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Mini-Review

Mutation-based detection and monitoring of cell-free tumor DNA in peripheral blood of cancer patients

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ABSTRACT

Prognosis of solid cancers is generally more favorable if the disease is treated early and efficiently. A key to long cancer survival is in radical surgical therapy directed at the primary tumor followed by early detection of possible progression, with swift application of subsequent therapeutic intervention reducing the risk of disease generalization. The conventional follow-up care is based on regular observation of tumor markers in combination with computed tomography/endoscopic ultrasound/magnetic resonance/positron emission tomography imaging to monitor potential tumor progression. A recent development in methodologies allowing screening for a presence of cell-free DNA (cfDNA) brings a new viable tool in early detection and management of major cancers. It is believed that cfDNA is released from tumors primarily due to necrotization, whereas the origin of nontumorous cfDNA is mostly apoptotic. The process of cfDNA detection starts with proper collection and treatment of blood and isolation and storage of blood plasma. The next important steps include cfDNA extraction from plasma and its detection and/or quantification. To distinguish tumor cfDNA from nontumorous cfDNA, specific somatic DNA mutations, previously localized in the primary tumor tissue, are identified in the extracted cfDNA. Apart from conventional mutation detection approaches, several dedicated techniques have been presented to detect low levels of cfDNA in an excess of nontumorous (nonmutated) DNA, including real-time polymerase chain reaction (PCR), "BEAMing" (beads, emulsion, amplification, and magnetics), and denaturing capillary electrophoresis. Techniques to facilitate the mutant detection, such as mutant-enriched PCR and COLD–PCR (coamplification at lower denaturation temperature PCR), are also applicable. Finally, a number of newly developed miniaturized approaches, such as single-molecule sequencing, are promising for the future.

Introduction

Although significant progress has been made in the development of new therapy approaches, cancer remains the leading cause of death worldwide [1]. Despite the availability of a number of screening schemes, in most cases cancer remains undetected until its advanced stages [2,3]. In a typical course of disease development, defective cellular adhesion allows malignant cells to be released and to travel to nearby structures or even migrate through the lymphatic or blood system to form malignant formations. If unnoticed, such micrometastases pose a serious risk for disease progression already in early stages of the primary tumor. Surgical treatment resulting in removal of the primary tumor, therefore, might not avert dissemination and generalization of the disease in the long term. Follow-up of cancer patients typically relies on computed tomography (CT),[1] positron emission tomography

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[1] *Abbreviations used:* CT, computed tomography; PET, positron emission tomography; cfDNA, cell-free DNA; PCR, polymerase chain reaction; LOH, loss of heterozygosity; DCE, denaturing capillary electrophoresis; ME–PCR, mutant-enriched PCR; BEAMing, beads, emulsion, amplification, and magnetics; CE, capillary electrophoresis; DCE, denaturing capillary electrophoresis; dHPLC, denaturing high-performance liquid chromatography; COLD–PCR, coamplification at lower denaturation temperature PCR.

(PET)–CT, or magnetic resonance imaging in combination with monitoring of serum tumor markers [4,5]. It is known that imaging methods typically spot objects on a millimeter scale (containing tens to hundreds of millions of cells). In addition, the widely established utility of tumor markers is sometimes inefficient due to sensitivity and specificity issues [6,7]. Therefore, there is great expectation in finding new diagnostic markers for better management of all major cancers. Among the few alternatives, there is growing interest in molecular diagnostics directed at nucleic acids released directly from the tumor and circulating in peripheral blood of patients.

The classic article on the occurrence of nucleic acids in human plasma was published back in 1948 by Mandel and Metais [8], followed by works of Bendich and coworkers in 1965 [9], Koffler and coworkers in 1973 [10], and Leon and coworkers in 1977 [11], who identified the importance of circulating tumor DNA as a vehicle of oncogenesis. With the use of then emerging methods such as radioimmunoassay, the presence of higher cell-free DNA (cfDNA) concentrations in serum in patients with carcinoma compared with healthy persons was observed along with a decrease after the administration of chemotherapy [10,11]. It was soon recognized that the circulating DNA could serve as a viable tool to monitor the efficiency of anticancer therapies by monitoring its levels in advanced cancers [12–14]. With the subsequent rapid development of modern polymerase chain reaction (PCR)-based techniques and their widespread availability, the interest in detecting circulating nucleic acids is steadily increasing, with colorectal, prostate, and lung cancers being the main focus of many published studies [15–19].

