Potential of modern circulating cell-free DNA diagnostic tools for detection of specific tumour cells in clinical practice

Jernej Gašperšič, Alja Videtič Paska*

Medical Centre for Molecular Biology, Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

*Corresponding author: alja.videtic@mf.uni-lj.si

Abstract

Personalized medicine is a developing field of medicine that has gained in importance in recent decades. New diagnostic tests based on the analysis of circulating cell-free DNA (cfDNA) were developed as a tool of diagnosing different cancer types. By detecting the subpopulation of mutated DNA from cancer cells, it is possible to detect the presence of a specific tumour in early stages of the disease. Mutation analysis is performed by quantitative polymerase chain reaction (qPCR) or the next generation sequencing (NGS), however, cfDNA protocols need to be modified carefully in preanalytical, analytical, and postanalytical stages.

To further improve treatment of cancer the Food and Drug Administration approved more than 20 companion diagnostic tests that combine cancer drugs with highly efficient genetic diagnostic tools. Tools detect mutations in the DNA originating from cancer cells directly through the subpopulation of cfDNA, the circular tumour DNA (ctDNA) analysis or with visualization of cells through intracellular DNA probes. A large number of ctDNA tests in clinical studies demonstrate the importance of new findings in the field of cancer diagnosis.

We describe the innovations in personalized medicine: techniques for detecting ctDNA and genomic DNA (gDNA) mutations approved Food and Drug Administration companion genetic diagnostics, candidate genes for assembling the cancer NGS panels, and a brief mention of the multitude of cfDNA currently in clinical trials. Additionally, an overview of the development steps of the diagnostic tools will refresh and expand the knowledge of clinics and geneticists for research opportunities beyond the development phases.

Keywords: cfDNA; NGS; personalized medicine; liquid biopsy; ctDNA

Submitted: March 10, 2020 Accepted: June 20, 2020
To search for genetic mutations in cancer, tissue samples are routinely obtained with tissue biopsy. Modern tissue biopsy is a low-risk procedure, but cannot usually be performed in the early stages of cancer due to the small size of an often heterogeneous tumour mass. In this case, the liquid biopsy, a body fluid collection, represents a beneficial alternative. It allows easy sampling, which can be used for mutation analysis of somatic or tumour cells. Namely, in body fluids apoptotic and necrotic tissue cells release DNA (genomic and mitochondrial) and RNA (5,7-13), that is fragmented into circulating cell-free DNA/RNA (cfDNA/cfRNA). In the presence of tumours, the circulating tumour DNA (ctDNA) is released into the surrounding fluid, which in most cases reaches the blood. Due to the transport from tissue to blood, cfDNA is usually degraded into fragments of 100-280 base pairs (bp), or 280-450 bp and 450-700 bp (di- or tri-lengths of nucleosomal DNA) (14). Cell-free DNA can be detected in plasma and serum, cerebrospinal fluid, saliva, stool, urine, and other body fluids (13,15-18).

Circulating tumour DNA contains information on somatic, hereditary, and acquired mutations. It is an important marker found in body fluids that can be detected during tumour cell apoptosis and necrosis. Cell-free DNA biomarkers are suitable for the detection of early disease stages, relapse control, treatment success, and the development of chemical resistance (19).

Sample preparation and sequencing for cfDNA is almost identical to genomic DNA (gDNA) in the analytical and postanalytical stages, while in the preanalytical stage it requires a completely different set of sampling and processing methods. Cell-free DNA is collected from blood plasma fraction, fragmentation step is not needed.

In the analytical stage, cfDNA analysis by quantitative polymerase chain reaction (qPCR) or next generation sequencing (NGS) is identical to gDNA. Problems can be attributed to DNA artifacts or usage of different internal controls or reference materials.

In the postanalytical stages, technical errors due to the application of different quantification algorithms or discrepancies in calculation, interpretation, and reporting of the results still remain a major problem.

The method for ctDNA analysis with increasingly growing preference is NGS, with which clinically relevant mutations in ctDNA samples from cancer patients have been successfully sequenced (20). Five hundred sixty eight mutations involved in non-small-cell lung cancer, gastrointestinal stromal tumour, colorectal carcinoma, and melanoma were searched for in DNA samples of 40 cancer patients (20). However, the introduction of new DNA technologies requires new genetic training of health care providers; new professions have to be introduced (21).

We have performed a review of modern genetic ctDNA diagnostics approaches for the detection of mutations in cancer associated genes. In order to give medical readers an overview of currently available clinical tests, we included approved cancer diagnostic and companion tests. Additionally, we prepared potential panels of cancer genes for future transition into clinical practice.

Genetic ctDNA diagnostic tools

Cancer is usually manually detected with mammography, colonoscopy, biopsy, and flexible sigmoidoscopy X-ray and computed tomographic (CT) colonography and later graded with histopathological imagery, which is time consuming and tedious task that requires considerable effort, expertise and experience of pathologists (22,23). Diagnosis is difficult due to late stage symptoms (24,25). New techniques use more high-tech approaches based on antibody specific labelling and DNA sequencing. The high-risk patients can be constantly monitored measuring the serum markers often in combination with ultrasonography (26).

Development of evidence based diagnostic methods, used to evaluate the test and guide the diagnosis, need to go through four stages: i) formulation of clinical question from patient’s disease is,
followed by, ii) search of relevant clinical articles, iii) evaluation of evidence for its validity and iv) usefulness is needed to implement the evolved disease diagnosis into clinical practice. To solve the disease diagnostic problem, a complete analytical process has to be implemented: all phases of pre-analytics (DNA sampling, dissolution, clean up, preconcentration and separation, storage), analytics (DNA mutation detection), and postanalytics (data analysis and interpretation) have to be developed. Preanalytical stage, sample collection, handling, and processing is an important step, as improper handling may lead to false diagnosis, while analytical and postanalytical problems are method dependent.

If tissue biopsy is possible, in situ hybridization (ISH) technique enables visual processing of mutation carrying cells through chromophore (chromogenic in situ hybridization - CISH) or fluorophore (fluorescence in situ hybridization - FISH). In situ hybridization technique is a technique where a probe – labelled single-stranded DNA or RNA – selectively binds to a specific target site of the cellular DNA or RNA (27). Detection can be performed through chromogenic or fluorescent signal analysis. Chromogenic in situ hybridization is used to determine gene amplification, gene deletion, chromosome translocation, and chromosome number (28). Fluorescence in situ hybridization additionally offers a multiplex option; it is possible to detect multiple targets in a single sample (29). In situ hybridization methods have certain advantages compared to other methods (Table 1).

Liquid biopsy enables easy sample collection, that can be used for mutation analysis of both somatic or tumour cells. Finger-stick capillary blood can be used as an alternative modern method for blood collection (18). Quantitative PCR or NGS enable fast, precise results. If the subpopulation of ctDNA is detected, tumour presence is confirmed. With the progression of the tumour, the share of ctDNA will increase (Figure 1).

