] However, while the image detection system was the bottleneck of the 454 GS JuniorTM and the reason of its limited scalability, Ion Torrent is much more scalable, thanks to the

digital electronic Ion Chip.[9] This is a specialised silicon chip similar to those of microprocessors, digital cameras and cell phones. The Ion Chip is the heart of the PGMTM, a relatively inexpensive, simple, computer-like instrument, which performs all the signal processing needed to produce base calls.[9] Individual nucleotides are provided to the open wells by flowing them over the Ion chip. However, in this instrument, the nucleotide flow is in a systematic order because there is no label to provide base-specific identity. In fact, upon each nucleotide incorporation, the Ion Chip detects the pH changes owing to the release of a hydrogen ion (H+) within any individual well. In the case of a homopolymeric template sequence, the multiple incorporations of the same base will proportionately result in more H+ releases, which may results in errors, especially when the sequence features insertions or deletions.[4] Conversely, substitution errors may occur at a very low frequency (i.e. = 0.5–1 in 100 bases). As billions of digital chips per year are collectively produced by the fabrication facilities (fabs), the application of digital electronics technology to sequencing make this technology relatively inexpensive and fast, and hence ideal for smaller laboratories that wish to use next-generation sequencing in their work.[10] The mass production of the Ion Chips also ensures a high level of scale according to the classical Moore's Law that has governed the semiconductor industry, which states that the number of transistors per chip will double every 2 years.[10] As a matter of fact, the number of sensors/wells of the Ion Chip, that sets the maximum number of reads producible per run, increased dramatically from 1.2 to 11.3 million from the earlier 314 to the later 318 chips.[10] The additional increase in average read length from 100 to 200 bp led to marked improvements in net-sequencing throughput. The very fast turn-around time of the PGM sequencing platform (3 h/run) perfectly matches with the rapid modality of multiplex PCR to generate a library.[11] To these ends, the Ion Torrent AmpliSeq(TM) gene panels, such as the 22 gene target panel called the Ion AmpliSeq Colon and Lung Cancer Panel[6] and the Cancer Mutation Hotspot Panel (46 genes)[12] enables in a cost-effective manner the implementation of the NGS in predictive molecular diagnostics, limiting the sample requirements to only 10 ng of DNA.

1.3 Data analysis

To generate interpretable results, the millions of produced reads need to be aligned to a reference human genome, which requires sufficient time and practice with a number of available informatics tools.[13] First, single reads are processed through filters that eliminate low-quality sequences and then mapped to the reference sequence; variants are called when differences occur between a base call and its aligned position to the reference sequence. The number of reads covering a given base position is described as the depth of coverage.[13] The average depth of coverage is the average number of overlapping reads within the total sequenced area. The uniformity of coverage is the distribution of coverage within specific targeted regions in which variant calling will occur. The combination of informatics tools used for processing, aligning and detecting variants in NGS data is usually termed as the bioinformatics pipelines. Different software programs, such as commercially available analysis packages or in-house developed analytical pipelines, can cause variability in the reported sequence of a given sample.[13] Thus, the appropriate software settings should be optimised during validation to ensure that a variant called by the bioinformatics pipeline is present in the sequence. After validation, quality maintenance is challenging, as informatics is rapidly evolving, and software updates are frequent; however, if only the informatics pipeline is altered, it may be sufficient to re-analyse the original validations raw data (FASTQ files).[5]

This thesis describes my research on the integration of NGS technologies in pathological diagnostics, both concerning histological and cytological specimens. Moreover, a research application of NGS on mouse xenograft cytological samples is described.

Chapter 2

Next-generation sequencing analysis of colon carcinomas

Antiepidermal growth factor receptor (EGFR) therapy is not effective in patients with metastatic colorectal cancer (CRC) harbouring mutations at codons 12 and 13 in KRAS exon 2.[1] More recent evidences showed that the so-called expanded RAS mutations (exon 3 and exon 4 of KRAS and exons 2, 3 and 4 of NRAS) also have negative predictive value.[2] The extension of community KRAS testing to all RAS mutations favoured the implementation of multitarget testing methodologies.

NGS, matched with multiplex capture of targeted gene regions and analysed by bioinformatics tools, enables the simultaneous detection of multiple mutations in multiple targetable genes. NGS may also identify rarer patient-specific somatic mutations. The latter are of unclear significance, as their incidence rates have not been established with certainty. In fact, while there is a wealth of data regarding RAS/RAF/PI3KA and TP53 gene mutations, the information on less frequently mutated genes is mostly derived by the genomic scale analysis of a limited number of CRC samples.[3]

Among the most popular NGS benchtop platforms is the Ion Torrent Personal Genome Machine (PGM; Life Technologies, Carlsbad, California, USA) which requires only a small amount of gDNA (10 ng).[4] This NGS platform has employed very broad panels containing around 50 cancer driver gene targets[5–8] and a 22 gene target panel called the Ion AmpliSeq Colon and Lung Cancer Panel. The performance of this panel has previously been evaluated for clinical cancer testing by other groups retrospectively on archival [9] and clinical trial specimens.[10]

A first study was conducted to verify the AmpliSeq Colon and Lung Cancer assay in the different setting of routine diagnostics; a subsequent study focuses on less frequently mutated genes potentially relevant for prognostic assessments or for actionable treatments.

2.1 Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients

Prospectively, a large number of consecutive and unselected samples were simultaneously processed by Sanger sequencing and by the AmpliSeq Colon and Lung Cancer assay. Diagnostic accuracy, cost, time around testing (TAT) and the overall practicality of the two methodologies were evaluated. The aim was to assess whether the AmpliSeq Colon and Lung Cancer Panel on the Ion Torrent PGM could be an alternative to Sanger sequencing for genotyping KRAS, NRAS and BRAF genes in our laboratory.

2.1.1 MATERIALS AND METHODS

Routine clinical samples

Following approval from the institutional review board, the relative performance of the Ion Torrent assay and of Sanger sequencing on prospectively collected and processed routine diagnostic samples was assessed. Routine samples were simultaneously processed by our current technology, based on Sanger sequencing, and by Ion Torrent PGM sequencing. To this end, 114 unselected consecutive (including 99 surgical samples and 15 endoscopic biopsies) FFPE samples, referred from 18 different institutions, were prospectively processed by both methods. Only one single tumour sample from a given location (primary tumour n=105; metastases n=9) was tested for each patient. After obtaining the patient's consent, oncologists and the primary pathologists from outside institutions record the clinical and pathological data (including the original pathology report) on a dedicated website. Then, the corresponding tissue sample is express-mailed to our central laboratory. Upon receipt of each sample, a representative H&E stained slide is reviewed by a pathologist and the area with the highest density of neoplastic cells is marked, annotating the percentage of neoplastic cells. Depending on the complexity of histology and on the density of the tumour, DNA was extracted using the QIAamp DNA Mini Kit (Qiagen, Crawley, West Sussex, UK) from two (resection specimens) or three (biopsy specimens) 10 µm-thick serial sections. An additional section (biopsy specimens only) was stained by H&E to confirm tumour cell percentage.

Sanger sequencing

DNA targets for exons 2, 3 and 4 of KRAS and NRAS, and exon 15 of BRAF were amplified using laboratory developed primer pair.[18,19] The concentration of reagents was optimised using 80 ng of DNA, 0.4 mM of each primer and 0.5 U of 5 PRIME Taq DNA Polymerase (Eppendorf, Milan, Italy) in a total volume of 25 µL. PCR conditions were as follows: initial denaturation for 5 min at 95°C, cyclic denaturation at 94°C for 30 s, annealing at 57°C for 30 s, elongation at 65°C for 30 s for 35 cycles, and final extension at 72°C for 2 min. Following PCR, the fragments were purified using the QIAQuick DNA purification kit (Qiagen) according to the manufacturer's instructions. Sequencing reactions were performed for both DNA strands by the Big Dye Terminator V.1.1 (Applied Biosystems, Monza, Italy) on a total of 10 ng of purified PCR products. Dye purification was carried out by alcohol/sodium acetate precipitation. Sequence analysis was performed on an Applied Biosystems 310 genetic analyser. The files obtained were aligned to the reference sequence and examined for mutations by the CodonCode software.

Ion Torrent sequencing

DNA was extracted from clinical tissue samples using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. DNA was suspended in 30 µL of molecular biology water. DNA quantity and quality were assessed using the Qubit photometer (Life Technologies) and the Qubit dsDNA HS (High Sensitivity) Assay Kit according to the manufacturer's instructions. According to the manufacturer's protocols, 10 ng of DNA for each sample was used for library preparation with the Ion AmpliSeq Library 96LV Kit 2.0 (Life Technologies) and the Colon and Lung Cancer Panel (Life Technologies). This panel gives 90 amplicons covering 504 mutational hotspot regions in 22 genes (AKT1, ALK, BRAF, CTNNB1, DDR2, EGFR, ERBB2, ERBB4, FBXW7, FGFR1, FGFR2, FGFR3, KRAS, MAP2K1, MET, NOTCH1, NRAS, PIK3CA, PTEN, SMAD4, STK11, TP53), with performance of at least 500× sequence coverage for eight samples on one Ion 316 chip. For samples yielding less than 10 ng DNA input, additional cycling conditions were used for library preparation as recommended by the manufacturer. Each library was barcoded with the Ion Xpress Barcode Adapters 1–16 Kit (Life Technologies). Barcoded libraries were combined to a final concentration of 100 pM. Template preparation by emulsion

PCR (emPCR) was performed on the Ion OneTouch 2 system (Life Technologies). Library quality control was performed using the Ion Sphere Quality Control Kit according to the manufacturer's instructions, ensuring that 10–30% of template positive Ion Sphere particles (ISP) were targeted in the emPCR reaction. Sequencing primer and polymerase were added to the final enriched ISPs prior to loading onto 316 (100 Mb output) chips. Sequencing was carried out on the PGM (Life Technologies). Data analysis was carried out with Torrent Suite Software V.3.2 (Life Technologies), considering only KRAS, NRAS and BRAF, while all other genes were masked. After alignment to the hg19 human reference genome, the Variant Caller plug-in was applied using the Colon and Lung hotspot file as a reference (downloaded from Ion Community, http://www.ioncommunity.lifetechnologies.com, last accessed 15 September 2014). The Ion Reporter suite (Life Technologies) was used to filter polymorphic variants. In addition, all nucleotide variations with less than a 5% variant frequency were masked. All detected variants were manually reviewed with the Integrative Genomics Viewer (IGV V.2.1, Broad Institute, Cambridge, Massachusetts, USA).

Evaluation of TAT and consumable costs in order to evaluate the overall practicality of performing Ion Torrent sequencing in our referral centre, TAT and consumable costs were taken into account. The TAT (the period from sample receipt to interpretation of the results) was recorded for every sample for both Sanger and Ion Torrent sequencing. The first step of our routine testing algorithm is evaluation of KRAS exon 2, where most resistance mutations are clustered. Only KRAS exon 2 wild-type cases undergo direct sequencing of PCR products of the remaining RAS and BRAF exons. For Sanger sequencing, consumable cost was evaluated for a single exon analysis and multiplied for the number of reactions needed for any given gene. For Ion Torrent sequencing, each patient analysis cost was estimated considering the fact that eight barcoded samples were loaded for each 316 chip.

2.1.2 RESULTS

Ion Torrent sequencing in routine settings

Most of the routine samples (109/114; 95.6%) processed on the PGM yielded an adequate library for subsequent sequencing, although library preparation failed in five

cases. Three of the failed cases did not yield adequate results by Sanger sequencing either. In most of the adequate cases (85/109), amplification for library generation was carried out without major technical problems; in a minority of cases (24/109), the low level of library concentration (<100 pM) required DNA reamplification.

Mutations detected by Ion Torrent with at least a 5% variant frequency were annotated (figure 1). Ten BRAF mutant cases (V600E, n=7; G596R, n=1; K601E, n=1; D594G, n=1) were detected by both Sanger and Ion Torrent sequencing. In addition, two BRAF mutations (G466E, n=1; G469A, n=1) not covered by our Sanger sequencing-based assay were only detected by Ion Torrent. A total of 38 KRAS mutations (exon 2, n=32; exon 3, n=2; exon 4, n=4) were detected by both techniques (figure 1). One KRAS Q22K mutation detected by Ion Torrent at a 5.5% mutant allele level was missed by Sanger sequencing (figure 1). Eight cases harbouring NRAS mutations (exon 2, n=2; exon 3, n=6) were detected by both sequencing methods. One G13R mutation with a 5.2% mutant allele frequency was only detected by Ion Torrent.

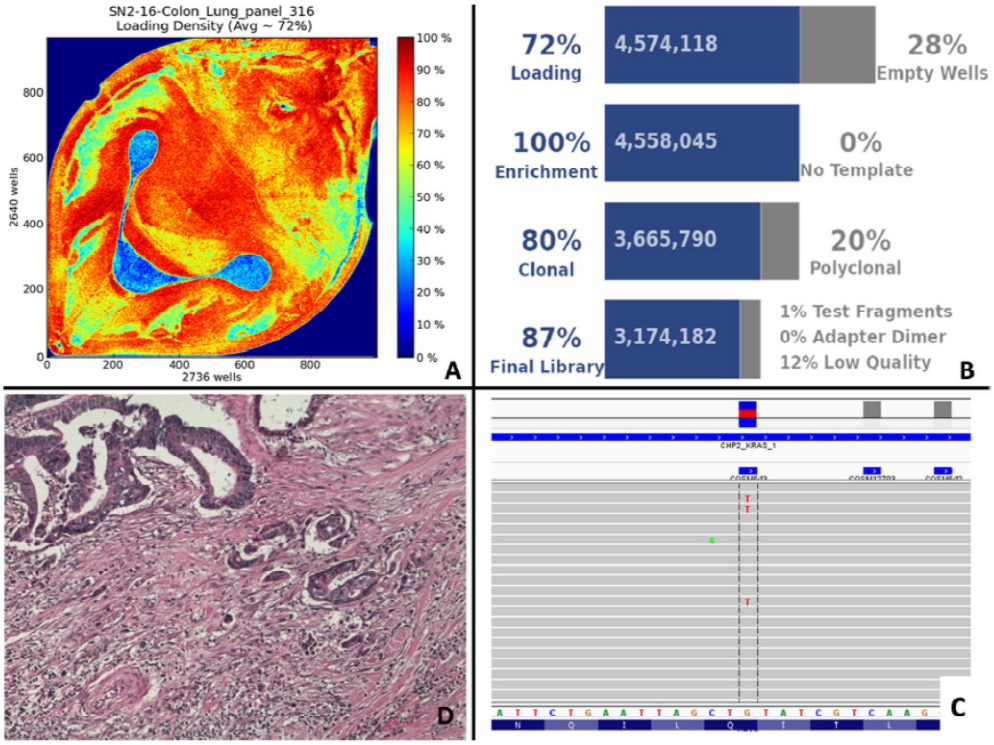

Figure 1 Loading density (A) and performance parameters (B) of an Ion Torrent sequencing run, carried out using a 316 chip are shown. A low frequency of a KRAS Q22K mutant allele (C) was observed with an integrated genetics viewer in a case of colorectal cancer (D) with 30% neoplastic cells.