Origin of free circulating nucleic acids

Circulating nucleic acids, often referred to as cell-free DNA, emphasize their exogenous nature in comparison with DNA originating from nuclei of the blood cells. Whereas cfDNA detection is currently at the forefront of molecular oncology community interest, the detail mechanism of cfDNA release from its native cell is yet to be fully elucidated. In 2001, a study by Jahr and coworkers revealed a combined contribution of apoptotic and necrotic processes to the overall production of cfDNA [20]. The idea was extended in further detail by Diehl and coworkers [21]. These authors considered that DNA fragments present in the circulation originate from the necrotic neoplastic cells phagocytized by macrophages, and these also engulf nontumor (apoptotic) cells, which is the reason why a particular level of nontumor cfDNA occurs in healthy individuals, as confirmed by others [22]. The two proposed hypothetical mechanisms for necrotic and apoptotic release of DNA are illustrated in Fig. 1. Fig. 1A depicts a mucous membrane of the colon affected by a growing tumor with a layer of necrotic cells on the surface. The necrotic tumor cell is released, and its fragments are captured by the macrophage pseudopodia. An engulfed fragment forms a phagosome, which fuses with lysosome to form a phagolysosome. Subsequently, the ingested particles, including tumor DNA fragment of various lengths, are released into the environment. Fig. 1B shows an alternative mechanism with the mucous membrane of the colon with a normal epithelium layer releasing a cell undergoing apoptosis. The cell forms apoptotic particles captured by the macrophage pseudopodia. The engulfed particle forms a phagosome, which fuses with lysosome to form a phagolysosome. Subsequently, the ingested particles, including equally sized DNA, are released into the environment.

Necrotic cells arise in invasive tumors, where tissue deterioration occurs as a result of hypoxia [23]. Benign tumors do not have this property, and the amount of fragmented DNA produced is minimal [24,25]. This implies that malignity of the tumor leads tumor cells as well, leading to a parallel release of nontumorous cfDNA into the circulation, resulting in an increase in concentrations of both tumorous and nontumorous DNA in plasma. Thus, the fact that in patients with malignant disease the volume of free DNA is increased, regardless of its origin, can in some circumstances be used for monitoring of cancer.

Extraction of cfDNA

To successfully detect the presence of tumor cfDNA in plasma or serum, a suitable methodology needs to be selected. This consists of several essential steps. The first step is to process collected blood while avoiding the rupture of blood cell membranes (i.e., hemolysis) and subsequent plasma contamination with DNA derived from the blood cell nuclei. The next step is cfDNA isolation when it is necessary to select the most appropriate method to gain a sufficient amount of quality DNA for further analysis. Here, the essential tool is PCR, and the detection of amplified products can be done either directly in real time (real-time PCR) or following amplification by electrophoresis on a slab gel or capillary format.

Blood collection, cfDNA isolation, and quantification

Blood samples collected in an anticoagulant solution must be processed within 2 h after the collection to avoid damage to nucleated blood cells and release of their DNA [26]. In some studies sampling was performed in heparinized test tubes [27,28], and in others it was performed in tubes containing EDTA (ethylenediaminetetraacetic acid) solution [29]. Immediately after blood collection, plasma needs to be separated from the blood cells by centrifugation. The centrifugal speed must not be too high (to avoid causing cell lysis), and recentrifugation may be performed following primary elimination of blood cells at lower speeds [27]. Alternatively, plasma may be extracted by filtration through membranes with a 0.45-μm pore size [29]. Extracted plasma may be stored at −20 °C for extended periods of time before subsequent processing. Some authors have reported the use of serum rather than plasma [30]. It was noted that serum is a less suitable material because it becomes readily contaminated with DNA from the leukocytes when blood coagulum is formed [26].