**Quantitative and droplet digital PCR**

A real-time polymerase chain reaction (real-time PCR), also referred to as qPCR, is a polymerase enzyme-based technique (30). The limit of validated qPCR methods is above 1% ct/cfDNA (13). Low detection is associated with method error; mostly it is attributed to technical error of preanalytical stage (standardization of sampling, sample storage, and preparation), analytical stage (use of different internal controls, reference material, for as-

|                | Sanger sequencing | NGS | qPCR | FISH | CISH |
|----------------|-------------------|-----|------|------|------|
| Tumour biopsy  | gDNA              | gDNA| gDNA | gDNA in fixed cells | gDNA in fixed cells |
| Liquid biopsy  | cfDNA             | cfDNA| cfDNA | / | /     |
| Sequence information | partial sequence | sequence | partial sequence | point mutation | point mutation |
| Time of analysis | 7 days | 3 days | 4h | 4h | 4h |
| Precision      | nucleotide resolution | nucleotide resolution | mutation resolution | mutation resolution | mutation resolution |
| Possibility of simultaneous sample analysis | - | + | + | - | - |
| Possibility of simultaneous gene analysis | + | + | - | + (a few) | - |
| Costs of a few sample analysis | high | high | low | medium | low |
| Costs of high throughput analysis | high | low | low | medium | medium |

+ – possible, - – not possible. NGS – next generation sequencing, qPCR – quantitative polymerase chain reaction, FISH – fluorescence in situ hybridization, CISH – chromogenic in situ hybridization, gDNA – genomic DNA, cfDNA – circulating cell-free DNA.
Gašperšič J, Videtič Paska A. Modern cancer DNA diagnostic tools

sessing the analytical performance, running analysis on different systems/platforms) or postanalytical stage (using different quantification algorithms, discrepancies in calculation and interpretation of the results, differences in reporting results) (30). The major problem (comparison of preanalytical, analytical, and postanalytical phases) of qPCR is that only a small number of genes can be analysed (Table 1). With an alternative method – the droplet digital PCR (ddPCR) we can determine ctDNA concentrations quantitatively and sensitively with better accuracy (31). The sample is sprayed into drops, where only one or zero copies of DNA exists (32). Droplet digital PCR then measures the signals in the absolute way as positive or negative (binary system). It is cheap, fast, but mutations must be tested sequentially; discovery of new mutations is impossible (13) (Table 1).

Sanger sequencing

Sanger sequencing has been the main DNA sequencing technology for more than 30 years (33,34). This method is based on synthesizing DNA on a single strand DNA matrix, randomly integrating dideoxi-nucleotide chain terminators (34). In 1990, the method was upgraded to label terminators with different coloured dyes, so that all can be integrated into a single reaction (35). Sanger sequencing is perfect for DNA sequencing of tissue samples, while a small amount of ctDNA in liquid biopsy prevents sequential analysis of multiple target genes (13). Major problems of Sanger sequencing are high costs, low sample processibility, and long analysis time (Table 1). High background noise (associated with undesired priming, contamination, frame shift mutation, etc.) may lead to DNA sequence determination error.
Next generation sequencing technology

Next generation sequencing methods are new technologies that are able to sequence a large number of samples with index-labelled DNA oligonucleotides (multiplexing). Next generation sequencing detectors monitor the addition of labelled nucleotides to already synthesized immobilized complementary DNA templates generated from the source DNA. Next generation sequencing systems offer reading lengths of 30-400 bp (13). Due to a large amount of information, the alignment of the sequences must be handled with the software. The software then automatically annotates the data with a variation/mutation database. The main NGS platforms are: Illumina, Thermoscientific, BGI Genomics, Agilent Technologies, Qiagen, Macrogen, Pacific Biosciences California, Genewiz, 10x Genomics, Oxford Nanopore Technologies (13). In recent years, the affordability of NGS sequencing technology has lowered the price of whole genome sequencing (WGS). The costs for the WGS – entire genome sequence fell from 2.7 billion euros in 2003 to only 200 euros (on black Friday) and are even expected to fall (36).

From body fluid, blood cells or tissue, DNA can be isolated and further processed with special NGS preparation kits. DNA must be fragmented, repaired and adapter marked. There are several fragmentation methods that use ultrasound, enzymes, or chemicals. In the case of cfDNA, due to its fragmented state, no additional fragmentation procedure is needed.

The targets may be specific genes enriched with an NGS panel of cancer-related gene primers. The NGS panels can be custom-made or ordered (e.g. Illumina, Agilent, LifeTechnologies). In this way, instead of WGS, sequencing is limited only to parts of the human chromosomes.

The NGS technique has several advantages over other methods (Table 1). It can be applied to all pathological conditions as it also enables the discovery of new DNA mutations. The major problem, the disadvantage of NGS is limited analytical ctDNA sensitivity (13), but the technology is evolving and sensitivity is expected to increase (Table 1). Low detection is associated with method error (incorrect calling of DNA bases or sequence variants), where artefacts in DNA sequence originate from preanalytical sample preparation, sequencing system platforms, or post sequencing data analysis. NGS enables the detection of somatic mutations under 5% (37). With the improvement of processes involved with sequencing, we can increase ctDNA detection in samples.

In clinical settings the NGS technology is already tested, but, is for now still too expensive to be used worldwide, due to high initial investment. The extra costs of specifically educated personnel and technology (material and service) are slowing down the possibility of global hospital use.

All methods have to undergo rather long way to achieve standardization, pass through quality control in order to transfer into clinical practice (Figure 2).

Cancer related candidate genes with potential of NGS panel assembly

The NGS cancer detection panel can be composed of a set of primers for genes involved in the specific tumour formation or tumour group. The sequencing of selected genes allows higher coverage and reduces analysis costs compared to whole genome sequencing. The advantage of the panel is that new genes can be easily added (38). Panels for breast, colon, hepatocellular pancreatic, and non-small cell lung cancer can be designed from the listed genes (Supplementary Table 2-6). Described cancers are spread worldwide and very difficult to detect at an early stage.

Breast cancer

Breast cancer is the most common type of cancer in women worldwide (39). It is the second most common cancer. It is usually manually discovered on mammograms and later graded with histopathological images, which is a time-consuming and lengthy task that requires considerable effort, expertise, and experience from pathologists (22). New techniques use more high-tech approaches for cancer detection, such as antibody-specific la-
Gašperšič J, Videtič Paska A. Modern cancer DNA diagnostic tools

Belling or sequencing of DNA. There is a connection between the degree of breast cancer and the mutation that caused it (40). However, enormous differences were found between inter- and intra-laboratory interpretations of the classification of breast cancer (41).