Evaluation of TAT and consumable costs

The Ion Torrent mean TAT was 13.0 working days (range 7–14). The mean TAT for Sanger sequencing evaluation of KRAS exon 2, was 4.2 working days (range 3–6); in the cases also evaluated for the remaining KRAS, NRAS and BRAF exons, the entire process had a mean TATof 10.4 working days. The cost of consumables for any single exon analysis by Sanger sequencing was €28. Consequently, the consumable cost of testing KRAS, NRAS and BRAF, including seven exons, was €196. For Ion Torrent sequencing, as eight barcoded samples were loaded for each 316 chip, the cost for each patient analysis was €187.23. This amount was slightly higher (€262.20) in a minority of cases (24/109) where the low level of library concentration required DNA re-amplification. Similarly, initialization failures, occurring twice for a total of 16 samples, led to an increase of €7.80 per sample.

2.1.3 DISCUSSION

Ion Torrent NGS assays have been retrospectively evaluated on previously characterised positive and negative archival control samples.[10,13,14,20] However, in this study, routine clinical samples have been prospectively received by our central laboratory from several local pathology laboratories. Sanger sequencing and Ion Torrent NGS were performed simultaneously, unlike in previous reports where these techniques were performed at different times in different laboratories and on different histological sections.[17,21] In the diagnostic setting, challenges include the less than optimal DNA quality of some samples due to formalin over-fixation, the low tumour cell content in tumour tissues with abundant inflammatory cells, and insufficient starting material, for example, minimal biopsy fragments. The Ion AmpliSeq Colon and Lung Cancer Panel failed in a small minority of cases (4.4%), in contrast to the 100% success rate of a recent clinical trial whose design included preliminary sample selection.[17] All of the 56 point mutations detected by Sanger sequencing were also correctly identified by Ion Torrent NGS, confirming the high level of specificity of the Ion Ampliseq Colon and Lung Cancer Panel.[16] In addition, the NGS assay detected two BRAF mutations in gene regions not covered by Sanger sequencing. The differential sensitivity of methods can differ; the NGS technique is able to detect mutations with low variant frequencies.[17] To avoid false positive results,

the 5% variant frequency threshold is generally recommended for AmpliSeq.[13,14] In our series, two mutations (KRAS Q22K and NRAS G13R) with variant frequency just above the 5% threshold were missed by Sanger sequencing. These discordant results have a number of technical and clinical implications. From a technical point of view, a laboratory that adopts NGS in clinical practice should consider having an in-house validated single gene assay with at least 5% sensitivity to confirm mutations occurring at a low level, in particular for the most clinically relevant hotspots. In this study, however, one of the discordant mutations occurred in codon 22, which would also be missed by high-sensitive assays targeted at codons 12 and 13.[22] The occurrence of less common mutations is expected to increase, as referral laboratories adopt NGS as a screening tool.

Our data may show that NGS assays are not overly time consuming and expensive. We found that the Ion Torrent mean TAT for all clinically relevant analysis was only slightly longer than for Sanger sequencing (13.0 vs 10.4 working days), reflecting the long learning curve and more hands-on technical time required for library preparation, chip loading and data analysis. In addition, the NGS assay TAT will continue to improve with the implementation of a fully integrated robotic station. Consequently, more efficient sample batching will improve the cost effectiveness of the whole procedure. To date, our data have shown that the consumable cost for testing KRAS, NRAS and BRAF using Ion Torrent sequencing (€187.23) is comparable to that for Sanger sequencing (€196) and much cheaper than the total for individual FDA/CE IVD-approved single-gene tests. However, while the most expensive tests are the easiest to interpret, NGS data analysis requires more expertise than usually available in academic institutions or in large clinical hospitals.[24]

2.2 Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of 653 routine cases

The incidence of RAS/RAF/PI3KA and TP53 gene mutations in colorectal cancer (CRC) is well established.[1-3] Less information, however, is available on other components of the CRC genomic landscape, which are potential CRC prognostic/predictive markers. Following the previous validation study[4], NGS was employed in our laboratory to process 653 routine CRC samples by Ion AmpliSeq Colon and Lung Cancer Panel, generating a large database whose interrogation can be useful to better define the incidence rate of rare mutations. Thus, besides KRAS, NRAS, BRAF, PIK3CA and TP53 alterations, this study focuses on mutations occurring in other receptor tyrosine kinase (RTK) genes (ALK, EGFR, ERBB2, ERBB4, FGFR1, FGFR2, FGFR3, MET, DDR2), in RTK signaling genes (AKT1, PTEN, MAP2K1, STK11) and in other well-known cancer-related genes (NOTCH1, CTNNB1, SMAD4, FBXW7).

2.2.1 MATERIALS AND METHODS

This study includes a series of 653 CRC tissue samples (398 men and 255 women) referred from 18 institutions located all over South Italy between January 2014 and March 2015. Mean patient age was 66.8 years (range, 29–96 years). Following current international guidelines, one single tumour sample was tested for each patient.

2.2.2 RESULTS

One or more gene mutations were detected in 499/653 (76.4%) tumours in 17 of the 22 genes included in the panel (table 1), for a total of 796 mutations. A representative case is reported in figure 1. Only three genes (DDR2, FGFR1 and FGFR2) did not harbour any alteration, while two genes (FGFR3 and MET) only harboured germline. Single mutations were found in 274 patients (41.9%), double mutations in 177 patients (27.1%) and 3 or more mutations were found in 48 patients (7.4%). Mutations occurred in TP53 (n=240; 38.8%), KRAS (n=247; 37.8%), NRAS (n=30; 4.6%) and BRAF (n=63; 9.6%). KRAS and NRAS mutations were mutually exclusive.

Table 1 Twenty-two multiple gene mutation analysis by the Ion Torrent AmpliSeq Colon and Lung Cancer Panel in routine samples of colorectal cancer

Total cases analysed	n=653
Wild type in all 22 gene analysed	n=154 (23.6%)
Mutated at ≥1 of 22 genes analysed	n=499 (76.4%)
Total mutations	n=796
Mutated genes	17/22

KRAS and NRAS coexisted with BRAF mutations in four and in one instances, respectively. In most of these cases (4/5), BRAF mutations occurred outside of codon 600. PIK3CA gene mutations occurred in 98 (15%) cases. More frequently, PIK3CA mutations were detected together with other gene mutations; PIK3CA was the only mutated gene in 15/98 (15.3%) samples. Number and percentage of mutated cases of each gene are reported in table 2. Besides RAS/RAF/PI3KA and TP53 gene mutations, the Ion AmpliSeq Colon and Lung Cancer Panel provided information on additional targets, such as RTK genes, RTK signalling genes and other well-known cancer-related genes, as it follows.

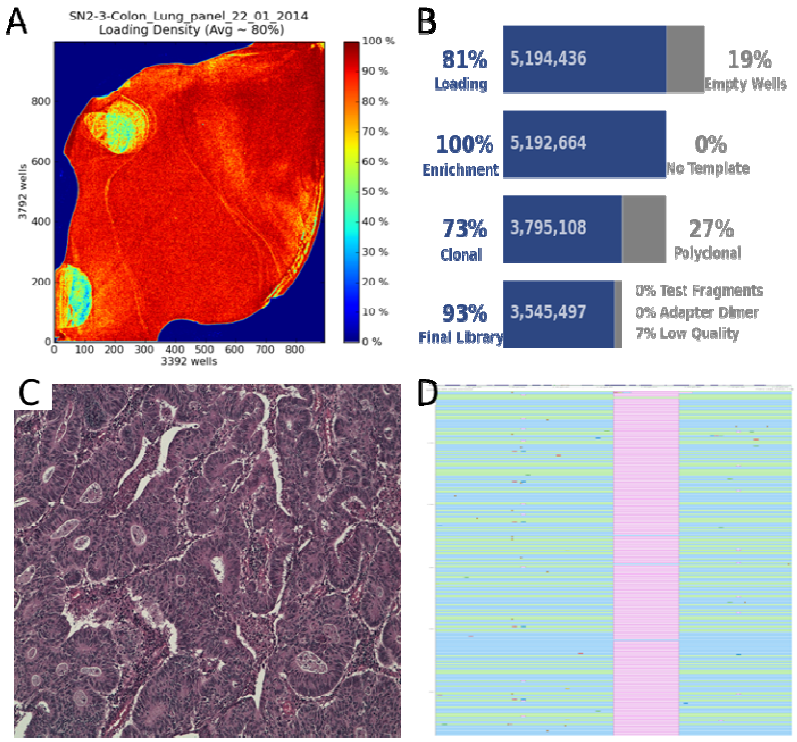

Figure 1 Loading density (A) and performance parameters (B) of an Ion Torrent sequencing run, carried out using a 316 chip, are shown. DNA extracted from the colorectal cancer (CRC) shown in (C) harboured an epidermal growth factor receptor p.E746_A750delELREA mutation. (D) was observed with a Genome Brower web app.

Table 2 Number and percentage of cases of each gene sequenced by the Ion Torrent AmpliSeq Colon and Lung Cancer Panel

Gene	Number of mutated cases (%)
KRAS	247* (37.8%)
TP53	240† (36.8%)
PIK3CA	98‡ (15%)
BRAF	63 (9.6%)
FBXW7	39 (6%)
NRAS	30 (4.6%)
PTEN	18 (2.8%)
SMAD4	14 (2.1%)
EGFR	8 (1.2%)
CTNNB1	7 (1.1%)
AKT1	6 (0.9%)
STK11	5 (0.8%)
ERBB4	4 (0.6%)
ERBB2	4 (0.6%)
NOTCH1	1 (0.2%)
ALK	1 (0.2%)
MAP2K1	1 (0.2%)

Note: *DDR2*, *FGFR1*, *FGFR2*, *FGFR3* and *MET* genes did not harbour any alteration.
*4/247 cases harboured 2 *KRAS* mutations.
†5/240 cases harboured 2 *TP53* mutations.
‡1/98 cases harboured 2 *PIK3CA* mutations.

RTK gene mutations

ALK: in one case (0.2%) the p.L1196M mutation was detected in association with two mutations of the TP53 gene. EGFR: mutations occurred in eight (1.2%) cases, with exon 19 deletion evident in four instances (n=3 p.E746_E749delELRE; n=1 p.E746_A750delELREA, as shown in figure 1). Most cases (7/8) were associated with other gene alterations; in particular, five cases harboured a KRAS mutation. ERBB2: mutations occurred in four (0.6%) cases, with the V842I being detected in three instances. ERBB4: mutations occurred in four cases (0.6%).

RTK signalling genes mutations

AKT1: the E17K mutation occurred in six cases (0.9%). PTEN: mutations occurred in 18 (2.8%) cases. MAP2K1: in one case (0.2%) the K57N mutation was associated with PIK3CA mutation. STK11: mutations occurred in five cases (0.8%).

Other cancer-related genes

NOTCH1: mutation occurred in one case (0.2%) and remarkably this case had five additional gene mutations occurring in TP53, KRAS, PTEN, ERBB4 and PIK3CA. CTNNB1: mutations were detected in seven cases (1.1%), being always associated with at least one

other concurrent mutation. In particular, CTNNB1 mutations were consistently associated with the constitutive activation of the RAF/MEK/ERK pathway by either KRAS (n=4) or BRAF (n=3) concurrent mutations. SMAD4: mutations were found in 14/653 (2.1%) samples, and in combination with other mutations (9/14). FBXW7: mutations were identified in 39/653 patients (6%), singly (n=7) and associated with KRAS (n=20).

