Technological advances in molecular pathology : a journey into the archives

Eijk, R. van

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Author: Eijk, Ronald van
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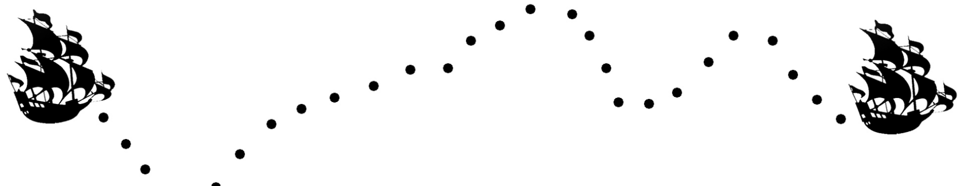
Chapter 7

Concluding remarks and future directions



As a result of revolutionary technological advances, the molecular analysis of cancer field is growing rapidly. Mutations in *KRAS*, *BRAF* and *EGFR* have been discovered, and these biomarkers appear to be pivotal in critical cancer pathways. This knowledge led to the discovery of specific inhibitors that have been implemented in personalized medicine, for example in colorectal cancer and non-small-cell lung carcinoma [1]. Cancer genomes from tumor subtypes have been sequenced, revealing a landscape of somatic mutations. Potential critical mutations have been identified which may be favorable prognostic markers [2]. Epigenetic and transcriptional profiling of tumors contributed to the development of validated molecular classification tests such as Oncotype DX and MammaPrint for breast cancer. Therefore, the expectation is high that technological advances and an understanding of the molecular basis of cancer will translate to benefits for cancer patients [3].
What will technological advances in the next 5, 10 or 25 years bring to pathology? In his book *De Toekomst van Gezondheid* (The future of health), futurologist and trendwatcher Adjiedj Bakas predicts that in 2025 90% of oncology patients will be cured. Kurzweil, an American futurist and the current director of engineering at Google, believes that people living in 2050 could be close to immortal as a result of the combination of biotechnology and nanotechnology. In his book*The Singularity is Near: When Humans Transcend Biology* he predicts that cell repair nanobots will flow through the bloodstream in an era when artificial intelligence has become reality. These predictions are tempting and promising although only future will tell if they become reality or remain science fiction. Still, the question can be prompted whether a general direction in pathology can be distinguished by current advances and directions in technology. From a clinical point of view, future decision making strategies in pathology will continue to depend on histopathological features, and tumor typing, grading and staging will remain critical [4].
Major advances will be made with the expected implementation of digital pathology within the next decade. As a successor to standard microscopy digital pathology will imply that ultra-high-resolution (fluorescent) scanners for microscopic imaging will become available, and that pathologists will perform diagnostics using high-resolution monitors with “Google Earth”-like zooming technology [5]. This field is expected to progress based on the development and application of specialized software for image analysis: “Apps” for digital pathology. These include algorithms for computer-assisted recognition of cells in which a plethora of cellular data (DNA, RNA, and protein) will be automatically assimilated in a form beyond the current scope of pathological diagnosis and the compass of the human eye [6]. In digital pathology genomic information can be accessed through the various techniques described in this thesis, while proteomic information may be accessed through new developments in spectral flow cytometry, Matrix-assisted laser desorption ionization (MALDI) imaging mass spectrometry (IMS), or a combination of mass spectrometry and flow cytometry [7–9]
Currently, metastatic cancer remains incurable and resistance or unresponsiveness to targeted therapies often develop. Therefore genetic screening should be

performed to identify new combined targeted agents and further efforts must be made to develop better anti-metastatic cancer drugs [10,11]. To identify patients who will benefit from novel, personalized therapies, more specialized methods must be continuously developed and adapted to routine diagnostic pathology [12]. Developments in research and progress in clinical practice make it essential to intensify collaborations between pathologists and clinicians as well as between molecular biologists, bio-informaticians and intermediaries. Database managers will be needed to organize and facilitate the dissemination of the massive amount of complex data.

Currently, the application of high-throughput sequencing in FFPE material to molecular tumor diagnostics and research is not yet widespread. Methods and approaches have been developed that should be applied and evaluated on DNA isolated from FFPE samples. The routine sequencing of whole-cancer genomes will be the ultimate goal in high-throughput sequencing [13,14]. In the meantime, many labs will study the exons of all protein-coding genes in the genome. Alternatively, whole transcriptomes [15] or the epigenome [16] will be analyzed. High-throughput sequencing in molecular tumor diagnostics will most likely begin with the rapid sequencing of smaller subsets of genes on multiple samples with sufficient sequence depth to identify rare somatic variants in heterogeneous tumors [17–19]. This demand can be fulfilled by making use of smaller, faster, bench-top sequencers, such as the Illumina Miseq™ and Life Technologies Ion PGM™ and Ion Proton™ sequencers, in combination with the targeted sequencing of “DNA barcoded” samples [20].

The currently available high throughput or next generation sequencing (HT-NGS) equipment is based on PCR, which may introduce artifacts that will be detected by this sensitive method. Therefore, methods that are suitable for sequencing DNA from a single molecule will eliminate the need for PCR and will be the next step in implementing “third-generation” sequencing in the laboratory. One of the first applications of single-cell sequencing was the Heliscope™ single molecule sequencer [21], and other approaches for single-cell sequencing, such as Single molecule real-time (SMRT™, Pacific Biosciences®) sequencing and *RNA polymerase* (RNAP) sequencing, have been developed [2,22].