Data mining was performed in the scientific literature bases and internet sites: clinicaltrials.gov and the genetic testing registry database (42-49). Mutations in \textit{BRCA1} and \textit{BRCA2} are responsible for 2/3 of familial breast cancer (50). The rest mostly cover mutations in genes \textit{ATM}, \textit{CHECK2}, \textit{PALB2}, \textit{PTEN}, \textit{TP53}. The \textit{ERBB2} (HER2) gene is the most sequenced gene in recent diagnostics. Genes involved in breast cancer, suitable for the development of diagnostic tools are \textit{TP53, CDH1, PALB2, ATM CHEK2, RAD51D, BARD1, BLM CDKN2A, FANC MRE11A, RAD50, APC HOXB13 and MITB} (51). Genes encode proteins that are involved in cell adhesion, cell growth, DNA repair mechanisms, and tumour suppression (Supplementary Table 2). Irreversible mutations in high-risk genes can cause damage that leads to the development of cancer cells and later somatic tumours (Supplementary Table 2). The database of clinical trials shows that several tests are waiting for approval. The genetic diagnostics were developed on the genes: \textit{KRAS, PD-L1, ER, PIK3CA, BRCA1, BRCA2, BRCA2, EGF, HER-2, C-MYC, PTEN, MET, IGFR-1}. Genes belong to the known oncogenes and tumour suppressors (clinicaltrials.gov). The Genetic Testing Registry NCBI database lists 34 genes used for breast cancer detection used in 613 tests: \textit{AKT1, AR, ATM, BAPM, BARD1, BRCA1, BRCA2, BRCA3, BRIP1, CASP8, CDH1, CHEK2, CYP2D6, ERBB2, ESRI, HMMR, IL1B, IL1RN, KISS1R, KRAS, LFS3, MKRN3, NQO2, PALB2, PHB, PIK3CA, PPM1D, PTEN, RAD51, RAD54L, RB1CC1, SLC22A18, TP53, XRCC3} (https://www.ncbi.nlm.nih.gov/gtr/).

**Colorectal cancer**

Colorectal carcinoma is one of the common widespread types of cancer (3rd most common diag-
nosed malignancy, 4th leading cause of cancer worldwide). It is usually treated with chemotherapy and EGFR antibodies (23). Early diagnostic methods for detection are colonoscopy, biopsy, and flexible sigmoidoscopy and computed tomographic (CT) colonography (www.nice.org.uk).

Gene candidates involved in cancer formation are stated in Supplementary Table 3 (52). Modern diagnostics for colorectal cancer detect mutations in KRAS, NRAS, and EGFR genes. Expressed proteins influence on cell proliferation and differentiation. High risk genes that can be used in the development of cancer diagnostics are APC, MLH1, MSH2, MSH6, POLE, TGFBR2, MLH3, POLD1, MUTYH, and AXIN2 (52). Mostly proteins act as tumour suppressors or are involved in DNA repair (Supplementary Table 3) (53-63).

Clinical trials database holds information regarding mutations of KRAS, NRAS, and BRAF. In NCBI database genetic testing registry 33 genes are listed for colorectal cancer detection in 584 tests: AKT1, APC, AXIN2, BUB1B, CRC56, CRC57, CTNNB1, DCC, DMC1, EP300, EPACAM; FGFR3, FLCN, G6PC, GPC3, GPC4, H19, HFE, HMBS, IFNAR2, IFN6, IFN13, IGF2, IGF2R, IL10RB, JAG1, JAK2, MET, MPV17, PDGFRL, PIK3CA, PT-PRC, RAS, SERPINA1, SLC25A13, SLC37A4, SPRTN, TJP2, TP53, UGT1A1 (https://www.ncbi.nlm.nih.gov/gtr/).

**Hepatocellular carcinoma**

Liver cancer is the sixth most common cancer. The most common type of liver cancer is hepatocellular carcinoma (24). High-risk patients are constantly monitored measuring the serum marker alpha-fetoprotein (AFP) often in combination with ultrasoundography (26).

The tumour appears to be regulated by the Wnt/β-catenin signalling pathway. In hepatitis-induced hepatocellular carcinoma, β-catenin mutations are present in 13–41% of cases. In more than 55% of the cases, the mutations are present in the GSK-3β region of the β-catenin gene (64).

Diagnostics for hepatocellular carcinoma can be developed by screening high risk cancer genes for mutations in CCNB1, CEP55, CHEK1, EZH2, KPN2A, LRRC1, PKB, RRM2, SLC7A11, SUCCO, ZWINT (Supplementary Table 4), that are up-regulated and ACLS1, CDC37L1 (Supplementary Table 4), that are down-regulated (65). Up-regulated genes are involved in the process of duplication, differentiation, and the biosynthesis. Down-regulated genes are involved in biosynthesis of lipids and transcription of RNA (Supplementary Table 4) (45,66-73).

The vast number of hepatocellular carcinoma tests waits Food and Drug Administration (FDA) approval. Clinical trials tests screen whole genomes or specific genes for mutations (clinicaltrials.gov). In NCBI database “genetic testing registry” 38 genes are listed for colorectal cancer detection in 94 tests: ABCB11, APC, ATF7B, AXIN1, BMP2, CASP8, CCR5, CTNNB1, F5, FAH, G6PC, GPC3, GPC4, H19, HFE, HMBS, IFNAR2, IFN6, IFN13, IGF2, IGF2R, IL10RB, JAG1, JAK2, MET, MPV17, PDGFRL, PIK3CA, PT-PRC, RAS, SERPINA1, SLC25A13, SLC37A4, SPRTN, TJP2, TP53, UROD, (https://www.ncbi.nlm.nih.gov/gtr/).

**Non-small cell lung cancer**

Lung cancer is the leading cause of death worldwide (25,74). It is graded as small cell and non-small cell lung cancer (NSCLC) types (25). NSCLC cancer is difficult to diagnose in early phases and first cancer signs are usually detected with X-ray and computed tomography (CT) (25).

Non-small cell lung cancer with mutations of epidermal growth factor receptor (EGFR) mutations, anaplastic lymphoma kinase (ALK) mutations, ROS proto-oncogene 1 (ROS1) rearrangement, mesenchymal-epithelial transition (MET) factor amplification, v-Raf murine sarcoma viral oncogene homolog B (BRAF) mutations, human epidermal growth factor receptor 2 (HER2) mutations, and RET rearrangement respond well to treatment (75).

Modern diagnostics for non-small cell lung cancer were developed on high risk cancer genes such as EGFR, PD-L1, ALK, BRAF (Supplementary Table 5), coding proteins involved in cell proliferation, and immune system evasion (Supplementary Table 5) (76-88).

More than 200 tests await in clinical trial settings. Genes MET, KRAS, NRAS, EGFR, FG, VEGF, PDGF, ALK, ROS1, HER2, HER3, BRAF are tested in diagnostic kits for mutations. Genes belong to known oncogenes, responsible for proliferation, tumour suppression
(Supplementary Table 5). In Supplementary Table 5 other candidate genes involved in NSCLC cancer formation are stated. In NCBI database “genetic testing registry” 51 tests are listed for NSCLC cancer detection on genes: ROS1, ALK, MET, ERBB2, KRAS, RET, EGFR, TYMS, RRM1, FGFR1, ERCC1, BRAF (https://www.ncbi.nlm.nih.gov/gtr/).