2.2.3 DISCUSSION

This study evaluated in CRC routine samples a broad set of genes for mutational events. Previous evidences regarding the RAS/RAF/PI3KA gene were confirmed. KRAS and NRAS mutations were always mutually exclusive,[5] whereas occasionally BRAF (mostly no V600E) mutations coexisted with an RAS gene alteration.[7] The frequent association of PIK3CA mutations with the RAS/RAF alterations was also confirmed.[5] Our data straighten the view that the simple distinction of tumours in RAS, BRAF or PIK3CA does not apply to CRC with combined RAS/RAF genetic changes.[7] We also confirmed that one of the most frequently mutated genes in CRC is TP53, whose mutation rate in our study was 38.8%.
Additional information was generated on other potentially actionable components of the CRC genomic landscape, such as RTK genes. Remarkably, the ALK p.L1196M gatekeeper mutation, which confers high-level resistance to crizotinib in lung cancer, was for the first time detected in CRC. EGFR mutations were also detected, as shown in figure 1, and their mutation rate (1.2%) was lower than that (4.5%) reported in the Tumor Cancer Genome Atlas (TCGA).[5] While KRAS and EGFR mutations are normally exclusive, concomitant KRAS and EGFR mutations were also detected, confirming previous NGS findings.[8] Other mutations include those involving ERBB2; in particular, the V842I ERBB2 mutation associated with breast cancer[9] was detected in three instances. Remarkably, in CRC preclinical models HER2 mutations were resistant to cetuximab and panitumumab and responsive to second-generation HER2/EGFR irreversible tyrosine, afatinib and neratinib.[10] Clinical trials targeting HER2 activating mutations in metastatic CRC are ongoing.[11] ERBB4 mutations occurring in 0.6% of the cases have an uncertain prognostic significance. In fact, the TCGA data set indicated a survival disadvantage in colorectal carcinoma with ERBB4,[5,12] whereas another study showed that the ERBB4 mutant clones are not selected in metastatic spread.[13] A number of rare mutations occurring in the PI3K/AKT/ mTOR pathway are potentially actionable. As an example, AKT1 mutations

were associated with primary resistance to anti-EGFR therapy.[14] In our study, AKT1 was mutated in 0.9% of cases, being mutually exclusive with PIK3CA alterations, as previously shown.[14] The recent association between E17K AKT1 and tumours with mucinous morphology was observed only in one of our six cases.[14] Previous studies showed a wide range of PTEN mutation rates (0.7%15 to 6%16). In our study, the mutation rate of PTEN was 2.8%. Interestingly, a total of 11 different mutations were found, according to the notion that mutations in tumour suppressor genes do not strongly cluster in single mutational hot spot.[17] Another RTK signalling gene included in our panel is the STK11 gene. We confirm that somatic STK11 mutations rarely occur in somatic CRC (0.8%).[18] Earlier studies reported that STK11 mutant neoplasms had alterations in nucleotide metabolism that confer hypersensitivity to deoxythymidylate kinase inhibition, proposing that deoxythymidylate kinase is a possible therapeutic target.[19] Interestingly, CTNNB1 mutations detected in 1.1% of the cases were always associated with at least one other concurrent mutation. In particular, CTNNB1 mutations were consistently associated with the constitutive activation of the RAF/MEK/ERK pathway by either KRAS (n=4) or BRAF (n=3) concurrent mutations, in keeping with the notion that CTNNB1 mutations are early events in CRC carcinogenesis.[20] Conversely, our data confirm that the occurrence of SMAD4 mutations (2.1%) is a late event.[21] In fact, in our study 64.3% of SMAD4 mutations occurred in combination with other alterations. SMAD4 loss of function was associated with a worse prognosis and decreased disease-free survival and with resistance to 5-fluorouracil chemotherapy.[22,23] In this present study, FBXW7, a major tumour suppressor gene crucial in promoting exit from the cell cycle, was mutated in 6% of cases, which is in line with the estimated 9% of CRCs containing FBXW7 mutations.[24,25] Preclinical data have suggested that inactivating mutations of FBXW7 could predict sensitivity either to the mTOR inhibitor rapamycin,[26] or to the histone deacetylase inhibitor MS-275.27 Noteworthy, as it was shown in previous reports FBXW7 were often (51.2%) associated with KRAS mutations.[28,29] Interestingly, concurrent molecular aberrations can contribute to limited therapeutic efficacy of mTOR inhibitors in the presence of FBXW7 mutations.

Certain genes included in our panel, such as MAP2K1, may have a future role in sensitivity, resistance or both, to a variety of preclinical drugs. Targeting of NOTCH signalling may be of therapeutic value in colon cancers, as activating mutations in NOTCH-1 have been previously reported in colon cancer.[30] In our study NOTCH mutation occurred

in one case (0.2%) and remarkably this case had five additional gene mutations occurring in TP53, KRAS, PTEN, ERBB4 and PIK3CA.

In conclusion, our data confirm that CRCs consist of a group of heterogeneous disorders with a large number of diverse sets of genetic changes in oncogenes and tumour suppressor genes. In a routine diagnostic setting, the Ion PGM and AmpliSeq colon and Lung Cancer Panel had the potential to exploit even a low-input DNA to uncover multiple common mutations simultaneously and to generate robust and comprehensive genetic information. Several updates of the Ion Torrent system may soon enable to detect also gene copy number alterations and translocations to more comprehensively cover the whole spectrum of genomic alterations refining the identification of reliable and reproducible biomarkers of response/resistance to the targeted treatment of CRC.

Chapter 3

Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective

Fine needle aspiration (FNA) samples are being increasingly exploited for the prediction of treatment response, in particular in advanced-stage cancer patients before the initiation of systemic treatment.[1-6,8] In 2009, Clark reviewing the current utilization of FNA-based molecular tests to inform targeted cancer therapy decisions, emphasized that FNAs were underutilized for such testing.[7] Today, with the development of more sensitive molecular techniques together with the increased awareness of cytopathologists to meet oncologists' requests has led to a widespread utilization of cytological samples to test a number of different oncogenes, including KIT, PDGFR, BRAF, EGFR, KRAS, NRAS, PIK3CA and others for somatic mutations, and to the recognition of this practice in standardized international biomarker testing guidelines.[1]

As the talent to do more with less is a special gift in cytopathology, in which diagnostic material is so often limited, NGS may offer cytopathologists the significant opportunity to fully exploit the material that can be provided by cytological tumour sampling. [9-24,33,34] Thus, genomics, having conventional and NGS as its central technologies, effectively interacts with modern cytopathology.[8]

However, NGS is a new tool that should be implemented cautiously, especially on cytology.[24] The potential of NGS to detect a very large number of possible alterations, i.e. the Cancer Mutation Hotspot Panel covers more than 2000 COSMIC mutations, makes it impossible to validate any single variant;[15] thus, a careful validation strategy should focus on the main genetic alterations of diagnostic interest, evaluating indicators of test performance such as sensitivity specificity and, precision in an end-to-end setting on routine samples.[23] As pointed out by Salto-Tellez et al.,[15] the validation strategy for single

mutation assays, aiming for a 99% statistical sensitivity (with a 95% confidence interval) would require testing at least 100 specimens (or greater than 300 variants) for a NGS panel analysing a 20-kb region. It is difficult, however, to provide a sizable collection of cytological specimens, homogeneous for the source, type, fixation, staining and tumour cell enrichment modalities, with a known mutational status for clinically relevant genes.[5] Commercial formalin-fixed, paraffin-embedded (FFPE) multiplex reference standards can represent a solution, at least when validating NGS assays on cell blocks.[26]

Establishing the minimum number of cells needed to allow a multigene massively parallel testing approach from cytology smears is also a crucial point.[23] The studies that applied NGS to cytological material (Table 3) had usually a retrospective design and only samples that featured at least 20% of neoplastic cells are usually selected, which may not fully reflect current practice. In any case, sample requirement depends on target capture and platform types. Illumina NGS required 15,000 cells[25] when following hybridation capture or 5000 cells[27] when preceded by PCR-based capture whereas, Ion Torrent NGS needed between 100 and 1000 cells.[24,28]

Table 1 Characteristics of findings in the literature for the application of next generation sequencing (NGS) to cytological specimens

Author (ref)	No. of cytological samples	Sample preparations	Site	NGS Platform	Panel	Study aims	Cellularity assessmnet	Inadequate rate
Buttitta *et al.*[37]	48	PAP SM	Lung	454 GS Junior	Single gene	Predicitive	ME	0
de Biase *et al.*[36]	66	SM	Lung	454 GS Junior	Single gene	Predicitive	ME (100 NC)	0
de Biase *et al.*[4]	60	PAP SM DQ SM NR	Pancreas	454 GS Junior	Single gene	Testing validation	ME (100 NC)	0
Karnes *et al.*[29]	5	PAP SM DQ SM	Lung	Hi-seq	Custom panel (27 genes)	Testing validation	NR	0
Young *et al.*[25]	39	CB	Pancreas Lung	Hi-seq	Custom panel (287 genes)	Testing validation	ME (20% NC on at least 15,000 C)	0
Gleeson *et al.*[28]	32	PAP SM DQ SM	Stomach	MiSeq	Cancer Hotspot Panel v2 (50 genes)	Testing validation	ME (20% NC on at least 1000 C)	0
Hadd *et al.*[45]	10	FC	Thyroid	GAIIx and PGM	Custom panel (35 genes)	Testing validation	NA	0
Kanagal-Shamanna *et al.*[30]	61	PAP DQ SM/CB	Thyroid, lung, melanoma	PGM	Cancer Hotspot panel v1 (46 genes)	Testing validation	ME (20% NC)	49%
Scarpa *et al.*[24]	38	PAP SM DQ SM NR	Lung	PGM	Colon and Lung cancer panel v1	Testing validation	ME (100 NC)	5%
Nikiforova *et al.*[34]	52	FC	Thyroid	PGM	Custom panel (13 genes)	Testing validation	NA	2%
Le Mercier *et al.*[33]	34	CB SM	Thyroid	PGM	Cancer Hotspot panel v2	Diagnostic	NR	0
Nikiforov *et al.*[44]	143	FC	Thyroid	PGM	Custom panel (13 genes)	Diagnostic	NA	0
Gailey *et al.*[46]	77	PAP DQ SM/TP/LBC	Lung, thyroid, colon, urothelium, nos	PGM	Cancer Hotspot panel v2 (50 genes)	Testing validation	ME (30% NC on at least 400C)	0
Gleeson *et al.*[27]	56	PAP DQ SM/TP	Adrenal gland	PGM	Cancer Hotspot panel v2 (50 genes)	Testing validation	ME (20% NC on at least 300C)	50%
Kubota *et al.*[39]	35	PAP SM DQ SM	Pancreas	PGM	Single gene	Diagnostic	NR	0

PAP, Papanicolaou stain; SM, smears; DQ, Diff Quik stain; CB, cell-blocks; FC, fresh cells; TP, Touch prep; LBC, liquid-based cytology; PGM, personal genome machine; ME, microscopic evaluation; NC, neoplastic cells; C, cells; NR, not reported; NA, not applicable.

As far as DNA input is concerned, Illumina NGS required from 50[25] to 170[29] ng, after hybridisation capture or 30 ng[27] downstream of multiplex PCR. Conversely, Ion Torrent sequencing of PCR products only needs 10 ng of DNA and precisely 12 µl of diluted DNA at a concentration of 0.8 ng/µl.[18,24,30]

Depth of coverage, average and uniformity of coverage should also be determined during the validation process. A high depth of coverage is especially important when the DNA input and the percentage of malignant cells are scant.[31] For a variant call, a minimum coverage of 500X with at least a 10% mutant allele frequency is generally used as cutoff for a variant to be considered true.[30] To examine the relationship between the percentage of amplicons adequately covered (500 reads) and cytological sample cellularity, we analysed using the Ion AmpliSeq Colon and Lung Cancer Panel 24 archival cytological samples (a single smear for patient), that had previously been sent to our referral laboratory for EGFR mutational testing. As reported in Figure 1, we observed that samples with less than 25% of neoplastic cells, mostly contributed (57%) to the group of cases with poorly covered amplicons and were less often associated (25%) with successfully amplicon-covered cases (Figure 2).

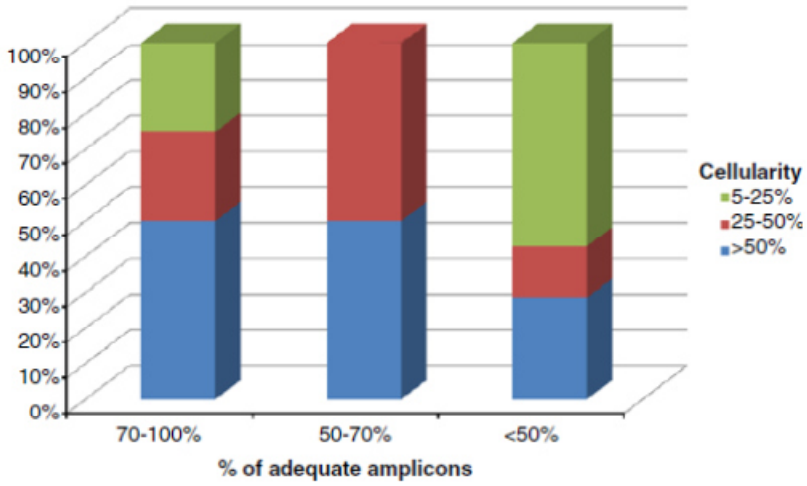

Figure 1. Relationship between the percentage of amplicons adequately covered (500 reads) and cytological sample (n = 24) cellularity (5–25%; 25–50%; greater than 50%) is shown. Overall, 14 (58%) cases showed less than 50% of adequate covered amplicons; two (8%) samples showed between 50% and 70% adequately covered amplicons and eight (33%) samples showed greater than 70% of covered amplicons. Samples with less than 25% of neoplastic cells, mostly contributed (57%) to the group of cases with poorly covered amplicons and were less often associated (25%) with successfully amplicon-covered cases.