Single-molecule nanopore sequencing does not require fluorescent labeling, which could further simplify sample preparation protocols, increase sequencing speed and reduce costs [23]. This method is patented by Oxford Nanopore Technologies® and is applied in the GridION and MinION sequencing systems. The MinION system is a miniaturized, portable device that is the size of a large USB flash drive and may bring next-generation sequencing to the operating room, bedside, or remote areas with few resources (http://www.nanoporetech.com/ accessed September 2012).

The implementation of this third-generation sequencing technology in molecular tumor diagnostics seems to be a matter of time and investment. It remains to be observed if next- or third-generation whole genome sequencing in clinical diagnostics will be cost effective, particularly if additional costs for analysis and data storage are taken into consideration [19]. Sanger sequencing has become

one of the first commercially exploited molecular technologies, and HT-NGS will likely become daily practice in sequencing efforts. In the near future, the basic steps in sample and library preparation will be performed in research and clinical laboratories, while the actual sequencing will be outsourced to commercial companies.

Other issues in the progression toward implementing whole genome sequencing in clinics concern ethical considerations. How will doctors be educated about these testing methods and the interpretation of results? How will we manage novel variants of uncertain significance? How will we address incidental findings? How will concerns regarding privacy, potential abuse and discrimination be tackled [18,24]? These are serious considerations, particularly during a time period when it is possible and affordable for individual persons to have their “own” genome sequenced by commercial companies without any a priori clinical questions. In summary, a challenging future lies ahead; 70 years after Prof. Lignac’s seminal publication, his question can be repeated: “Quo Vadis?”.

As a consequence of the major technological advances in molecular pathology, SNP arrays and MLPA have been developed for the simultaneous analysis of many genetic loci in a relatively limited number of specimens. Applications of hydrolysis probe assays, PCR with M13-tailed primers, HRM as a mutation prescreening method and Sanger sequencing have contributed to the implementation of a largely automated workflow to detect mutations in extended series of samples. The implementation of next-generation sequence technology in molecular pathology is at hand. However, the application of these technologies using degraded DNA isolated from tiny amounts of formalin-fixed and paraffin-embedded material remains a challenge. Specialized analytical tools have been developed by commercial companies. However, these software packages are not always appropriate for the analysis of tumors in which imbalances between wild-type and mutant alleles are often observed. Databases and laboratory information management systems (LIMSs) must be implemented and maintained in the analysis workflow. Original, “raw” and analyzed data should be archived and accessible at all times for data mining, review and future reference. Therefore, the development of dedicated software, analysis approaches and a reliable and secure data storage facility is critical to continuously keep pace with the newest developments and specific demands. In the near future, electronic pathological archives with microscopic images together with whole genome and whole transcriptome sequence data will become as important as the FFPE tissue archives have proven to be over the previous decades.

Finally, molecular outcomes are not as black and white as the pharmaceutical industry would like. Factors such as minimal input, allelic imbalance, tumor heterogeneity and the role of the stroma must be considered and may present major challenges to mutation detection. Small subpopulations of cells, individual circulating tumor cells or tumor DNA may direct future therapeutic decisions and biomarker development, and the stroma is a key element of the tumor microenvironment [25,26]. Consequently, genomic analyses on individual tumor and stromal cells should be performed with an understanding of the risk of

contamination. DNA contamination must be considered because minimal amounts of patient material are available and the sensitivity of the utilized methods is increasing. Standard hydrolysis probe assays detect a mutation content of 1%, and if the wildtype allele is inhibited, a content of 0.1% or less can be identified. High-throughput sequencers can detect one mismatched DNA copy in 1000 or 10,000 reads, depending on the methodology. Digital PCR, which has become the method of choice for detecting circulating tumor DNA or cells, has a detection limit of 1:100,000 [27]. In addition to the challenging question of how patients with a low copy mutant allele will respond to therapeutics, we must realize that DNA, contaminating or not, is present everywhere, and these are challenges for future molecular testing that will require high standards at the test facilities.
These constantly evolving biological and technological insights make it necessary to concentrate molecular analysis in specialized laboratories. Only technologies that are general and widely used and validated can be applied in non-specialized routine laboratories. For instance, at the end of the 19th century H&E staining was first described and a specialized technique that could only be performed in specialized laboratories, but over time, virtually every hospital implemented this technique. In the same way, for some molecular testing methods, it is only a matter of time before tests become available for use in every hospital. However, keeping pace with new biological insights and technological developments and the implementation and maintenance of laboratories for molecular pathology in each hospital will likely be impossible and a waste of resources. More seriously, striving to keep pace with all technological advancements may even contribute to unnoticed false-positive and false-negative diagnoses. Therefore, a more rational direction for molecular tumor diagnostics must be taken, and initiatives for a few specialized centers that can be utilized by many hospitals must be exploited further. These well-equipped centers should have specialized employees to perform the tests and should continuously implement and develop new methods. These combined efforts will contribute to future advances in molecular pathology and will improve and extend the use of archival material to benefit patients.

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