Pancreatic cancer

Pancreatic ductal adenocarcinoma (PDAC) is the most common type of pancreatic cancer. It is the twelfth most common cancer and the seventh most frequent cause of cancer-related death (89). Diagnosis is performed with CT or magnetic resonance (MR).

Familial pancreatic cancer mutations residues are located mostly on BRCA1, BRCA2, p16, PALB2 genes. BRCA2 mutations are highly associated with familial and sporadic pancreatic cancers (90).

Important genes determined to be involved in pancreatic cancer are stated in Supplementary Table 6 (91-95). Modern diagnostics for pancreatic cancer are still in the phase of clinical testing. Clinical research of diagnostics is being developed on high risk cancer genes such as APC, ATM, BARD1, BRCA1, BRCA2, BRIPI, BMPRT1A, CDH1, CDK4, CDKN2A, CHEK2, EPCAM, GREM1, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, NF1, PALB2, PMS2, POLD1, POLE, PTEN, RAD50, RAD51C, RAD51D, SMAD4, SMARCA4, STK11, TP53 (clinicaltrials.gov). Researched proteins belong to the family of oncogenes, tumor suppressors. Some are involved in cell proliferation. In NCBI database genetic testing registry 28 genes are listed for pancreatic cancer detection in 287 tests: AKT1, ATM, BARD1, BRCA1, BRCA2, BRIPI, CASP8, CDH1, CDKN2A, CHEK2, ESR1, HMMR, KRAS, NQO2, PALB2, PALLD, PHB, PIK3CA, PPM1D, RAD51 (https://www.ncbi.nlm.nih.gov/gtr/).

FDA approved cancer diagnostic tests

The drug has to go through 4 clinical phases to determine its safety, efficiency, and dosage in advance of FDA approval (www.fda.gov). After initial discovery, a lot of time has to be invested into its development. Development is concluded with preclinical in vitro laboratory tests and in vivo animal studies. Safety is afterwards performed with tests on human subjects. Each of the clinical phases sections take a specific time period to complete the defined tasks (Figure 2). When clinical phases are completed, active monitoring of the drug begins. FDA reviews the drug documentation and in parallel monitor’s safety of the drug.

According to the FDA, companion diagnostic is a device that gives information regarding the safe and effective usage of the corresponding drug or biological product. Different genetic tests for mutation detection were developed and later approved by the FDA for different types of cancers (Supplementary Table 1). Mutation detection kits are gaining influence in clinical trials, where together with antibody and probe detection kits (immunohistochemical staining – IHS, western blot – WB) represents the majority of detection kits for use in personalized medicine (Supplementary Table 1).

Cobas EGFR Mutation Test v2 (Roche Molecular Systems, Inc.) was approved on 1 June 2016 (FDA) for the detection of non-small cell lung cancer. The test is part of a companion diagnostic with the cancer drug Tarceva (erlotinib) (96). It detects epidermal growth factor receptor gene mutations in non-small cell lung cancer patients (10-20% of all lung cancer) (FDA) (96). Cobas EGFR Mutation Test v2 was tested on blood samples of positive patients for 42 EGFR mutations on exon 18, 19, 20, 21 mutations as determined by the Test v1 (FDA). Drug Tarceva should work if one of the mutations is found in tumor DNA. Mutations can be discovered on gDNA and cfDNA samples.

Similar test the RealTime IDH1 Assay was approved in July 2018. A companion diagnostic test can be used to detect specific mutations in the IDH1 gene in patients with acute myeloid leukemia (FDA). Tivosovo is an inhibitor of isocitrate dehydrogenase. The drug is administered if the test for gDNA mutation (isolated from white blood cells) comes out positive.

Approved diagnostic tests by FDA are optimized mostly for gDNA samples. Recently ctDNA technologies are gaining ground in the segment of companion diagnostics. In a few years, we expect...
a whole set of ctDNA diagnostics to be approved and enter clinical settings.

A large number of tests in clinical trials shows the importance of emerging technology. At the beginning of the 2020 year, 537 ctDNA and 368 cfDNA clinical tests for different diseases were found to be awaiting approval (clinicaltrials.gov). Easy sampling (liquid biopsy) and processing needed for ctDNA testing is a major advantage of tests. The majority (non-ISH) of the already approved tests (Supplementary Table 1) can be modified to use cfDNA or ctDNA instead of gDNA, due to identical DNA source (cellular gDNA). In order to switch to cfDNA analysis, protocols for DNA isolation and DNA preparation for sequencing, need to be modified. Major points that should be addressed before handling the cfDNA are associated with the ability to isolate very small amounts of cfDNA and the prevention of leukocyte lysis. Therefore, during the transport extreme high and low temperatures, and agitations should be avoided. Plasma preparation using filtration or centrifugation should be performed prior to leukocyte lysis, within 6 hours after the blood draw using anticoagulant tubes K$_2$EDTA. Furthermore, freeze-thaw cycles should be minimized to only one cycle in order to prevent nucleic acid degradation and the diminished capability of cfDNA detection. The isolation procedures vary, and numerous commercial kits are available. The versatility of purification protocols has an influence on the cfDNA purity and yield, which can reflect also on the downstream procedures, and therefore determination of optimal approach is crucial. In the sequential analytics step, it is of great importance to use appropriate reference material for the valuation of the analytical performance (97).

Conclusions

This review describes the breakthroughs in modern diagnostic techniques that are recently approved or are in clinical trials. The overview will help geneticists to refresh the knowledge of drug or diagnostic development phases from the beginning and show which segment will surely prosper in the future.

Liquid biopsy analysis is becoming one of the important less invasive analysis tools. Precise knowledge of gene function and their role in the particular disease will help to detect causes of disease and prescribe preventive action. Genetic and epigenetic studies of oncogenes, tumour suppressors, and associated genes will shed new light on cancer development and diagnosis. Next generation sequencing developed analytical methods will bring a new era in precise personalized treatment, improve the usefulness and effectiveness of the medication.

The vast number of ctDNA and cfDNA tests registered in clinical phases shows the importance of new emerging technologies. FDA approved the first set of companion diagnostic tool in 1998 (HER2-trustuzumab). For cancer diagnostic whole set of companion diagnostic tools was approved later on. The tests use immunohistochemistry (IHC), in situ hybridization technique (CISH and FISH), PCR, qPCR, Sanger, and new NGS technology to detect specific mutation or overexpressed proteins (IHC). The diagnostic is coupled to the pharmaceutical drug that is very efficient for curing specific target tumours. Innovations in personalized medicine, such as precise genetic analysis of genome and acquired mutations, give new information regarding predisposition to certain diseases, and predict the effectiveness of the discovered drug. This allows a higher chance of recuperation.

In the future, the evolution of personalized medicine will enable the personalized treatment of disease/condition with the predicted course of treatment. The development of analytical tools will lead to the approval of urine cfDNA tests, which will even further facilitate sampling and screening of the patients. Most likely NGS panels for detection of cancers will be improved with a complete set of disease associated genes.

Acknowledgements

This work was partially supported by Slovenian Research Agency (program P1-0390).