Thus, as neoplastic cell-rich FNA procurement is the first and crucial step,[3] on-site adequacy at the time of the procedure should be made for both morphological analysis

and molecular studies.[27,30] Interestingly, several studies showed that Diff-Quik_ smears are as good as cell blocks and Papanicolaou for NGS testing without significant differences in the total number of reads, the percentage of reads mapping to the target region, or the coverage of target regions in the gene set.[29,30]

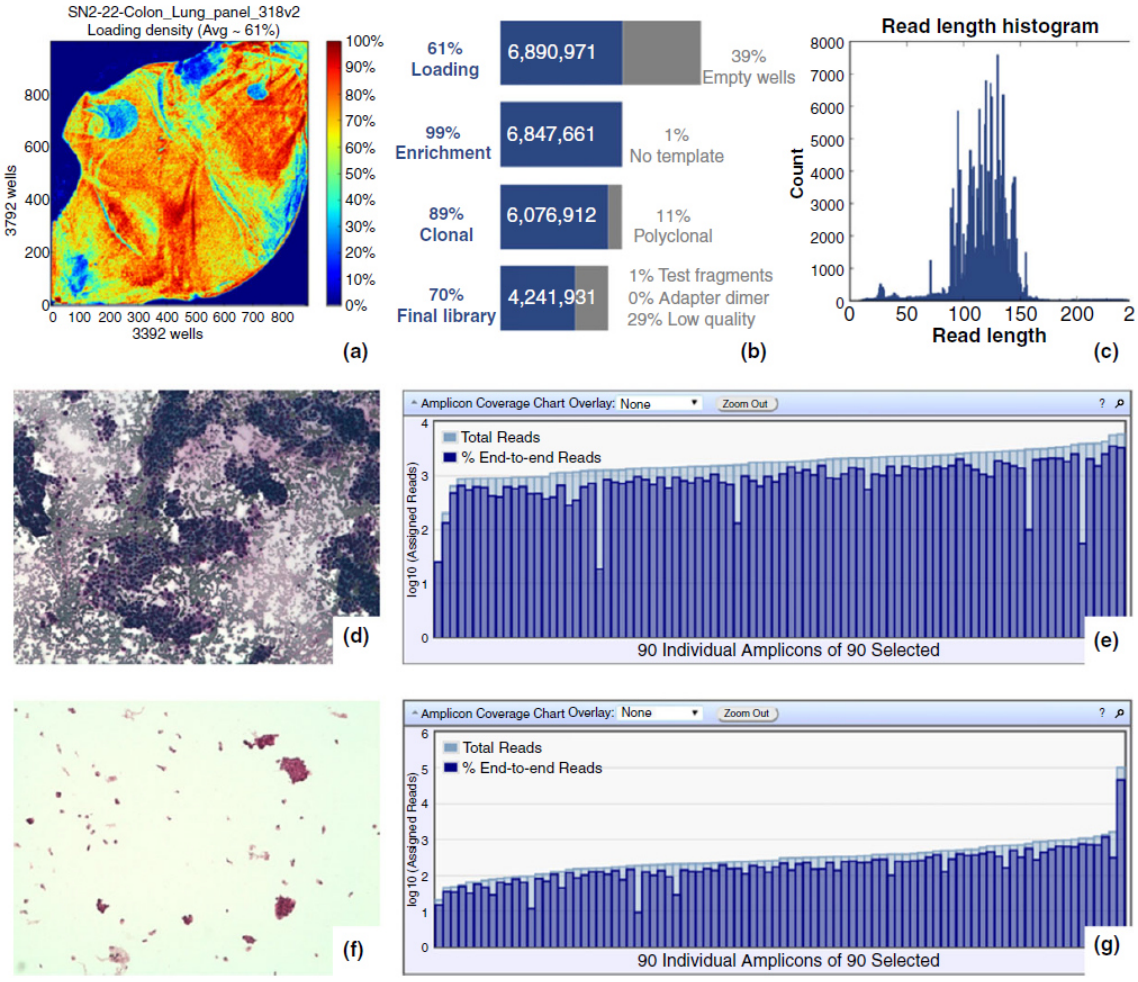

Figure 2. Representative examples of next-generation sequencing (NGS) performance (a–c) by the Ampliseq Colon Lung Cancer Panel on highly (d–e) and poorly (f–g) cellular lung cancer fine needle aspirations (FNAs). Efficient run parameters are shown by the Ion Torrent 316 chip high loading density (a) run metrics (b) and reads length of total cytological smears DNA barcoded libraries (c). The analysis of highly cellular smears (d) generated adequate (500 reads) coverage (e) for any of the 90 amplicons of the Colon Lung Cancer Panel. Conversely, a poorly cellular smear (f) is associated with insufficient amplicons coverage (g).

NGS to increase assay sensitivity

As it was also shown on small histological biopsies,[38] NGS increased sensitivity to detect EGFR[36,37] and KRAS[4] mutations on paucicellular cytological samples, eluding direct sequencing false negative results and avoiding cumbersome microdissection.[4,36,39] On bronchoalveolar lavages (BAL) and pleural fluids, Buttitta et al.[37] correctly classified as a

mutant, 81% of specimens using NGS compared with only 16% of cases detected by Sanger sequencing. Similarly, in the multicentre study by de Biase et al.,[36] the 454 NGS assays detected EGFR mutations on paucicellular lung cancer smears, that had been missed by Sanger sequencing.[4] In the same way, on endoscopic ultrasound (EUS) guided pancreatic FNAs, 454 NGS had a higher sensitivity (73.6%) than Sanger sequencing (42.1%) to detect exons 2 and 3 KRAS mutations.[4] It is noteworthy that in 20% of pancreatic cyst samples, despite the absence of diagnostic cells on the matched cytological specimens, a KRAS mutation was found by NGS.[4] Similarly, Buttitta et al.[37] using their highly sensitive assay were able to detect the presence of EGFR mutations even in five cytological samples, which had previously been deemed negative for neoplastic cells by morphological examination. In these cases, the presence of mutant alleles was limited only to 0.3–3.2% of the DNA molecules. The relevance of the preliminary validation of the threshold to reliably call a mutation cannot be overemphasised,[23] while the clinical relevance of a true positive lowlevel mutation in terms of its real significance needs further investigation.[40]

NGS multigene testing to refine uncertain diagnoses

The use of DNA mutational testing on thyroid cytological samples is a rapidly expanding practice currently useful to stratify further into high- and low-risk categories the indeterminate cytology classes identified by the Bethesda System.[56]

Although molecular profiling of cells sampled by FNA is the principal field of application of NGS to diagnostic cytology, new directions are also envisioned for exfoliative cytology. In particular, NGS can be applied to a standard liquid-based Pap smear to test for oncogenic somatic mutations (APC, AKT1, BRAF, CTNNB1, EGFR, FBXW7, KRAS, NRAS, PIK3CA, PPP2R1A, PTEN and TP53) as a tool to detect early gynaecological malignancies, such as endometrial and ovarian cancer.[47]

NGS multigene testing to inform targeted therapy

Driven by regulatory agencies' requirements and professional society's international guidelines, patient selection for targeted treatments is evolving to include a larger number of biomarkers aimed to personalize regimens even further. As an example, recently, the European Medicine Agency required, prior to anti-EGFR administration, to

test colon cancer patient for codons 12 and 13 in exon 2, codons 59 and 61 in exon 3, and codons 117 and 146 in exon 4 for both KRAS and NRAS.*12* Similarly, lung cancer international guidelines extended EGFR testing also to exons 18 and 20.[48]

Whereas FNA samples may be the only pathologic material obtained from advanced-stage cancer patients available for targeted analysis before the initiation of systemic treatment, the evaluation of multiple biomarkers by NGS becomes mandatory.

NGS in the cytological follow-up of targeted treatment

In patients with a progressive neoplastic disease, targeted treatment is often based on the genotyping analysis of the archived primary tumour. This, however, does not necessarily reflect the patient's current disease, that may feature the emergence of treatment-resistant subclones that might already be present at a minor frequency in the primary neoplasm. NGS can define the tumour 'molecular phenotype', discriminating whether mutations are present in the same population of cells or whether there is the existence of a dominant mutated neoplastic cell clone and a smaller neoplastic cell subsets carrying additional mutations.[53] Considering only EGFR, de Biase et al.[36] and Marchetti et al.[54] showed the presence of a different subpopulation of cells harbouring different EGFR alterations. Scarpa et al.[24] showed on a lung FNA that a major driver mutation (EGFR deletion in 73% of cells) coexisted with other variants displaying lower allele frequencies (TP53 in the 16% and MAP2K1 in 4% of cells). On indeterminate thyroid FNAs, Nikiforov et al. identified low level of KRAS, NRAS and TSHR gene mutations suggesting intra-tumour heterogeneity.[44] A close follow-up with repeat thyroid FNA and molecular testing to monitor for the expansion of the mutated clone within the nodule may be relevant.[44] Similarly, serial characterization of metastatic lesions through fine needle biopsy could be used to identify clonal evolution and to eradicate treatment-emergent clones. Compared with unidirectional core needle biopsy, FNA samples may be more representative of the tumour owing to sampling of multiple areas.

Two studies were conducted to investigate the usefulness of Ion AmpliSeq Cancer Hotspot Panel v2 for NGS of thyroid FNA samples in clinical application and of mouse xenograft FNA samples in research application.

3.1 Can the Ion AmpliSeq Cancer Hotspot Panel v2 be used for Next-generation sequencing of thyroid FNA samples?

Fine-needle aspiration (FNA) is the most accurate and cost-effective tool with which to evaluate thyroid nodules. The Bethesda 6-tiered reporting system has greatly improved the standardization of this diagnosis.[1] However, the malignancy risk rates associated with the diagnostic classes that fall within the "indeterminate" categories (namely atypia of undetermined significance/follicular lesion of undetermined significance, follicular neoplasm/suspicious for follicular neoplasm, and suspicious for malignancy) significantly differ among institutions,[2–4] thereby leading to ambiguity regarding the correct management of these patients. Molecular techniques may help to improve the diagnostic accuracy of indeterminate samples and thereby avoid the overtreatment of patients with benign nodules and increase the presurgical detection of malignancies.[5]

The integrated genomic characterization of papillary thyroid cancer has reduced the percentage of thyroid cancers with unknown oncogenic mutations to 3.5%.[6] In particular, it is now clear that the vast majority of thyroid cancers are either BRAF V600E-driven or RAS (NRAS, HRAS, or KRAS)-driven tumors. BRAF V600E-driven cancers are frequently papillary carcinomas of the classic type. Conversely, RAS-driven thyroid tumors are follicular patterned lesions (eg, follicular variants of papillary carcinoma, follicular carcinomas, and a subset of follicular adenomas). The RET gene usually is mutated in sporadic and familial medullary thyroid carcinoma.[6] Thus, a test that is able to assess the mutational status of the BRAF, RAS, and RET genes on FNA could improve the preoperative risk assessment of thyroid nodules.[7] In this context, the Ion Torrent PGM was reported to be suitable for the analysis of cytology specimens.[8]

To our knowledge to date, the only NGS panel customized on PGM for the molecular diagnosis of thyroid cytology is ThyroSeq (CBLPath, Rye Brook, NY).[9–11] However, this test is proprietary; it is centralized in the Division of Molecular and Genomic Pathology at the University of Pittsburgh Medical Center.[12] Thus, the widespread implementation of ThyroSeq is not practical. As an alternative, several groups have used the Ion AmpliSeq Cancer Hotspot Panel v2 (CHPv2; Thermo Fisher Scientific Inc), a commercially available primer pool for sequencing generic cancer genes, including the BRAF, NRAS, HRAS, KRAS,

and RET genes.[13–16] However, this approach is reported to have had a limited success rate. In fact, the requirement for at least 10 ng of DNA precluded the analysis of up to 49% of routine samples.[13–15] Moreover, not all samples with adequate DNA produce a sufficient number of high-quality sequences and reportable results.[13–15] In fact, as well as a low amount of DNA, the poor quality of the DNA also may preclude the success of NGS.[17]

In an attempt to increase the number of cases evaluable by CHPv2, we investigated whether it can be used for the NGS of "suboptimal" specimens, namely those containing a low amount of DNA and/or poor-quality DNA, and assessed the reliability of the results obtained with sequencing metrics that were less than optimal. To this end, we processed a retrospective series of thyroid FNA samples regardless of any pre-established criteria, and verified the NGS assessment of relevant thyroid cancer genes by pyrosequencing, which is an orthogonal sequencing platform widely used in the molecular diagnosis of thyroid cytology specimens.[18–20]

3.1.1 MATERIALS AND METHODS

To assess the performance of NGS and CHPv2 on routine thyroid FNA specimens, we selected 37 Diff-Quik stained smears from the cytopathology files of the University of Naples Federico II; the histological follow-up also was retrieved when available. The data set was enriched with malignant FNA specimens to increase the number of mutations. However, to avoid selection bias, we were blind to the cellularity of the slides examined. The smears were independently reviewed by 2 cytopathologists to verify the original diagnosis and the correct classification according to The Bethesda System for Reporting Thyroid Cytopathology.[21] Any discrepancy was resolved by consensus review with a multiheaded microscope. A total of 22 samples were classified as malignant, 8 as suspicious for malignancy, 4 as follicular neoplasm/suspicious for follicular neoplasm, 2 as atypia of undetermined significance/follicular lesion of undetermined significance, and 1 sample as benign. As far as the cellularity, by screening the whole slide at a magnification of 310, we categorized the study samples into 3 classes depending on the percentage of fields containing at least 25 to 50 cells: 1) poorly cellular (5%-25% of the total fields); 2) moderately cellular (25%-50% of the total fields); and 3) highly cellular (>50% of the total fields). Pyrosequencing was performed on 26 samples with residual DNA aliquot

availability at the Molecular Laboratory in the Division of Endocrinology and Nephrology at the University of Leipzig (Leipzig, Germany) to verify the variant call or wild-type status of the BRAF, NRAS, KRAS, HRAS, and RET genes.

3.1.2 RESULTS

Sequencing Performance

NGS was feasible in the majority of cases (34 of 37 cases; 91.8%); only samples 17, 25, and 29 failed. Samples 17 and 29 demonstrated a few on-target reads (21.24% and 3.01%, respectively) and a low library concentration (1.29 ng/µL and 1.36 ng/µL, respectively) most likely due to DNA degradation (Figure 1). In particular, the BRAF, HRAS, NRAS, KRAS, and RET genes demonstrated a low number of reads (<10) in the majority of amplicons targeted by CHPv2 . Conversely, sample 25 yielded a sufficient library (>600 ng/µL), and thus the NGS failure was most likely due to improper handling during barcoding or sample processing. The mean and median DNA yields of the successfully processed samples were 7.1 ng/µL and 5.5 ng/µL, respectively (range, 1.07-35.5 ng/µL). It is interesting to note that 20 of 34 successfully processed samples (58.8%) featured suboptimal DNA. In fact, in 1 sample (sample 12) the DNA concentration was below the requested concentration of 1.6 ng/µL, whereas 20 of 34 samples (including sample 12) had a 260/230 ratio<1, which indicates poor DNA quality due to organic contamination.