The basis for successful cfDNA detection is selection of an isolation method that ensures extraction of a sufficient amount of quality DNA. A classic phenol–chloroform extraction or commercial kits based on the principle of membrane columns can be applied. The advantage of the phenol–chloroform method is an unlimited amount of input material; thus, the yield can be higher with an increased volume of isolated plasma, whereas in commercial kits the amount of input material is limited. However, the use of commercial kits is considerably easier, and special kits designed specifically for cfDNA isolation are already available. Kuang and coworkers [28] compared three isolating kits: QIAamp DNA Micro Kit (Qiagen), NucleoSpin Plasma XS (Macherey–Nagel), and Wizard (Promega). In this study, the authors used a method described previously [31] based on a principle that the majority of tumor DNA in plasma derived from necrotic cells occurs in unequally sized fragments ranging from 185 to 926 bp, whereas the DNA from apoptotic (i.e., nontumor) cells is usually present in relatively uniform sizes ranging from 185 to 200 bp. Based on these facts, they examined the amounts of both cfDNA types using real-time PCR by amplifying two different length fragments of Alu sequences, namely, 115 and 247 bp. Alu 115 captured the concentration of short DNA fragments derived from apoptotic cells as well as DNA fragments from tumor cells, whereas Alu 247 captured only

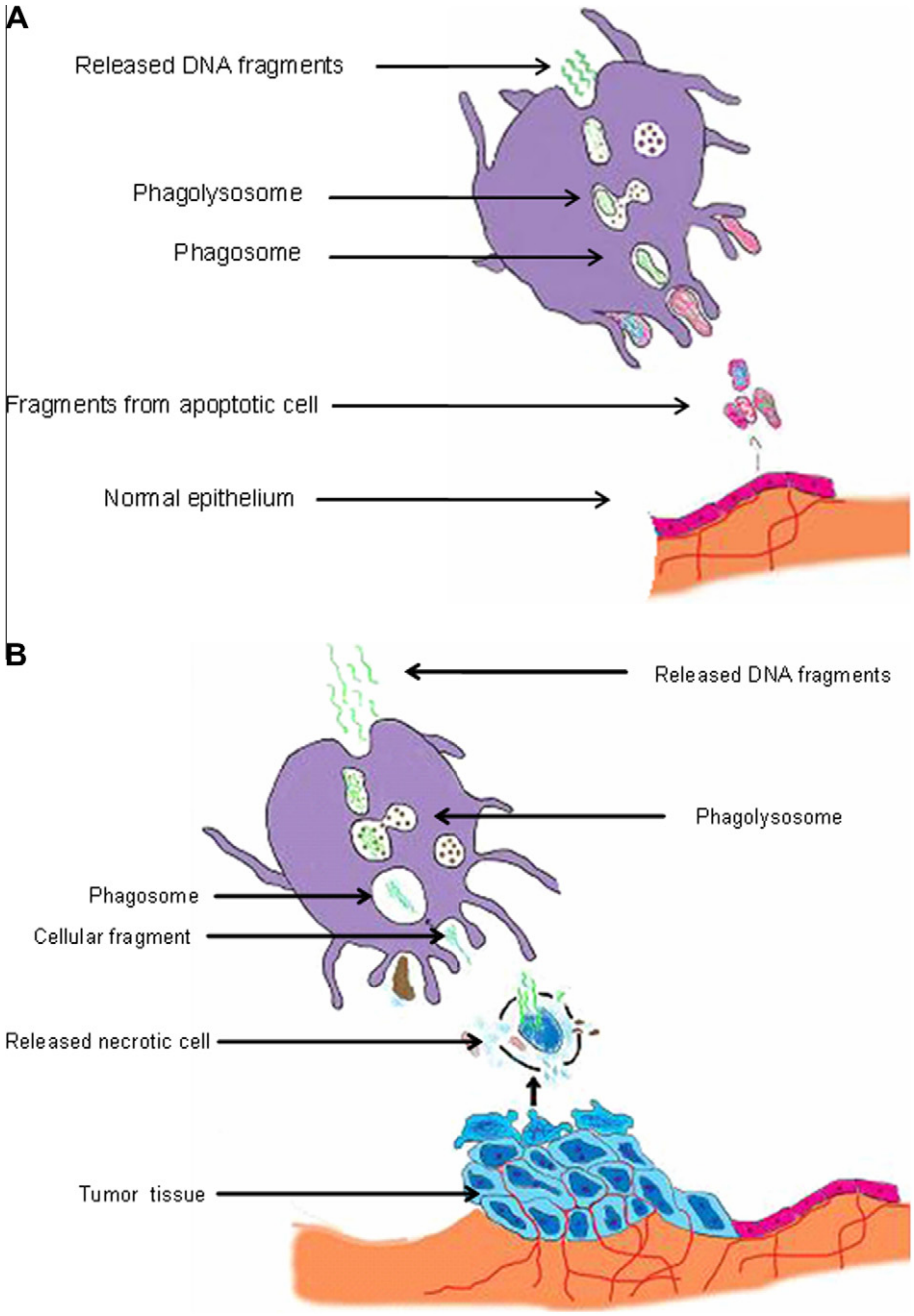

Fig.1. Alternative mechanisms of cfDNA release during phagocytosis. Unequally sized DNA fragments result from phagocytosis of a necrotic cell (A), whereas uniformly sized DNA fragments are released by macrophage from apoptotic cell (B).

tumor cells. This led to a finding that although the greatest concentration of total cfDNA was obtained using the NucleoSpin Plasma kit, for extracting fragments derived from tumor cells, the QIAamp DNA Micro Kit was more suitable. The results are summarized in Table 1. In another study, a QIAamp MinElute Virus Vacuum Kit (Qiagen) was used for cfDNA extraction. Its advantage was a greater input volume of isolated plasma [29]. In some other studies, column kits were used for isolation from blood [32]. Their disadvantage was a loss of small cfDNA fragments through membrane pores, leading to reduced detection sensitivity [33].

Table 1
Comparison results of isolation kits for cfDNA detection.

DNA extraction protocol	Total DNA concentration (ng/μl)	Fraction of tumor cfDNA (% ratio of Alu 247/115)