Potential conflict of interest

None declared.
References

1. Oellerich M, Schutz E, Beck J, Kanzow P, Plowman P N, Weiss G J, et al. Using circulating cell-free DNA to monitor personalized cancer therapy. Crit Rev Clin Lab Sci. 2017;54:205-18. https://doi.org/10.1080/10408363.2017.1299683

2. Ehrich M, Tyman J, Mazloom A, Almasri E, McCullough R, Boomer T, et al. Genome-wide cfDNA screening: clinical laboratory experience with the first 10,000 cases. Genet Med. 2017;19:1332-7. https://doi.org/10.1038/gim.2017.56

3. Neal AJ, Hoskin PJ. eds Clinical oncology. Boca Raton, London, New York: CRC press; 2012.

4. Novaković S, Hočevar M, Novaković BJ, et al., eds. [Onkologia]. Ljubljana: emka.si; 2009 (in Slovenian).

5. Shackleton M, Quintana E, Fearon ER, Morrison SJ. Heterogeneity in cancer: cancer stem cells versus clonal evolution. Cell. 2009;138:822-9. https://doi.org/10.1016/j.cell.2009.08.017

6. Jacobs K B, Yeager M, Zhou W, Wacholder S, Wang Z, Rodriguez-Santiago B, et al. Detectable clonal mosaicism and its relationship to aging and cancer. Nat Genet. 2012;44:651-8.

7. Otandault A, Anker P, Al Amir Dache Z, Guillaumon V, Meddeb R, Pastor B, et al. Recent advances in circulating nucleic acids in oncology. Ann Oncol. 2019;30:374-84. https://doi.org/10.1093/annonc/mdz031

8. Boussiós S, Ozturk MA, Moschetta M, Karathanasi A, Smith N, Kota K, et al. Whole genome amplification of cell-free DNA enables detection of circulating tumor DNA mutations from fingerstick capillary blood. Sci Rep. 2018;8:17313. https://doi.org/10.1038/s41598-018-35470-9

9. Gai W, Sun K. Epigenetic biomarkers in cell-free DNA and applications in liquid biopsy. Genes (Basel). 2019;10:32. https://doi.org/10.3390/genes10010032

10. Novaković S, Hočevar M, Novaković BJ, et al. Using circulating cell-free DNA to monitor personalization in exercise physiology. Sports Medicine. 2018;42:565-86. https://doi.org/10.2165/11631380-00000000-0000-0

11. Podlesny S, Figueiro-Silva J, Llado A, Antonell A, Sanchez-Valle R, Alcolea D, et al. Low cerebrospinal fluid concentration of mitochondrial DNA in preclinical Alzheimer disease. Ann Neurol. 2013;74:655-68. https://doi.org/10.1002/ana.23955

12. Cheng TH, Jiang PY, Tam JCW, Sun X, Lee WS, Yu SCY, et al. Genomewide bisulfite sequencing reveals the origin and time-dependent fragmentation of urinary cfDNA. Clin Biochem. 2017;50:496-501. https://doi.org/10.1016/j.clinbiochem.2017.02.017

13. Volckmar AL, Sultmann H, Riediger A, Fioretos T, Schirmer P, Jordana-Ariza N, et al. Development of a gene panel for next generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients. Br J Cancer. 2017;116:802-10. https://doi.org/10.1038/bjc.2017.8

14. Keppens C, Dufraing K, van Krieken HJ, Siebers AG, Kafatos G, Lowe K, et al. European follow-up of incorrect biomarker results for colorectal cancer demonstrates the importance of quality improvement projects. Virchows Arch. 2019;475:25-37. https://doi.org/10.1007/s00428-019-02525-9

15. Hemken PM, Sokoll LJ, Yang XQ, Dai JL, Elliott D, Gawel SH, et al. Validation of a novel model for the early detection of hepatocellular carcinoma. Clin Proteomics. 2019;16:2. https://doi.org/10.1186/s13104-019-4121-7

16. Blandin Knight S, Crosbie PA, Balata H, Chudziak J, Hulset T, Dive C. Progress and prospects of early detection in lung cancer. Open Biology. 2017;7:170070. https://doi.org/10.1098/rsob.170070

17. Aksac A, Demetrick DJ, Ozyer T, Alhajj R. BreCaHAD: a data-set for breast cancer histopathological annotation and diagnosis. BMC Res Notes. 2019;12:82. https://doi.org/10.1186/s13104-019-0548-4

18. Volckmar AL, Sultmann H, Riediger A, Fioretos T, Schirmer P, Jordana-Ariza N, et al. Development of a gene panel for next generation sequencing of clinically relevant mutations in cell-free DNA from cancer patients. Br J Cancer. 2017;116:802-10. https://doi.org/10.1038/bjc.2017.8

19. Keppens C, Dufraing K, van Krieken HJ, Siebers AG, Kafatos G, Lowe K, et al. European follow-up of incorrect biomarker results for colorectal cancer demonstrates the importance of quality improvement projects. Virchows Arch. 2019;475:25-37. https://doi.org/10.1007/s00428-019-02525-9

20. Hemken PM, Sokoll LJ, Yang XQ, Dai JL, Elliott D, Gawel SH, et al. Validation of a novel model for the early detection of hepatocellular carcinoma. Clin Proteomics. 2019;16:2. https://doi.org/10.1186/s13104-019-4122-0

21. Blandin Knight S, Crosbie PA, Balata H, Chudziak J, Hulset T, Dive C. Progress and prospects of early detection in lung cancer. Open Biology. 2017;7:170070. https://doi.org/10.1098/rsob.170070

22. Bialecki ES, DiBisceglie MA. Diagnosis of hepatocellular carcinoma. HPB (Oxford). 2005;7:26-34. https://doi.org/10.1080/13651820410024049

23. Atabati H, Raoofi A, Amini A, Farahani RM. Evaluating HER2 gene amplification using chromogenic in situ hybridization (CISH) method in comparison to immunohistochemistry method in breast carcinoma. Open Access Maced J Med Sci. 2018;6:1977-81. https://doi.org/10.3889/oamjms.2018.455
28. Nitta H, Kelly B. Chromogenic tissue-based methods for detection of gene amplifications (or rearrangements) combined with protein overexpression in clinical samples. Methods Mol Biol. 2019;1953:301-14. https://doi.org/10.1007/978-1-4939-9145-7_19

29. Jiang J. Fluorescence in situ hybridization in plants: recent developments and future applications. Chromosome Res. 2019;27:153-65. https://doi.org/10.1007/s10577-019-09607-z

30. Quan PL, Sauzade M, Brouzes E. dPCR: A technology review. Sensors (Basel). 2018;18:1271. https://doi.org/10.3390/s18041271

31. Wan JCM, Massie C, Garcia-Corbacho J, Mouliere F, Brenton JD, Caldas C, et al. Liquid biopsies come of age: towards implementation of circulating tumour DNA. Nat Rev Cancer. 2017;17:223-38. https://doi.org/10.1038/nrc.2017.7