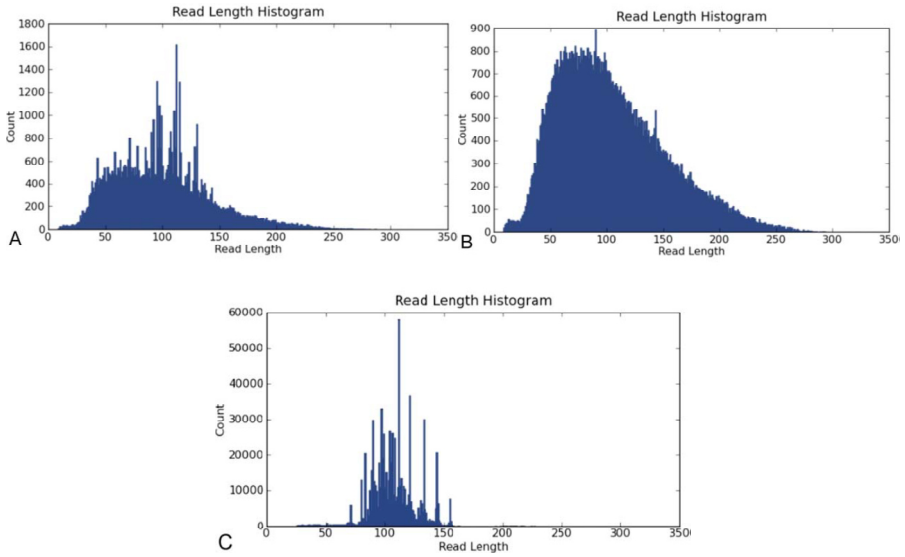

Figure 1. Histograms representing the read distribution (count) per amplicon size (read length). Samples (A) 17 and (B) 29 had a low number of reads distributed within a wide range of amplicon sizes; this may reflect DNA degradation. (C) Histogram of an "ideal" case. Note the distribution of a high number of reads in the expected amplicon size range (75-150 base pairs).

The cellularity in the successfully processed group was high in the majority of cases (23 of 34 cases; 67.6%), moderate in 6 of 34 cases (17.6%), and scarce in 5 of 34 cases (14.7%). In the failed NGS group, 2 samples were moderately cellular and 1 sample had a high cellularity. The postsequencing metrics (ie, the number of mapped reads, average base coverage,percentage of reads on target, and uniformmity of reads) are summarized in Table 1.

TABLE 1. Mean and Ranges of Postsequencing Metrics in Cytology Samples Successfully Processed by NGS

	Mean	Maximum	Minimum
Mapped reads	160,120.26	732,933	963
On-target reads, %	76.05	99.15	3.06
Average base coverage	967.91	3,740	4.13
Uniformity, %	78.93	99.99	40.23

Abbreviation: NGS, next-generation sequencing.

Molecular Analysis

BRAF, RAS, and RET somatic variants were identified in 22 of 34 samples. In 18 of these 22 samples (81.8%), DNA was available for pyrosequencing to verify the mutational calls (Table 2). Seventeen of 18 mutation calls (94.4%) were confirmed by pyrosequencing, whereas an uncommon BRAF mutation (P453T) was not confirmed in sample 3. No variants were called for the BRAF, RAS, and RET genes in the remaining 12 cases. Among the latter, 6 of 8 cases (75%) with residual DNA were found to be wild-type, whereas the orthogonal sequencing of BRAF exon 15 in samples 11 and 36 demonstrated a V600E mutation (Table 2). Thus, with standard orthogonal sequencing, NGS and CHPv2 were found to have a high sensitivity (89.4%), a moderate specificity (85.7%), and an accuracy of 88.4% for the BRAF, RAS, and RET genes. We also analyzed other genes that may be associated with aggressive behavior (eg, TP53 and PIK3CA) and we did not find any variants.

TABLE 2. Bethesda Category, Mutation Type, Analytical Characteristic and Histology Follow-Up of The 34 Samples Processed by NGS

Sample ID	Bethesda Category	Gene	Mutation	Allele Frequency, %	Amplicon Coverage	Pyrosequencing	Histology
1	Suspicious	WT	-	-	-	Confirmed	NA
2	Malignant	BRAF	p.V600E (c.1799T>A)	39.8	1099	Confirmed	CvPTC
3	AUS/FLUS	BRAF	p.P453T (c.1357C>A)	100	9	Not confirmed	FvPTC
4	Malignant	BRAF	p.V600E (c.1799T>A)	7.8	615	Confirmed	CvPTC
5	Malignant	BRAF	p.V600E (c.1799T>A)	55	20	DNA NA	NA
6	FN/SFN	WT	-	-		Confirmed	FA
7	Malignant	BRAF	p.V600E (c.1799T>A)	33.2	1517	Confirmed	CvPTC
8	FN/SFN	WT	-	-	-	Confirmed	NA
9	Malignant	RET	p.C634W (c.1902C>G)	6.4	45	Confirmed	MTC[a]
10	FN/SFN	WT	-	-	-	Confirmed	NA
11	Suspicious	WT	-	-	-	BRAFV600E	CvPTC
12	Malignant	BRAF	p.V600E (c.1799T>A)	55.4	1994	Confirmed	CvPTC
13	Malignant	BRAF	p.V600E (c.1799T>A)	35.3	116	Confirmed	Cv-FvPTC
14	AUS/FLUS	WT	-	-	-	DNA NA	NA
15	Malignant	BRAF	p.V600E (c.1799T>A)	27.4	241	DNA NA	CvPTC
16	Malignant	BRAF	p.V600E (c.1799T>A)	42.5	1080	Confirmed	NA
18	Malignant	WT	-	-	-	Confirmed	NA
19	Malignant	BRAF	p.V600E (c.1799T>A)	25.7	855	Confirmed	NA
20	Suspicious	WT	-	-	-	DNA NA	Cv-FvPTC
21	Malignant	BRAF	p.V600E (c.1799T>A)	35	1147	Confirmed	FvPTC
22	Malignant	BRAF	p.V600E (c.1799T>A)	34.7	72	Confirmed	CvPTC
23	Suspicious	KRAS	p.Q61K (c.180_181TC>AA)	20.4	1972	Confirmed	NA
24	Malignant	BRAF	p.V600E (c.1799T>A)	20	15	Confirmed	CvPTC
26	Malignant	BRAF	p.V600E (c.1799T>A)	16.6	331	Confirmed	NA
27	Malignant	WT	-	-	-	Confirmed	CvPTC
28	Malignant	BRAF	p.V600E (c.1799T>A)	23.8	1655	Confirmed	NA
30	Suspicious	BRAF	p.V600E (c.1799T>A)	6.5	248	DNA NA	CvPTC
31	FN/SFN	WT	-	-	-	DNA NA	NA
32	Malignant	BRAF	p.V600E (c.1799T>A)	7.1	84	Confirmed	CvPTC
33	Malignant	BRAF	p.V600E (c.1799T>A)	39.9	28	DNA NA	NA
34	Malignant	BRAF	p.V600E (c.1799T>A)	2.7	263	Confirmed	CvPTC
35	Suspicious	NRAS	p.Q61R (c.182A>G)	36.2	271	Confirmed	FvPTC
36	Malignant	WT	-	-	-	BRAFV600E	CvPTC-FvPTC
37	Suspicious	WT	-	-	-	DNA NA	NA

Abbreviations: AUS/FLUS, atypia of undetermined significance/follicular lesion of undetermined significance; CvPTC, classic variant of papillary thyroid carcinoma; FA, follicular adenoma; FN/SFN, follicular neoplasm/suspicious for follicular neoplasm; FvPTC, follicular variant of papillary thyroid carcinoma; ID, identification; MTC, medullary thyroid carcinoma; NA, not available; NGS, next-generation sequencing.

[a] Case was confirmed on cell block by immunocytochemistry.

Discordant NGS/Pyrosequencing Test Results

The results of NGS and pyrosequencing were discordant in samples 3, 11, and 36. In sample 3, the BRAF P453T mutation was detected only by NGS and not by pyrosequencing. An in-depth evaluation demonstrated that the amplicon was covered by only 9 reads, all bearing the P453T mutation (Fig. 2). Because the amount of DNA was low (4.88 ng/μL) and the number of PCR cycles had been increased, it is conceivable that a nucleotide misincorporation occurred during the first PCR cycle, and was homogeneously carried by all PCR molecules generated during subsequent cycles. Conversely, a BRAF V600E mutation was detected only by pyrosequencing in samples 11 and 36. A detailed analysis of these samples revealed a low BRAF exon 15 amplicon coverage; in fact, only 34 and 56 reads, respectively, were available for samples 36 and 11. Notably, visual inspection of the corresponding BAM files demonstrated very a low abundance of the V600E mutation, which was not detected by the software. In fact, only 1 read in sample

11 and 2 reads in sample 36 carried the mutation. Last, V600E was identified in only 1 strand in sample 36 (Figs. 3A and 3B).

Figure 2. Visual inspection of the BAM file from case 3. The P453T substitution was detected on all 9 reads. However, this variant call most likely was an artifact because its coverage was low and it was detected mainly on the forward strand (blue reads).

Figure 3. Visual inspection of BAM files from cases (A) 11 and (B) 36. In both cases, the amplicon coverage was low and the T1799A substitution was detected in only (A) 1 read and (B) 2 reads on the same strand

(green indicates reverse; blue, forward). Thus, these alterations were below the software requirements for variant calling.

3.1.3 DISCUSSION

To our knowledge to date, the use of NGS with CHPv2 to analyze cytology samples has been limited by the perception that only "ideal" samples, namely samples with abundant, high-quality DNA and satisfactory postsequencing metrics, could be processed.[13–15] In the current study, we demonstrated that NGS also can be informative on routine smears that have suboptimal DNA quality and postsequencing metrics. There are different methods with which to determine DNA requirements for NGS, including qualitative and quantitative (functional) measurements such as quantitative PCR assays.[26] Despite a poor DNA quality revealed by NanoDrop in the majority of cases (58.8%), we were able to satisfactorily process approximately 91.8% of the specimens using NGS. This result expands previous experiences that have demonstrated the feasibility of suboptimal DNA cases on NGS.[14] Thus, the Ion Torrent platform can be applied successfully to those samples yielding <10 ng of or low-quality DNA. Similarly, the stringent postsequencing metrics criteria (ie, mapped reads, average base coverage, amplicon reads, and mutant allele frequency) to report somatic variants ensure high accuracy but rule out many cases.[13–15] As shown in Table 3, had we adopted rigid postsequencing metrics thresholds, the majority of the NGS results would not have been reported. Notwithstanding the suboptimal postsequencing metrics, approximately 94.4% of the variant calls in the relevant thyroid genes included in the current study were confirmed by pyrosequencing. CHPv2 is a generic cancer gene panel that, in addition to BRAF, RAS, and RET hotspot mutations, covers many other genes involved in thyroid carcinogenesis and malignant progression (ie, AKT1, APC, ATM, CTNNB1, PI3CKA, PTEN, RB1, and TP53).[6] Thus, CHPv2 could be a better rule-in test than targeted PCR-based commercial panels such as the 7-gene panel test, which covers a limited, albeit informative, number of gene alterations (BRAF and NRAS/HRAS/KRAS point mutations and RET/PTC and PAX8/PPARg translocations). This test may be performed either by outsourcing the sample or in house using a commercially available kit distributed by EntroGen Inc (Los Angeles, Calif).[5] However, an important drawback of using a large NGS panel on cytology specimens is that coverage may not be evenly distributed across all clinically informative genes. Therefore, when applying CHPv2 to thyroid FNA samples, an adaptive validation approach

must be used to detect and report variants when either the entire sample library or a single amplicon do not reach the validated postsequencing metric thresholds.[27] This strategy requires long-term monitoring of NGS results and frequent confirmation of variant calls using an orthogonal single-gene test, such as pyrosequencing, to implement NGS technology into the routine molecular diagnostics of thyroid FNA samples.[27] It is interesting to note that the unsupervised variant calling of BRAF, RAS, and RET hotspots resulted in a high sensitivity and specificity, and an accuracy of 88.4%. However, this adaptive approach must include an expert "dry bench" operator to examine the sequencing data and the corresponding metrics on a case by-case basis, and integrate the results generated by informatics pipeline analysis with visual inspection of sequences. Indeed, in 3 samples in the current study, the discordance between NGS and pyrosequencing was resolved by visual inspection of the BAM files (Figs. 2 and 3), thereby highlighting a limited (<60 reads) BRAF amplicon coverage, largely below the thresholds reported in the literature.[13–15] Thus, we can argue that a limited number of reads yielded in the amplicons of interest may led to a "reflex" confirmatory assay. However, in the setting of suboptimal specimens and when orthogonal validation could be not achieved, a statement specifying these limitations should be added to the final clinical report, in particular when dealing with wild-type findings.

The current study, which was conducted to validate CHPv2 on NGS for the detection of gene mutations in thyroid FNA specimens, was enriched for malignant FNA samples. To our knowledge, the clinical role of this panel in indeterminate cases remains to be established, as well as in light of the recent proposal to reclassify encapsulated, noninvasive follicular variants of papillary carcinoma as a "noncancerous or premalignant neoplasm" (ie, "noninvasive follicular thyroid tumor with papillary-like nuclear features" or NIFTP).[28] In fact, this reclassification appears to impact significantly on the risk of malignancy of the 3 indeterminate categories.[29] The molecular alterations of NIFTP cases are typical of follicular patterned lesions (eg, NRAS, HRAS, and KRAS) and, when detected in patients with an indeterminate FNA diagnosis and low-risk ultrasonographic features, could lead to a limited surgical resection with no further treatment if the histopathological diagnosis is NIFTP.[30] In conclusion, given the lack of a commercially available, thyroid-specific NGS gene panel, the CHPv2 cancer generic gene panel is a valid option for the molecular evaluation of thyroid FNA specimens. This approach is accurate

and effective, even when applied to routine cytology samples that do not meet stringent preanalytical (DNA input quality and quantity) and postsequencing requirements.