QIAamp DNA Micro Kit (Qiagen)	0.064	50.9
NucleoSpin Plasma XS (Macherey–Nagel)	0.086	10.9

Analysis of cfDNA based on tumor-specific mutations

There are two basic approaches to cfDNA analysis: quantitative analysis and analysis based on DNA-specific mutations. The first approach is based solely on quantification of cfDNA, including both tumor and nontumorous cfDNA [34]. Increased DNA levels in plasma of cancer patients compared with healthy controls indicate the presence of tumor cfDNA. The actual cfDNA amount is typically determined by amplification of Alu sequences or other specific markers (e.g., β-globin, β-actin) [35]. This method yields high sensitivity but has rather low specificity because both tumor and nontumorous DNA is amplified. The specificity can, alternatively, be enhanced by relating amplification of two unequally sized fragments, one of which is expressed in both tumor and nontumorous cfDNA and the other of which is only reflecting tumor cfDNA [31].

An alternative approach is based on locating a tumor-specific mutation in the primary tumor, followed by detection of the same marker in isolated cfDNA. The most commonly used tumor-specific mutations include single-point substitutions or short deletions of proto-oncogenes and tumor suppressor genes as well as extensive deletions and DNA hypermethylation of tumor suppressor gene promoters. Detection of extensive deletions is performed by analysis of microsatellite markers using the LOH (loss of heterozygosity) method. LOH has considerable limitations, primarily due to the need for simultaneous amplification of multiple microsatellite markers from a limited material and a relatively complicated results interpretation [36]. Hypermethylation can be analyzed in several ways, including methylation-specific PCR [37], quantitative or fluorescent methylation-specific PCR [38,39], and methylation-specific restriction analysis [40]. Although the sensitivity of cfDNA detection based on methylation is relatively high, its specificity is limited. One of the reasons is the dynamic process of DNA methylation, which may lead to a variation in methylation status among DNA molecules derived from the same tumor or, indeed, a primary tumor versus metastasis [41] or a primary tumor versus circulating cfDNA [42]. Another cause or a lower specificity in this case is a possible coincidence of a given methylation in healthy cells or in cells stemming from other defects [43].