32. Pabinger S, RÄdiger S, Krieger A, Vierlinger K, Weinhausel A. A survey of a tools for the analysis of quantitative PCR (qPCR) data. Biomol Detect Quantif. 2014;1:23-33. https://doi.org/10.1016/j.bdq.2014.08.002

33. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci. 1977;74:5463-7. https://doi.org/10.1073/pnas.74.12.5463

34. Hall N. Advanced sequencing technologies and their wider impact in microbiology. J Exp Biol. 2007;210:1518-25. https://doi.org/10.1242/jeb.013170

35. Smith LM, Sanders JZ, Kaiser RJ, Hughes P, Dodd C, Connell JD, Caldas C, et al. Targeted sequencing of established and candidate colorectal cancer genes in the Colon Cancer Family Registry Cohort. Oncotarget. 2017;8:93450-63. https://doi.org/10.18632/oncotarget.18596

36. Molteni M. Now You Can Sequence Your Whole Genome for Just $200. Wired. 2018.

37. Page K, Guttery DS, Fernandez-Garcia D, Hills A, Hastings M, Hart SN, Vijai J, Schrader KA, Slavin TP, Thomas M, et al. Targeted sequencing of established and candidate colorectal cancer genes in the Colon Cancer Family Registry Cohort. Oncotarget. 2017;8:93450-63. https://doi.org/10.1016/j.celrep.2018.12.071

38. Tskin D, Yan D, Bademci G, Feng Y, Guo S, Foster J, et al. A next-generation sequencing gene panel (MiamiOtoGene) for comprehensive analysis of deafness genes. Hear Res. 2016;333:179-84. https://doi.org/10.1016/j.heares.2016.01.018

39. Al Hannan F, Keogh MB, Taha S, Al Buainain L. Characterization of BRCA1 and BRCA2 genetic variants in a cohort of Bahraini breast cancer patients using next-generation sequencing. Mol Genet Genomic Med. 2019;7:e00771. https://doi.org/10.1002/mgg3.771

40. Purrington KS, Slettedahl S, Bolla MK, Michailidou K, Czene K, Nevanlinna H, et al. Genetic variation in mitotic regulatory pathway genes is associated with breast tumor grade. Hum Mol Genet. 2014;23:6034-46.

41. van Dooijeweert C, van Diest PJ, Willems SM, Kuipers C, van der Wall E, Overbeek LH, et al. Significant inter- and intra-laboratory variation in grading of invasive breast cancer: A nationwide study of 33,043 patients in the Netherlands. Int J Cancer. 2020;146:769-780. https://doi.org/10.1002/ijc.32330

42. Jeong EH, Lee TG, Ko YJ, Kim SY, Kim HR, Kim H, et al. Anti-tumor effect of CDK inhibitors on CDK2A-defective squamous cell lung cancer cells. Cell Oncol (Dordr). 2018;41:663-75. https://doi.org/10.1007/s13402-018-0404-6

43. Jezek M, Green E M. Histone modifications and the maintenance of telomere integrity. Cells. 2019;8:199. https://doi.org/10.3390/cells8020199

44. de Almeida BP, Apolônio JD, Botta M, Castelo-Branco P. Roadmap of DNA methylation in breast cancer identifies novel prognostic biomarkers. BMC Cancer. 2019;19:219. https://doi.org/10.1186/s12885-019-5403-0

45. Asplund E, Raskin L, Guo Y, Du L P, Clendenning M, Lindor N, et al. Epigenetic suppression of the T-box subfamily 2 (TBX2) in human non-small cell lung cancer. Int J Mol Sci. 2019;20:11159. https://doi.org/10.3390/ijms20051159

46. Oussalah A, Rischer S, Bensenane M, Conroy G, Filhkreterrius P, Debard R, et al. Plasma mSEPT9: A novel circulating cell-free DNA-based epigenetic biomarker to diagnose hepatocellular carcinoma. EBioMedicine. 2018;30:138-47. https://doi.org/10.1016/j.ebiom.2018.03.029

47. Park JW, Baek IH, Kim YT. Preliminary study analyzing the methylated genes in the plasma of patients with pancreatic cancer. Scand J Surg. 2012;101:38-44. https://doi.org/10.1177/145749691210100108

48. Jarman EJ, Ward C, Tumbull AK, Martinez-Perez C, Meehan J, Xintapoulou C, et al. HER2 regulates HIF-2α and drives an increased hypoxic response in breast cancer. Breast Cancer Res. 2019;21:10. https://doi.org/10.1186/s13058-019-1097-0

49. Santoro A, Vlachou T, D'Elia E, et al. p53 loss in breast cancer leads to Myc activation, increased cell plasticity, and expression of a mitotic signature with prognostic value. Cell Rep. 2019;26:624–38.e8. https://doi.org/10.1016/j.celrep.2018.12.071

50. Catana A, Apestou AP, Antoniou RG. Multi gene panel testing for hereditary breast cancer - is it ready to be used? Med Pharm Rep. 2019;92:220-5. https://doi.org/10.15136/mpr-1083

51. Maxwell KN, Hart SN, Vijai J, Schrader KA, Slavin TP, Thomas M, et al. Evaluation of ACMG-guideline-based variant classification of cancer susceptibility and non-cancer-associated genes in families affected by breast cancer. Am J Hum Genet. 2016;98:801-17. https://doi.org/10.1016/j.ajhg.2016.02.024

52. Sokin L, Guo Y, Du L P, Clendenning M, Rosty C, Lindor N M, et al. Targeted sequencing of established and candidate colorectal cancer genes in the Colon Cancer Family Registry Cohort. Oncotarget. 2017;8:93450-63. https://doi.org/10.18632/oncotarget.18596

53. Wang D, Liang S, Zhang X, Dey SK, Li Y, Xu C, et al. Targeted next-generation sequencing approach for molecular genetic diagnosis of hereditary colorectal cancer: Identification of a novel single nucleotide germline insertion in adenomatous polyposis coli gene causes familial adenomatous polyposis. Mol Genet Genomic Med. 2019;7:e00505. https://doi.org/10.1002/mgg3.505

54. Arshita N, Lestari RV, Hutajulu SH, Ghozali A, Paramita DK. The tendency of having MSH2 and MSH6 instability among clinicopathological features in patients with...
colorectal cancer. Asian Pac J Cancer Prev. 2018;19:3147-52. https://doi.org/10.3357/APCP.2018.19.11.3147

55. Gallo G, Sena G, Vescio G, Pappadreata M, Sacco R, Trompetto M, et al. The prognostic value of KRAS and BRAF in stage I-III colorectal cancer. A systematic review. Ann Ital Chir. 2019;90:127-37.