3.2 Next-generation sequencing and cytopathology in basic science

FNA cytology is now a first-line diagnostic procedure in many nonneoplastic and neoplastic settings worldwide. Because it is easy to use and cost-effective, FNA also has become an essential tool in experimental pathology as an alternative to open biopsies and mouse necropsies, and as a means to sample human tumor mouse xenografts as well as mouse models of various types of human tumors. Cytopathologists were instrumental in translating FNA from the clinical setting to the laboratory. They encouraged investigators to handle experimental specimens using the sampling and processing procedures used in the cytology clinic. Today, this integrated approach is being developed even further, particularly in the field of cancer drug development. Indeed, with the widespread use of routine cytological samples to study molecular targeted therapies, cytopathologists are becoming increasingly familiar with the modern tools of genomics, and consequently interactions between cytopathologists and basic scientists have become more effective. Thus, from the simple detection of therapy-mediated protein changes in target signaling pathways,[1,2] the application of NGS to mouse FNA extends the contribution of modern cytopathology to genomic biomarkers.

3.2.1 CYTOLOGICAL SAMPLES OF PATIENT-DERIVED XENOGRAFTS

The preclinical evaluation of novel cancer drugs is conducted in conditions that simulate clinical conditions with the highest possible fidelity. Patient-derived xenografts ("xenopatients") are used to reproduce the spectrum of tumor heterogeneity and the complexity of signal transduction networks inherent to human neoplasms. To obtain these models, surgically resected tumor samples are engrafted directly into immune-compromised mice and propagated through several generations to obtain numbers suitable for the evaluation of multiple treatments. The outcome of a given treatment can be predicted and monitored by serial FNA sampling of the tumor of the same animal before, during, and at the end of the experimental treatment. FNA biopsy is usually performed on mice after inhaled general anesthesia. However, this step is not necessary when using a fine needle (23-gauge to 25-gauge needle) to sample, for example, a subcutaneous flank mass. Before analysis, the first FNA pass is smeared onto glass slides that are stained with Diff-Quik and/or the Papanicolaou method for morphologic

assessment to ensure that a sufficient number of cells have been sampled and that necrotic areas have been avoided. Various techniques have been used to phenotype these cytological samples: flow cytometry, immunohistochemistry, immunofluorescence, and nanoimmunoassay. In particular, Diff-Quik-stained smears yielded highquality proteins that are required for studies of the phosphorylation status of enzymes involved in cell growth by Western blot analysis and enzyme-linked immunoadsorbent assays.[1,2] Thanks to these methodologies, ex vivo and in vivo sensitivity assays can be performed to predict and assess the efficacy of therapy in xenograft models of human cancer.

3.2.2 NGS

The recent emergence of several potentially therapeutic genomic targets raised the issue of accurate genomic annotation not only of patients, but also of preclinical models. Close interaction between investigators and molecular cytopathologists is crucial for profiling human tumor mouse xenografts on FNA samples. Cytopathologists can apply their expertise during FNA sampling, by fixing the mass with fingers of one hand, and radiating needle passes with the other to obtain a representative cellular sample. After sampling, Diff-Quik-stained smears are completely scraped because mouse DNA derived from contaminating benign cells does not affect the subsequent analysis.

We recently implemented NGS on cytological samples for routine molecular diagnostics and research applications.[3,4] A preliminary validation of the platform and of the test and informatics pipeline indicated that a magnification of 500 was the appropriate threshold of coverage (ie, the number of reads covering a given base position necessary to ensure reliable variant calling). On this basis, mouse FNAs can be sufficient for NGS mutational analysis. Figure 1 illustrates the molecular profiling of the HCC827 cell line (CRL2868; American Type Culture Collection, Manassas, Va) on mouse xenograft FNAs by the Hot Spot Cancer Panel (Life Technologies) performed on a Personal Genomic Machine platform (Life Technologies). The NGS of scraped Diff-Quik smears enabled the detection, with a coverage of greater than 3500, of the exon 19 epidermal growth factor receptor (EGFR) deletion (c.2236_2250del15, p.E746_A750delELREA) and of the exon 10 KIT point mutation (c.1621A>C, p.M541L) detected in the cell line before inoculation. Thus, NGS of mouse FNA samples was found to be as efficient as the procedure used to evaluate HCC827 cell culture-derived DNA. Because it is now possible to reliably define the

mutation status of cancer-related genes by performing NGS on mouse FNA samples, necropsy can be avoided.

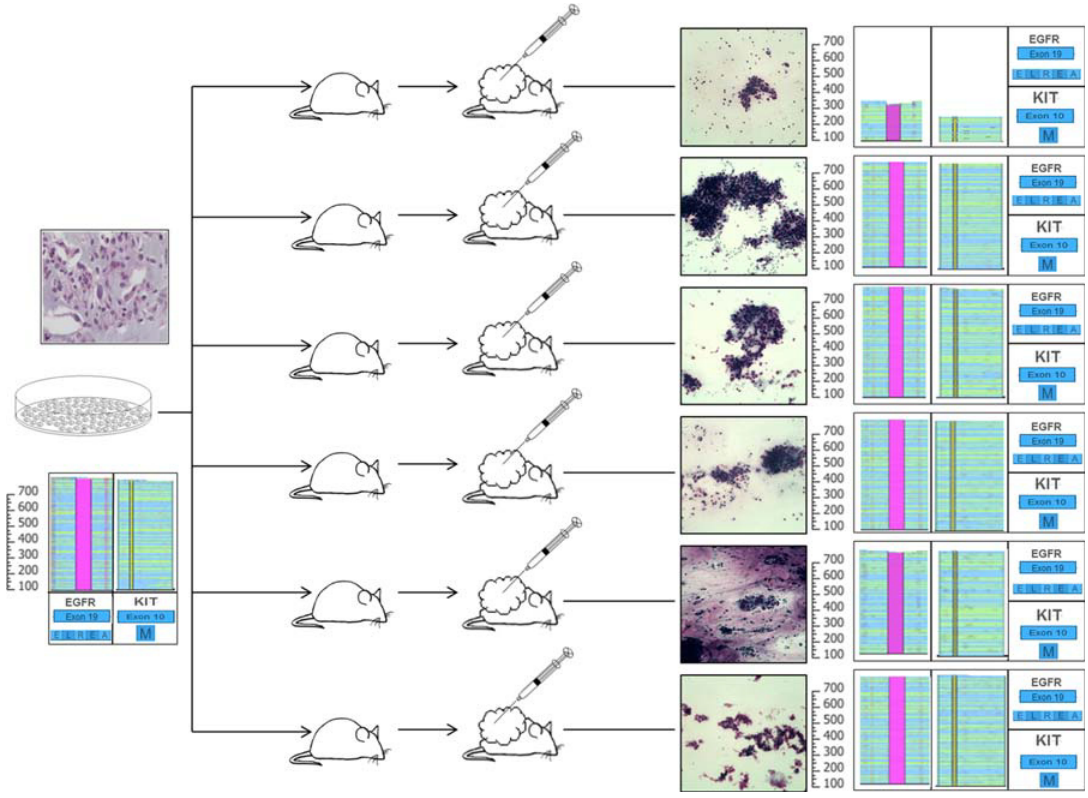

Figure 1. Next-generation sequencing profiling of Diff-Quik-stained mouse xenograft fine-needle aspiration samples. On the left is a hematoxylin and eosin-stained cell block section of the HCC827 cell line, which was grown in vitro. The extracted DNA, profiled using the Hot Spot Cancer Panel on a Personal Genome Machine platform, revealed an epidermal growth factor receptor (EGFR) deletion (c.2236_2250del15, p.E746_A750delELREA) in exon 19, and a KIT point mutation (c.1621A>C, p.M541L) in exon 10. The HCC827 cell line was inoculated in 6 mice and the corresponding xenograft was sampled by fine-needle aspiration. The aspirated material was smeared and stained using Diff-Quik before whole-slide scraping for DNA extraction. The EGFR deletion and KIT mutation were detected in all instances as in the cell line before inoculation. In only one instance (top row, last 3 columns) was coverage less than 3500.

In this setting, FNA also can be used to serially assess the genomic profile of the same animal in a dynamic fashion to determine whether a given experimental drug causes specific genomic alterations. This approach also can be implemented in co-clinical trials to monitor clonal dynamics during treatment and to eradicate treatment-emergent clones.

In conclusion, the modern cytopathologist will become increasingly involved in research protocols, both to provide clinical expertise and to acquire knowledge that can be translated into the burgeoning world of molecular predictive medicine.

Bibliography

Chapter 1 - Introduction

1. International Human Genome Sequencing Consortium, Finishing the euchromatic sequence of the human genome, Nature 431 (2004) 931–945.

2. Xuan J, Yu Y, Qing T, Guo L, Shi L. Next-generation sequencing in the clinic: promises and challenges. Cancer Lett 2013;340:284–95.

3. Metzker ML. Sequencing technologies - the next generation. Nat Rev Genet. 2010;11(1):31-46

4. Pant S, Weiner R, Marton MJ. Navigating the rapids: the development of regulated next-generation sequencing-based clinical trial assays and companion diagnostics. Front Oncol 2014;4:78.

5. Salto-Tellez M, de Castro DG. Next generation sequencing: a change of paradigm in molecular diagnostic validation. J Pathol 2014;234:5–10

6. Malapelle U, Vigliar E, Sgariglia R et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol 2015;68:64–8.

7. Rothberg JM, Leamon JH. The development and impact of 454 sequencing. Nat Biotechnol 2008;26:1117–24.

8. Mardis ER. Next-generation sequencing platforms. Annu Rev Anal Chem (Palo Alto Calif) 2013;6:287–303.

9. Rothberg JM, Hinz W, Rearick TM et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011;475:348–52

10. Merriman B, Ion Torrent R, Team D, Rothberg JM. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis 2012;33:3397–417.

11. Beadling C, Neff TL, Heinrich MC et al. Combining highly multiplexed PCR with semiconductor-based sequencing for rapid cancer genotyping. J Mol Diagn 2013;15:171–6.

12. Tsongalis GJ, Peterson JD, de Abreu FB et al. Routine use of the Ion Torrent AmpliSeq Cancer Hotspot Panel for identification of clinically actionable somatic mutations. Clin Chem Lab Med 2014;52:707–14.

13. Gargis AS, Kalman L, Berry MW et al. Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 2012;30:1033–6

Chapter 2 - Next-generation sequencing analysis of colon carcinomas

1. Malapelle U, Carlomagno C, de Luca C, et al. KRAS testing in metastatic colorectal carcinoma: challenges, controversies, breakthroughs and beyond. J Clin Pathol 2014;67:1–9.

2. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol 2010;28:4697–705.

3. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–7.

4. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011;475:348–52.

5. Beadling C, Neff TL, Heinrich MC, et al. Combining highly multiplexed PCR with semiconductor-based sequencing for rapid cancer genotyping. J Mol Diagn 2013;15:171–6. 11 Sikkema-Raddatz B, Johansson LF, et al. Targeted next-generation sequencing can replace Sanger sequencing in clinical diagnostics. Hum Mutat 2013;34:1035–42.

6. Zhang L, Chen L, Sah S, et al. Pro filing cancer gene mutations in clinical formalin-fixed, paraffin-embedded colorectal tumor specimens using targeted next-generation sequencing. Oncologist 2014;19:336–43.

7. Tsongalis GJ, Peterson JD, de Abreu FB, et al. Routine use of the Ion Torrent AmpliSeq Cancer Hotspot Panel for identification of clinically actionable somatic mutations. Clin Chem Lab Med 2013;52:707–14.

8. Hadd AG, Houghton J, Choudhary A, et al. Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. J Mol Diagn 2013;15:234–47.

9. Normanno N, Petraroli R, Rico A, et al. The OncoNetwork Consortium: a European collaborative research study on the development of an Ion AmpliSeq gene panel targeting hot spots in colon and lung cancers. Cancer Research 2013; 73(8, Suppl. 1):36.

10. Ciardiello F, Normanno N, Maiello E, et al. Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by next generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 2014;25:1756–61.

Chapter 2.1 - Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients

1. Malapelle U, Carlomagno C, de Luca C, et al. KRAS testing in metastatic colorectal carcinoma: challenges, controversies, breakthroughs and beyond. J Clin Pathol 2014;67:1–9.

2. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol 2010;28:4697–705.

3. Karapetis CS, Khambata-Ford S, Jonker DJ, et al. K-ras mutations and benefit from cetuximab in advanced colorectal cancer. N Engl J Med 2008;359:1757–65.

4. De Roock W, Jonker DJ, Di Nicolantonio F, et al. Association of KRAS p.G13D mutation with outcome in patients with chemotherapy-refractory metastatic colorectal cancer treated with cetuximab. JAMA 2010;304:1812–20.

5. Allegra CJ, Jessup JM, Somerfield MR, et al. American Society of Clinical Oncology provisional clinical opinion: testing for KRAS gene mutations in patients with metastatic colorectal carcinoma to predict response to anti-epidermal growth factor receptor monoclonal antibody therapy. J Clin Oncol 2009;27:2091–6.

6. van Krieken JH, Jung A, Kirchner T, et al. KRAS mutation testing for predicting response to anti-EGFR therapy for colorectal carcinoma: proposal for an European quality assurance program. Virchows Arch 2008;453:417–31.

7. Malapelle U, Bellevicine C, Salatiello M, et al. Sanger sequencing in routine KRAS testing: a review of 1720 cases from a pathologist's perspective. J Clin Pathol 2012;65:940–4.

8. Douillard JY, Oliner KS, Siena S, et al. Panitumumab-FOLFOX4 treatment and RAS mutations in colorectal cancer. N Engl J Med 2013;369:1023–34.

9. Salto-Tellez M, de Castro DG. Next generation sequencing: a change of paradigm in molecular diagnostic validation. J Pathol 2014;234:5–10.