56. Damin F, Galbiati S, Soriani N, Burgio V, Ronzoni M, Ferrari M, et al. Analysis of KRAS, NRAS and BRAF mutational profile by combination of in-tube hybridization and universal tag-microarray in tumor tissue and plasma of colorectal cancer patients. Plos One. 2018;13:e0207876. https://doi.org/10.1371/journal.pone.0207876

57. Cocco E, Benhamida J, Middha S, Zehr A, Mullaney K, Shia J, et al. Colorectal carcinomas containing hypermethylated MLH1 promoter and wild type BRAF/KRAS are enriched for targetable kinase fusions. Cancer Res. 2019;79:1047-53. https://doi.org/10.1158/0008-5472.CAN-18-3126

58. Vande Perre P, Siegfried A, Corsini C, Bonnet D, Toulas C, Cocco E, Benhamida J, et al. Germline mutation p.N363K in POLE is associated with an increased risk of colorectal cancer and giant cell giglusteroma. Fam Cancer. 2019;18:173-8. https://doi.org/10.1007/s10689-018-0102-6

59. Kamiza AB, Wang WC, You JF, Tang R, Wang YT, Chien HT, et al. Modern cancer DNA diagnostic tools — construction of a competing endogenous RNA regulatory network in hepatocellular carcinoma. Onco Targets Ther. 2019;12:561-76. https://doi.org/10.2147/OTT.S188913

60. Widy L, Huang JX, Jiang MH. RRM2 computational phosphoprotein network construction and analysis between no-tumor hepatities/cirrhotic liver tissues and human hepatocellular carcinoma (HCC). Cell Physiol Biochem. 2010;26:303-10. https://doi.org/10.1159/000320553

61. Bugide S, Green MR, Wajapeyee N. Inhibition of Enhancer of zeste homolog 2 (EZH2) induces natural killer cell-mediated eradication of hepatocellular carcinoma cells. Proc Natl Acad Sci U S A. 2018;115:E3509-E18. https://doi.org/10.1073/pnas.1802691115

62. Gu J, Liu XR, Li J, He Y. MicroRNA-144 inhibits cell proliferation, migration and invasion in human hepatocellular carcinoma by targeting CCNB1. Cancer Cell Int. 2019;19:15. https://doi.org/10.1186/s12935-019-0729-x

63. Li M, Gao J, Li DF, Yin YC. CEPP5 promotes cell motility via JAK2-STAT3-MMPs cascade in hepatocellular carcinoma. Cells. 2018;7:99. https://doi.org/10.3390/cells7080099

64. Liu Y, Zhou B, Dai JH, Liu R F, Han ZG. Aberrant upregulation of LRR1C contributes to human hepatocellular carcinoma. Mol Biol Rep. 2013;40:4543-51. https://doi.org/10.1007/s11033-013-2549-8

65. Hong J, Hu KS, Yuan YF, Sang Y, Bu QG, Chen GH, et al. CHK1 targets spleen tyrosine kinase (L) for proteolysis in hepatocellular carcinoma. J Clin Invest. 2012;122:2165-75. https://doi.org/10.1172/JCI61380

66. Jiang P, Tang YQ, He L, Tang H, Liang M, Mai C, et al. Aberrant expression of nuclear KPN2A is correlated with early recurrence and poor prognosis in patients with small hepatocellular carcinoma after hepatectomy. Med Oncol. 2014;31:131. https://doi.org/10.1007/s12032-014-0131-4

67. He F, Yan QG, Fan LN, Liu YX, Cui JH, Wang JH, et al. PBK/TOPK in the differential diagnosis of cholangiocarcinoma from hepatocellular carcinoma and its involvement in prognosis of human cholangiocarcinoma. Hum Pathol. 2010;41:415-24. https://doi.org/10.1016/j.humpath.2009.05.016

68. Weller DP, Peake MD, Field JK. Presentation of lung cancer in primary care. NPJ Prim Care Respir Med. 2019;29:21. https://doi.org/10.1038/s41533-019-0133-y

69. Liu WJ, Du Y, Wen R, Yang M, Xu J. Drug resistance to targeted therapeutic strategies in non-small cell lung cancer. Pharmacol Ther. 2020;206:107438. https://doi.org/10.1016/j.pharmthera.2019.107438

70. Auliac JB, Bayle S, Vergnenegre A, Le Caer H, Falchero L, Gervais R, et al. Patients with non-small-cell lung cancer harbouring a BRAF mutation: a multicentre study exploring clinical characteristics, management, and outcomes in a real-life setting: EXPLORE GFPC 02-14. Curr Oncol 2018;25:e398-402. https://doi.org/10.3747/co.25.3945

71. Yamaguchi O, Kaira K, Mouri A, Shiono A, Hashimoto K. AXIN2 polymorphisms and their association with colorectal cancer in patients with Lynch syndrome. International Journal of Molecular Sciences. 2020;21:11430-11444. https://doi.org/10.3390/ijms210411430

72. Bugide S, Green MR, Wajapeyee N. Inhibition of Enhancer of zeste homolog 2 (EZH2) induces natural killer cell-mediated eradication of hepatocellular carcinoma cells. Proc Natl Acad Sci U S A. 2018;115:E3509-E18. https://doi.org/10.1073/pnas.1802691115

73. Gu J, Liu XR, Li J, He Y. MicroRNA-144 inhibits cell proliferation, migration and invasion in human hepatocellular carcinoma by targeting CCNB1. Cancer Cell Int. 2019;19:15. https://doi.org/10.1186/s12935-019-0729-x

74. Li M, Gao J, Li DF, Yin YC. CEPP5 promotes cell motility via JAK2-STAT3-MMPs cascade in hepatocellular carcinoma. Cells. 2018;7:99. https://doi.org/10.3390/cells7080099

75. Liu Y, Zhou B, Dai JH, Liu R F, Han ZG. Aberrant upregulation of LRR1C contributes to human hepatocellular carcinoma. Mol Biol Rep. 2013;40:4543-51. https://doi.org/10.1007/s11033-013-2549-8

76. Hong J, Hu KS, Yuan YF, Sang Y, Bu QG, Chen GH, et al. CHK1 targets spleen tyrosine kinase (L) for proteolysis in hepatocellular carcinoma. J Clin Invest. 2012;122:2165-75. https://doi.org/10.1172/JCI61380

77. Jiang P, Tang YQ, He L, Tang H, Liang M, Mai C, et al. Aberrant expression of nuclear KPN2A is correlated with early recurrence and poor prognosis in patients with small hepatocellular carcinoma after hepatectomy. Med Oncol. 2014;31:131. https://doi.org/10.1007/s12032-014-0131-4

78. He F, Yan QG, Fan LN, Liu YX, Cui JH, Wang JH, et al. PBK/TOPK in the differential diagnosis of cholangiocarcinoma from hepatocellular carcinoma and its involvement in prognosis of human cholangiocarcinoma. Hum Pathol. 2010;41:415-24. https://doi.org/10.1016/j.humpath.2009.05.016

79. Weller DP, Peake MD, Field JK. Presentation of lung cancer in primary care. NPJ Prim Care Respir Med. 2019;29:21. https://doi.org/10.1038/s41533-019-0133-y

80. Liu WJ, Du Y, Wen R, Yang M, Xu J. Drug resistance to targeted therapeutic strategies in non-small cell lung cancer. Pharmacol Ther. 2020;206:107438. https://doi.org/10.1016/j.pharmthera.2019.107438