10. Beadling C, Neff TL, Heinrich MC, et al. Combining highly multiplexed PCR with semiconductor-based sequencing for rapid cancer genotyping. J Mol Diagn 2013;15:171–6.

11. Sikkema-Raddatz B, Johansson LF, et al. Targeted next-generation sequencing can replace Sanger sequencing in clinical diagnostics. Hum Mutat 2013;34:1035–42.

12. Rothberg JM, Hinz W, Rearick TM, et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011;475:348–52.

13. Zhang L, Chen L, Sah S, et al. Pro filing cancer gene mutations in clinical formalin-fixed, paraffin-embedded colorectal tumor specimens using targeted next-generation sequencing. Oncologist 2014;19:336–43.

14. Tsongalis GJ, Peterson JD, de Abreu FB, et al. Routine use of the Ion Torrent AmpliSeq Cancer Hotspot Panel for identification of clinically actionable somatic mutations. Clin Chem Lab Med 2013;52:707–14.

15. Hadd AG, Houghton J, Choudhary A, et al. Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. J Mol Diagn 2013;15:234–47.

16. Normanno N, Petraroli R, Rico A, et al. The OncoNetwork Consortium: a European collaborative research study on the development of an Ion AmpliSeq gene panel targeting hot spots in colon and lung cancers. Cancer Research 2013; 73(8, Suppl. 1):36.

17. Ciardiello F, Normanno N, Maiello E, et al. Clinical activity of FOLFIRI plus cetuximab according to extended gene mutation status by

next generation sequencing: findings from the CAPRI-GOIM trial. Ann Oncol 2014;25:1756–61.

18. Troncone G, Cozzolino I, Fedele M, et al. Preparation of thyroid FNA material for routine cytology and BRAF testing: a validation study. Diagn Cytopathol 2010;38:172–6.

19. Troncone G, Malapelle U, Cozzolino I, et al. KRAS mutation analysis on cytological specimens of metastatic colo-rectal cancer. Diagn Cytopathol 2010;38:869–73.

20. Lin MT, Mosier SL, Thiess M, et al. Clinical validation of KRAS, BRAF, and EGFR mutation detection using next-generation sequencing. Am J Clin Pathol 2014;141:856–66.

21. Kothari N, Schell MJ, Teer JK, et al. Comparison of KRAS mutation analysis of colorectal cancer samples bystandard testing and next-generation sequencing. J Clin Pathol 2014;67:764–7.

22. Janakiraman M, Vakiani E, Zeng Z, et al. Genomic and biological characterization of exon 4 KRAS mutations in human cancer. Cancer Res 2010;70:5901–11.

23. Malapelle U, Carlomagno C, Salatiello M, et al. KRAS mutation detection by high-resolution melting analysis significantly predicts clinical benefit of cetuximab in metastatic colorectal cancer. Br J Cancer 2012;107:626–31.

24. Pant S, Weiner R, Marton MJ. Navigating the rapids: the development of regulated next-generation sequencing-based clinical trial assays and companion diagnostics. Front Oncol 2014;4:78.

Chapter 2.2 - Less frequently mutated genes in colorectal cancer: evidences from next-generation sequencing of 653 routine cases

1. Malapelle U, Carlomagno C, de Luca C, et al. KRAS testing in metastatic colorectal carcinoma: challenges, controversies, breakthroughs and beyond. J Clin Pathol 2014;67:1–9.

2. Douillard JY, Siena S, Cassidy J, et al. Randomized, phase III trial of panitumumab with infusional fluorouracil, leucovorin, and oxaliplatin (FOLFOX4) versus FOLFOX4 alone as first-line treatment in patients with previously untreated metastatic colorectal cancer: the PRIME study. J Clin Oncol 2010;28:4697–705.

3. Tops BB, Normanno N, Kurth H, et al. Development of a semi-conductor sequencing-based panel for genotyping of colon and lung cancer by the Onconetwork consortium. BMC Cancer 2015;15:26.

4. Malapelle U, Vigliar E, Sgariglia R, et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol 2015;68:64–8.

5. Cancer Genome Atlas Network. Comprehensive molecular characterization of human colon and rectal cancer. Nature 2012;487:330–7.

6. Wong NA, Gonzalez D, Salto-Tellez M, et al. RAS testing of colorectal carcinoma-a guidance document from the Association of Clinical Pathologists Molecular Pathology and Diagnostics Group. J Clin Pathol 2014;67:751–7.

7. Normanno N, Rachiglio AM, Lambiase M, et al. Heterogeneity of KRAS, NRAS, BRAF and PIK3CA mutations in metastatic colorectal cancer and potential effects on therapy in the CAPRI GOIM trial. Ann Oncol 2015;26:1710–4.

8. Chevrier S, Arnould L, Ghiringhelli F, et al. Next-generation sequencing analysis of lung and colon carcinomas reveals a variety of genetic alterations. Int J Oncol 2014;45:1167–74.

9. Weigelt B, Reis-Filho JS. Activating mutations in HER2: neu opportunities and neu challenges. Cancer Discov 2013;3:145–7.

10. Greulich H, Kaplan B, Mertins P, et al. Functional analysis of receptor tyrosine kinase mutations in lung cancer identifies oncogenic extracellular domain mutations of ERBB2. Proc Natl Acad Sci USA 2012;109:14476–81.

11. Kavuri SM, Jain N, Galimi F, et al. HER2 activating mutations are targets for colorectal cancer treatment. Cancer Discov 2015;5:832–41.

12. Williams CS, Bernard JK, Demory Beckler M, et al. ERBB4 is over-expressed in human colon cancer and enhances cellular transformation. Carcinogenesis 2015;36:710–18.

13. Kogita A, Yoshioka Y, Sakai K, et al. Inter- and intra-tumor profiling of multi-regional colon cancer and metastasis. Biochem Biophys Res Commun 2015;458:52–6.

14. Hechtman JF, Sadowska J, Huse JT, et al. AKT1 E17K in colorectal carcinoma is associated with BRAF V600E but not MSI-H status: a clinicopathologic comparison to PIK3CA helical

and kinase domain mutants. Mol Cancer Res 2015;13:1003–8.

15. Lan YT, Jen-Kou L, Lin CH, et al. Mutations in the RAS and PI3K pathways are associated with metastatic location in colorectal cancers. J Surg Oncol 2015;111:905–10.

16. Day FL, Jorissen RN, Lipton L, et al. PIK3CA and PTEN gene and exon mutation-specific clinicopathologic and molecular associations in colorectal cancer. Clin Cancer Res 2013;19:3285–96.

17. Stachler MD, Rinehart E, Lindeman N, et al. Novel molecular insights from routine genotyping of colorectal carcinomas. Hum Pathol 2015;46:507–13.

18. Avizienyte E, Roth S, Loukola A, et al. Somatic mutations in LKB1 are rare in sporadic colorectal and testicular tumors. Cancer Res 1998;58:2087–90.

19. Liu Y, Marks K, Cowley GS, et al. Metabolic and functional genomic studies identify deoxythymidylate kinase as a target in LKB1-mutant lung cancer. Cancer Discov 2013;3:870–9.

20. Fearon ER. Molecular genetics of colorectal cancer. Annu Rev Pathol 2011;6:479–507.

21. Fleming NI, Jorissen RN, Mouradov D, et al. SMAD2, SMAD3 and SMAD4 mutations in colorectal cancer. Cancer Res 2013;73:725–35.

22. Zhang B, Zhang B, Chen X, et al. Loss of Smad4 in colorectal cancer induces resistance to 5-fluorouracil through activating Akt pathway. Br J Cancer 2014;110:946–57.

23. Alhopuro P, Alazzouzi H, Sammalkorpi H, et al. SMAD4 levels and response to 5-fluorouracil in colorectal cancer. Clin Cancer Res 2005;11:6311–6.

24. Akhoondi S, Sun D, von der Lehr N, et al. FBXW7/hCDC4 is a general tumor suppressor in human cancer. Cancer Res 2007;67:9006–12.

25. Bamford S, Dawson E, Forbes S, et al. The COSMIC (Catalogue of Somatic Mutations in Cancer) database and website. Br J Cancer 2004;91:355–8.

26. Wang Y, Liu Y, Lu J, et al. Rapamycin inhibits FBXW7 loss-induced epithelial-mesenchymal transition and cancer stem cell-like characteristics in colorectal cancer cells. Biochem Biophys Res Commun 2013;434:356–6.

27. Yokobori T, Yokoyama Y, Mogi A, et al. FBXW7 mediates chemotherapeutic sensitivity and prognosis in NSCLCs. Mol Cancer Res 2014;12:32–7.

28. Jardim DL, Wheler JJ, Hess K, et al. FBXW7 mutations in patients with advanced cancers: clinical and molecular characteristics and outcomes with mTOR inhibitors. PLoS ONE 2014;9:e89388.

29. Bai J, Gao J, Mao Z, et al. Genetic mutations in human rectal cancers detected by targeted sequencing. J Hum Genet 2015;60:589–96.

30. Fender AW, Nutter JM, Fitzgerald TL, et al. Notch-1 promotes stemness and epithelial to mesenchymal transition in colorectal cancer. J Cell Biochem 2015;116:2517–27.

Chapter 3 - Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective

1. Bellevicine C, Vita GD, Malapelle U, Troncone G. Applications and limitations of oncogene mutation testing in clinical cytopathology. Semin Diagn Pathol 2013;30:284– 97.

2. Troncone G, Cozzolino I, Fedele M, Malapelle U, Palombini L. Preparation of thyroid FNA material for routine cytology and BRAF testing: a validation study. Diagn Cytopathol 2010;38:172–6.

3. Clark DP. Molecular diagnostics on thyroid fine-needle aspirations: the pathway to value creation. Cancer Cytopathol 2010;118:14–6.

4. de Biase D, Visani M, Baccarini P et al. Next generation sequencing improves the accuracy of KRAS mutation analysis in endoscopic ultrasound fine needle aspiration pancreatic lesions. PLoS ONE 2014;9:e87651.

5. Bellevicine C, Malapelle U, de Luca C et al. EGFR analysis: current evidence and future directions. Diagn Cytopathol 2014;42:984–92.

6. Malapelle U, Bellevicine C, Zeppa P, Palombini L, Troncone G. Cytology-based gene mutation tests to predict response to anti-epidermal growth factor receptor therapy: a review. Diagn Cytopathol 2011;39:703–10.

7. Clark DP. Seize the opportunity: underutilization of fine-needle aspiration biopsy to inform targeted cancer therapy decisions. Cancer Cytopathol 2009;117:289–97.

8. Idowu MO. Epidermal growth factor receptor in lung cancer: the amazing interplay of

molecular testing and cytopathology. Cancer Cytopathol 2013;121:540–3.

9. Sanger F, Coulson AR. A rapid method for determining sequences in DNA by primed synthesis with DNA polymerase. J Mol Biol 1975;94:441–8.

10. Ronaghi M. Pyrosequencing for SNP genotyping. Methods Mol Biol 2003;212:189–95.

11. Smouse JH, Cibas ES, Janne PA et al. EGFR mutations are detected comparably in cytologic and surgical pathology specimens of nonsmall cell lung cancer. Cancer Cytopathol 2009;117:67–72.

12. Malapelle U, Vigliar E, Sgariglia R et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol 2015;68:64–8.

13. Rothberg JM, Leamon JH. The development and impact of 454 sequencing. Nat Biotechnol 2008;26:1117–24.

14. Pant S, Weiner R, Marton MJ. Navigating the rapids: the development of regulated next-generation sequencing-based clinical trial assays and companion diagnostics. Front Oncol 2014;4:78.

15. Salto-Tellez M, de Castro DG. Next generation sequencing: a change of paradigm in molecular diagnostic validation. J Pathol 2014;234:5–10.

16. Loman NJ, Misra RV, Dallman TJ et al. Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 2012;30:434–9.

17. Mardis ER. Next-generation sequencing platforms. Annu Rev Anal Chem (Palo Alto Calif) 2013;6:287–303.

18. Beadling C, Neff TL, Heinrich MC et al. Combining highly multiplexed PCR with semiconductor-based sequencing for rapid cancer genotyping. J Mol Diagn 2013;15:171–6.

19. Rothberg JM, Hinz W, Rearick TM et al. An integrated semiconductor device enabling non-optical genome sequencing. Nature 2011;475:348–52.

20. Merriman B, Ion Torrent R, Team D, Rothberg JM. Progress in ion torrent semiconductor chip based sequencing. Electrophoresis 2012;33:3397–417.

21. Bentley DR, Balasubramanian S, Swerdlow HP et al. Accurate whole human genome sequencing using reversible terminator chemistry. Nature 2008;456:53–9.

22. Tsongalis GJ, Peterson JD, de Abreu FB et al. Routine use of the Ion Torrent AmpliSeq Cancer Hotspot Panel for identification of clinically actionable somatic mutations. Clin Chem Lab Med 2014;52:707–14.

23. Gargis AS, Kalman L, Berry MW et al. Assuring the quality of next-generation sequencing in clinical laboratory practice. Nat Biotechnol 2012;30:1033–6.

24. Scarpa A, Sikora K, Fassan M et al. Molecular typing of lung adenocarcinoma on cytological samples using a multigene next generation sequencing panel. PLoS ONE 2013;8:e80478.

25. Young G, Wang K, He J et al. Clinical next-generation sequencing successfully applied to fine-needle aspirations of pulmonary and pancreatic neoplasms. Cancer Cytopathol 2013;121:688–94.

26. Le Mercier M, D'Haene N, De Neve N et al. Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. Histopathology 2015;66:215–24.

27. Gleeson FC, Kipp BR, Levy MJ et al. Lung cancer adrenal gland metastasis: optimal fine-needle aspirate and touch preparation smear cellularity characteristics for successful theranostic next-generation sequencing. Cancer Cytopathol 2014;122:822–32.

28. Gleeson FC, Kipp BR, Kerr SE et al. Kinase genotype analysis of gastric gastrointestinal stromal tumor cytology samples using targeted next-generation sequencing. Clin Gastroenterol Hepatol 2015;13:202–6.