81. Auliac JB, Bayle S, Vergnenegre A, Le Caer H, Falchero L, Gervais R, et al. Patients with non-small-cell lung cancer harbouring a BRAF mutation: a multicentre study exploring clinical characteristics, management, and outcomes in a real-life setting: EXPLORE GFPC 02-14. Curr Oncol 2018;25:e398-402. https://doi.org/10.3747/co.25.3945

82. Yamaguchi O, Kaira K, Mouri A, Shiono A, Hashimoto K, Miura Y, et al. Re-challenge of afatinib after 1st generation EGFR-TKI failure in patients with previously treated non-small cell lung cancer harboring EGFR mutation. Cancer Chemo Therapeut. 2019;83:817-25. https://doi.org/10.1007/s00280-019-03790-w
Gašperšič J, Videtič Paska A. Modern cancer DNA diagnostic tools

78. Gupta R, Amanam I, Rahmanuddin S, Mambetsariy I, Wang Y, Huang C, et al. Anaplastic lymphoma kinase (ALK)-positive tumors: Clinical, radiographic and molecular profiles, and uncommon sites of metastases in patients with lung adenocarcinoma. Am J Clin Oncol. 2019;42:337-44. https://doi.org/10.1097/COC.0000000000000508

79. Shin DH, Jo JY, Han JY. Dual Targeting of ERBB2/ERBB3 for the treatment of SLC3A2-NRG1-mediated lung cancer. Mol Cancer Ther. 2018;17:2024-33. https://doi.org/10.1158/1535-7163.MCT-17-1178

80. Kurppa KJ, Denessiouk K, Johnson MS, Elenius K. Activating ERBB4 mutations in non-small cell lung cancer. Oncogene. 2016;35:1283-91. https://doi.org/10.1038/onc.2015.185

81. Si X, Zhang L, Wang HP, Zhang Z, Han BH, et al. Quality of life results from a randomized, double-blind, placebo-controlled, multi-center phase III trial of anlotinib in patients with advanced non-small cell lung cancer. Lung Cancer. 2018;122:32-7. https://doi.org/10.1016/j.lungcan.2018.05.013

82. Qin A, Johnson A, Ross JS, Miller VA, Ali SM, Schrock AB, et al. Detection of known and novel FGFR fusions in non-small cell lung cancer by comprehensive genomic profiling. J Thorac Oncol. 2019;14:54-62. https://doi.org/10.1016/j.jtho.2018.09.014

83. Wang R, Yang L, Zhang CQ, Wang RJ, Zhang Z, He QY, et al. Th17 cell-derived IL-17A promoted tumor progression via STAT3/NF-kappa B/Notch1 signaling in non-small cell lung cancer. Oncogener. 2019;17:e1461303. https://doi.org/10.1038/s41392-017-0005-2

84. Fathi Z, Mousavi SAJ, Roudi R, Ghazi F. Distribution of KRAS, DDR2, and TP53 gene mutations in lung cancer: An analysis of Iranian patients. Plos One. 2018;13:e0200633. https://doi.org/10.1371/journal.pone.0200633

85. Shi R, Li M, Raghavan V, Tam S, Cabanero M, Pham NA, et al. Targeting the CDK4/6-Rb pathway enhances response to PI3K inhibition in PIK3CA-mutant lung squamous cell carcinoma. Clin Cancer Res. 2018;24:5990-6000. https://doi.org/10.1158/1078-0432.CCR-18-0717

86. Mueck K, Rebholz S, Harati MD, Rodemann HP, Toullany M. Akt1 stimulates homologous recombination repair of DNA double-strand breaks in a Rad51-dependent manner. Int J Mol Sci. 2017;18:2473. https://doi.org/10.3390/ijms18112473

87. Liu L, Huang L, He JJ, Cai SW, Weng YM, Huang SH, et al. PTEN inhibits non-small cell lung cancer cell growth by promoting G0/G1 arrest and cell apoptosis. Oncol Lett. 2019;17:1333-40. https://doi.org/10.3892/ol.2018.9719

88. Kim SH, Go SI, Song DH, Park SW, Kim HR, Jang I, et al. Prognostic impact of CD8 and programmed death-ligand 1 expression in patients with resectable non-small cell lung cancer. Br J Cancer. 2019;120:547-54. https://doi.org/10.1038/s41416-019-0398-5

89. Kanno A, Masamune A, Hanada K, Kikuyama M, Kitano M. Advances in early detection of pancreatic cancer. Diagnostics. 2019;9:18. https://doi.org/10.3390/diagnostics9010018

90. Barbara M, Tsen A, Tenner L, Rosenkranz L. Talking genes in breast and pancreatic malignancies. Mater Sociomed. 2019;31:146-9. https://doi.org/10.5455/msm.2019.31.146-149

91. Tan N, Wong M, Nannini MA, Hong R, Lee LB, Price S, et al. Bcl-2/Bcl-x(L) inhibition increases the efficacy of MEK inhibition alone and in combination with PI3 kinase inhibition in lung and pancreatic tumor models. Mol Cancer Ther. 2013;12:853-64. https://doi.org/10.1158/1535-7163.MCT-12-0949

92. Xi T, Zhang G Z. Integrated analysis of tumor differentiation genes in pancreatic adenocarcinoma. Plos One. 2018;13:e0193427. https://doi.org/10.1371/journal.pone.0193427

93. Rozengurt E, Sinnett-Smith J, Eibl G. Yes-associated protein (YAP) in pancreatic cancer: at the epicenter of a targetable signaling network associated with patient survival. Signal Transduct Target Ther. 2018;3:11. https://doi.org/10.1038/s41392-018-0005-2

94. Chiou SH, Dorsch M, Kusch E, Narango S, Kozak MM, Koong AC, et al. Hmga2 is dispensable for pancreatic cancer development, metastasis, and therapy resistance. Sci Rep. 2018;8:14008. https://doi.org/10.1038/s41598-018-32159-x

95. Neill KG, Saller J, Al Diffalha S, Centeno BA, Malafa MP, Coppola D. EGFR L861Q mutation in a metastatic solid-pseudopapillary neoplasm of the pancreas. Cancer Genomics Proteomics. 2018;15:201-5. https://doi.org/10.21873/cgp.20078

96. Wu YL, Lee V, Liang CK, Lu S, Park K, Srimuninnimit V, et al. Clinical utility of a blood-based EGFR mutation test in patients receiving first-line erlotinib therapy in the ENSURE, FASTACT-2, and ASPIRATION studies. Lung Cancer. 2018;126:1-8. https://doi.org/10.1016/j.lungcan.2018.10.004

97. Merker JD, Oxnard GR, Compton C, Diehn M, Hurley P, Lazar AJ, et al. Circulating Tumor DNA Analysis in Patients With Cancer: American Society of Clinical Oncology and College of American Pathologists Joint Review. J Clin Oncol. 2018;36:1631-41. https://doi.org/10.1200/JCO.2017.76.8671