29. Karnes HE, Duncavage EJ, Bernadt CT. Targeted nextgeneration sequencing using fine-needle aspirates from adenocarcinomas of the lung. Cancer Cytopathol 2014;122:104–13.

30. Kanagal-Shamanna R, Portier BP, Singh RR et al. Nextgeneration sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics. Mod Pathol 2014;27:314–27.

31. Lin MT, Mosier SL, Thiess M et al. Clinical validation of KRAS, BRAF, and EGFR mutation

detection using next-generation sequencing. Am J Clin Pathol 2014;141:856–66.

32. McCall CM, Mosier S, Thiess M et al. False positives in multiplex PCR-based next-generation sequencing have unique signatures. J Mol Diagn 2014;16:541–9.

33. Le Mercier M, D'Haene N, De Neve N et al. Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. Histopathology 2015;66:215–24.

34. Nikiforova MN, Wald AI, Roy S, Durso MB, Nikiforov YE. Targeted next-generation sequencing panel (ThyroSeq) for detection of mutations in thyroid cancer. J Clin Endocrinol Metab 2013;98:E1852–60.

35. Xuan J, Yu Y, Qing T, Guo L, Shi L. Next-generation sequencing in the clinic: promises and challenges. Cancer Lett 2013;340:284–95.

36. de Biase D, Visani M, Malapelle U et al. Next-generation sequencing of lung cancer EGFR exons 18-21 allows effective molecular diagnosis of small routine samples (cytology and biopsy). PLoS ONE 2013;8: e83607.

37. Buttitta F, Felicioni L, Del Grammastro M et al. Effective assessment of egfr mutation status in bronchoalveolar lavage and pleural fluids by next-generation sequencing. Clin Cancer Res 2013;19:691–8.

38. Moskalev EA, Stoehr R, Rieker R et al. Increased detection rates of EGFR and KRAS mutations in NSCLC specimens with low tumour cell content by 454 deep sequencing. Virchows Arch 2013;462:409–19.

39. Kubota Y, Kawakami H, Natsuizaka M et al. CTNNB1 mutational analysis of solid-pseudopapillary neoplasms of the pancreas using endoscopic ultrasound-guided fine-needle aspiration and next-generation deep sequencing. J Gastroenterol 2015;50:203–10.

40. Malapelle U, Carlomagno C, Salatiello M et al. KRAS mutation detection by high-resolution melting analysis significantly predicts clinical benefit of cetuximab in metastatic colorectal cancer. Br J Cancer 2012;107:626– 31.

41. Ohori NP, Singhal R, Nikiforova MN et al. BRAF mutation detection in indeterminate thyroid cytology specimens: underlying cytologic, molecular, and pathologic characteristics of papillary thyroid carcinoma. Cancer Cytopathol 2013;121:197–205.

42. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. Cell 2014;159:676–90.

43. Kelly LM, Barila G, Liu P et al. Identification of the transforming STRN-ALK fusion as a potential therapeutic target in the aggressive forms of thyroid cancer. Proc Natl Acad Sci USA 2014;111:4233–8.

44. Nikiforov YE, Carty SE, Chiosea SI et al. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer 2014;120:3627–34.

45. Hadd AG, Houghton J, Choudhary A et al. Targeted, high-depth, next-generation sequencing of cancer genes in formalin-fixed, paraffin-embedded and fine-needle aspiration tumor specimens. J Mol Diagn 2013;15:234–47.

46. Gailey MP, Stence AA, Jensen CS, Ma D. Multiplatform comparison of molecular oncology tests performed on cytology specimens and formalin-fixed, paraffinembedded tissue. Cancer Cytopathol 2015;123:30–9.

47. Kinde I, Bettegowda C, Wang Y et al. Evaluation of DNA from the Papanicolaou test to detect ovarian and endometrial cancers. Sci Transl Med 2013;5:167ra4.

48. Lindeman NI, Cagle PT, Beasley MB et al. Molecular testing guideline for selection of lung cancer patients for EGFR and ALK tyrosine kinase inhibitors: guideline from the College of American Pathologists, International Association for the Study of Lung Cancer, and Association for Molecular Pathology. Arch Pathol Lab Med 2013;137:828–60.

49. Beadling C, Heinrich MC, Warrick A et al. Multiplex mutation screening by mass spectrometry evaluation of 820 cases from a personalized cancer medicine registry. J Mol Diagn 2011;13:504–13.

50. Magdaleno SM, Cheng A, Petraroli R et al. Abstract 3575: The OncoNetwork Consortium: a global collaborative research study on the development and verification of an Ion AmpliSeq RNA gene lung fusion panel. Cancer Res 2014;74(19 Suppl):3575.

51. Vigliar E, Malapelle U, Bellevicine C, de Luca C, Troncone G. Outsourcing cytological samples to a referral laboratory for EGFR testing in non-small cell lung cancer: does theory meet

practice? Cytopathology 2014. doi: 10.1111/cyt.12221

52. Tripathy D, Harnden K, Blackwell K, Robson M. Next generation sequencing and tumor mutation profiling: are we ready for routine use in the oncology clinic? BMC Med 2014;12:140.

53. Bedard PL, Hansen AR, Ratain MJ, Siu LL. Tumour heterogeneity in the clinic. Nature 2013;501:355–64.

54. Marchetti A, Del Grammastro M, Filice G et al. Complex mutations & subpopulations of deletions at exon 19 of EGFR in NSCLC revealed by next generation sequencing: potential clinical implications. PLoS ONE 2012;7:e42164.

55. Malapelle U, Bellevicine C, de Luca C et al. EGFR mutations detected on cytology samples by a centralized laboratory reliably predict response to gefitinib in non small cell lung cancer patients. Cancer Cytopathol 2013;121:552–60.

56. Ohori NP, Nikiforova MN, Schoedel KE et al. Contribution of molecular testing to thyroid fine-needle aspiration cytology of "follicular lesion of undetermined significance/atypia of undetermined significance". Cancer Cytopathol 2010;118:17–23.

Chapter 3.1 - Can the Ion AmpliSeq Cancer Hotspot Panel v2 Be Used for Next-Generation Sequencing of Thyroid FNA Samples?

1. Bongiovanni M, Spitale A, Faquin WC, Mazzucchelli L, Baloch ZW. The Bethesda System for Reporting Thyroid Cytopathology: a meta-analysis. Acta Cytol. 2012;56:333-339.

2. Onder S, Firat P, Ates D. The Bethesda system for reporting thyroid cytopathology: an institutional experience of the outcome of indeterminate categories. Cytopathology. 2014;25:177-184.

3. Ho AS, Sarti EE, Jain KS, et al. Malignancy rate in thyroid nodules classified as Bethesda category III (AUS/FLUS). Thyroid. 2014;24:832-839.

4. Straccia P, Rossi ED, Bizzarro T, et al. A meta-analytic review of the Bethesda System for Reporting Thyroid Cytopathology: has the rate of malignancy in indeterminate lesions been underestimated? Cancer Cytopathol. 2015;123:713-722.

5. Nishino M. Molecular cytopathology for thyroid nodules: a review of methodology and test performance. Cancer Cytopathol. 2016; 124:14-27.

6. Cancer Genome Atlas Research Network. Integrated genomic characterization of papillary thyroid carcinoma. Cell. 2014;159:676-690.

7. Nikiforov YE, Ohori NP, Hodak SP, et al. Impact of mutational testing on the diagnosis and management of patients with cytologically indeterminate thyroid nodules: a prospective analysis of 1056 FNA samples. J Clin Endocrinol Metab. 2011;96:3390-3397.

8. Vigliar E, Malapelle U, de Luca C, Bellevicine C, Troncone G. Challenges and opportunities of next-generation sequencing: a cytopathologist's perspective. Cytopathology. 2015;26:271-283.

. Nikiforova MN, Wald AI, Roy S, Durso MB, Nikiforov YE. Targeted next-generation sequencing panel (ThyroSeq) for detection of mutations in thyroid cancer. J Clin Endocrinol Metab. 2013;98:E1852-E1860.

10. Nikiforov YE, Carty SE, Chiosea SI, et al. Highly accurate diagnosis of cancer in thyroid nodules with follicular neoplasm/suspicious for a follicular neoplasm cytology by ThyroSeq v2 next-generation sequencing assay. Cancer. 2014;120:3627-3634.

11. Nikiforov YE, Carty SE, Chiosea SI, et al. Impact of the multi-gene ThyroSeq next-generation sequencing assay on cancer diagnosis in thyroid nodules with atypia of undetermined significance/follicular lesion of undetermined significance cytology. Thyroid. 2015;25:1217-1223.

12. University of Pittsburgh Medical Center. ThyroSeqV R -thyroid cancer next-generation sequencing panel specimen requirements. http://mgp.upmc.com/Applications/mgp/Home/ Test/ThyroSeq_ Details. Accessed September 10, 2016.

13. Kanagal-Shamanna R, Portier BP, Singh RR, et al. Next-generation sequencing-based multi-gene mutation profiling of solid tumors using fine needle aspiration samples: promises and challenges for routine clinical diagnostics. Mod Pathol. 2014;27:314-327.

14. Roy-Chowdhuri S, Goswami RS, Chen H, et al. Factors affecting the success of next-generation sequencing in cytology specimens. Cancer Cytopathol. 2015;123:659-668.

15. Le Mercier M, D'Haene N, De Neve N, et al. Next-generation sequencing improves the

diagnosis of thyroid FNA specimens with indeterminate cytology. Histopathology. 2015;66:215-224.

16. Gailey MP, Stence AA, Jensen CS, Ma D. Multiplatform comparison of molecular oncology tests performed on cytology specimens and formalin-fixed, paraffin-embedded tissue. Cancer Cytopathol. 2015;123:30-39.

17. Simbolo M, Gottardi M, Corbo V, et al. DNA qualification workflow for next generation sequencing of histopathological samples. PLoS One. 2013;8:e62692.

18. Rehfeld C, Munz S, Krogdahl A, et al. Impact of different methodologies on the detection of point mutations in routine air-dried fine needle aspiration (FNA) smears. Horm Metab Res. 2013;45: 513-517.

19. Eszlinger M, Krogdahl A, Munz S, et al. Impact of molecular screening for point mutations and rearrangements in routine airdried fine-needle aspiration samples of thyroid nodules. Thyroid. 2014;24:305-313.

20. Eszlinger M, Piana S, Moll A, et al. Molecular testing of thyroid fine-needle aspirations improves presurgical diagnosis and supports the histologic identification of minimally invasive follicular thyroid carcinomas. Thyroid. 2015;25:401-409.

21. Cibas ES, Ali SZ; NCI Thyroid FNA State of the Science Conference. The Bethesda System For Reporting Thyroid Cytopathology. Am J Clin Pathol. 2009;132:658-665.

22. Bellevicine C, Malapelle U, Vigliar E, de Luca C, Troncone G. Epidermal growth factor receptor test performed on liquid-based cytology lung samples: experience of an academic referral center. Acta Cytol. 2014;58:589-594.

23. Dumur CI, Almenara JA, Powers CN, Ferreira-Gonzalez A. Quality control material for the detection of somatic mutations in fixed clinical specimens by next-generation sequencing. Diagn Pathol. 2015;10:169.

24. Dijkstra JR, Tops BB, Nagtegaal ID, van Krieken JH, Ligtenberg MJ. The homogeneous mutation status of a 22 gene panel justifies the use of serial sections of colorectal cancer tissue for external quality assessment. Virchows Arch. 2015;467:273-278.

25. Malapelle U, Vigliar E, Sgariglia R, et al. Ion Torrent nextgeneration sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol. 2015; 68:64-68.

26. Sah S, Chen L, Houghton J, et al. Functional DNA quantification guides accurate next-generation sequencing mutation detection in formalin-fixed, paraffin-embedded tumor biopsies. Genome Med. 2013;5:77.

27. Salto-Tellez M, Gonzalez de Castro D. Next-generation sequencing: a change of paradigm in molecular diagnostic validation. J Pathol. 2014;234:5-10.

28. Nikiforov YE, Seethala RR, Tallini G, et al. Nomenclature revision for encapsulated follicular variant of papillary thyroid carcinoma: a paradigm shift to reduce overtreatment of indolent tumors. JAMA Oncol. 2016;2:1023-1029.

29. Faquin WC, Wong LQ, Afrogheh AH, et al. Impact of reclassifying noninvasive follicular variant of papillary thyroid carcinoma on the risk of malignancy in The Bethesda System for Reporting Thyroid Cytopathology. Cancer Cytopathol. 2016;124:181-187.

30. Baloch ZW, Seethala RR, Faquin WC, et al. Noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP): a changing paradigm in thyroid surgical pathology and implications for thyroid cytopathology [published online ahead of print Mary 20, 2016]. Cancer Cytopathol. doi: 10.1002/cncy.21744.

Chapter 3.2 - Next-generation sequencing and cytopathology in basic science

1. Jimeno A, Kulesza P, Wheelhouse J, et al. Dual EGFR and mTOR targeting in squamous cell carcinoma models, and development of early markers of efficacy. Br J Cancer. 2007;96:952-959.

2. Rubio-Viqueira B, Mezzadra H, Nielsen ME, et al. Optimizing the development of targeted agents in pancreatic cancer: tumor fine-needle aspiration biopsy as a platform for novel prospective ex vivo drug sensitivity assays. Mol Cancer Ther. 2007;6:515-523.

3. Malapelle U, Vigliar E, Sgariglia R, et al. Ion Torrent next-generation sequencing for routine identification of clinically relevant mutations in colorectal cancer patients. J Clin Pathol. 015;68:64-68.

4. Bellevicine C, Vigliar E, Malapelle U, et al. Lung adenocarcinoma and its thyroid metastasis

characterized on fine-needle aspirates by cytomorphology, immunocytochemistry, and next-generation sequencing [published online ahead of print April 21, 2015]. Diagn Cytopathol.