Examination of DNA variations of single-point mutations or short indels is challenging due to the presence of high levels of normal (wild-type) DNA originating either in apoptotic or necrotic tissues lacking the detected mutation or in an inherent background presence of wild-type DNA from leukocytes. The situation with a mutated DNA copy screened by an overwhelming background of wild-type DNA is often referred to as a needle in a haystack [44,45]. The protocol usually consists of initial PCR followed by detection of amplified products. This can be performed using several methods, beginning with sequencing with limited sensitivity, through methods based on conformational changes and electrophoresis (e.g., denaturing capillary electrophoresis, DCE), alternatively increasing the mutant fraction by mutant-enriched PCR (ME–PCR), and opting for a dedicated approach such as "BEAMing" (beads, emulsion, amplification, and magnetics), digital PCR, or single-molecule sequencing.

DCE

The separation power of capillary electrophoresis (CE) has long been extensively used in DNA analyses. During the 1990s, a family of mutation detection techniques was introduced combining a classic principle of differential melting known from classic DGGE (denaturing gradient gel electrophoresis) [46] with the separation power of CE [47]. The separation was performed either at the predetermined temperature or during a temperature gradient [48]. Following a variety of abbreviations from the original CDCE (constant denaturant capillary electrophoresis) [47] and TGCE (temperature gradient capillary electrophoresis) [49] through further improvements by cycling the temperature gradient in CGCE (cycling gradient capillary electrophoresis) [50] or CTCE (cycling temperature capillary electrophoresis) [51], the terminology recently settled on DCE (denaturing capillary electrophoresis) [52] in an apparent analogy to dHPLC (denaturing high-performance liquid chromatography) widely adapted in routine DNA testing laboratories [53]. Similar to dHPLC, the basic principle of DCE lies in separation of partially denatured double-stranded PCR fragments. Homoduplexes are fragments with perfect sequence complementarity, whereas heteroduplexes have sequence complementarity except at the mutation position containing a mismatch due to a presence of mutation. Both homo- and heteroduplexes are formed by a random combination of individual single strands during a process of full denaturation (melting) and a slow reannealing performed at the very end of PCR amplification. Each homo- and heteroduplex fragment has an individual melting temperature reflecting its actual composition of bases at the mutation site. When the fragment mixture is electrophoresed at an optimal temperature in a gel matrix, fragments with higher melting temperatures (homoduplexes) will remain in nondenatured (double-stranded) conformation, and thus migrate faster, compared with fragments with lower melting temperatures (heteroduplexes), which will adopt a denatured structure [54]. A subtle change in melting temperature of ±0.1 °C is translated into a significant difference in electrophoretic migration, giving DCE great separation power for resolving all mutations within a given target sequence [47]. Unlike in dHPLC, where individual mutations are recognized based on different shapes of peaks in chromatograms, each fragment in DCE is usually observed as an individual peak. Naturally, DCE was applied to detect somatic mutations in cancerous tissues [54–56]. The potential of high mutation sensitivity of the approach, usable in detecting mutated DNA fragments circulating in patients' plasma, has long been recognized [57,58].

cfDNA detection in plasma of colorectal cancer patients using DCE has been presented over the past 3 years [59,60]. It was clearly demonstrated that for the advanced stages of the disease, a mutation found in primary tumor tissue can be readily confirmed in local lymph nodes and distant metastases as well as detected in cfDNA. Fig. 2 demonstrates such a result. The DCE electropherogram of a PIK3CA amplification control from healthy tissue contains a single peak from the nonmutated DNA, as seen in line A of Fig. 2. A PIK3CA mutation is detected in the presence of additional fragment peaks in the mutation-specific region. In the current case, the mutation was detected in rectal adenocarcinoma from a primary tumor biopsy collected during an endoscopic polypectomy (Fig. 2, line B). A subsequent CT scan uncovered progression, with the disease spreading into lymph nodes and liver metastases, both also containing the PIK3CA mutation (Fig. 2, lines C and D, respectively). The presence of high levels of cfDNA was subsequently confirmed in peripheral blood of the patient (Fig. 2, line E). A relative amount of tumor DNA can be estimated from the ratio of the normal PCR fragment (Fig. 2, N) and the mutation-specific fragments (Fig. 2, M).

ME–PCR and COLD–PCR approaches

ME–PCR methods are also useful in mutation-based detection of cfDNA. They are based on suppression of nonmutated (wild-type) DNA or, alternatively, a preferential amplification of mutated DNA during PCR. An important distinction from a number of al-

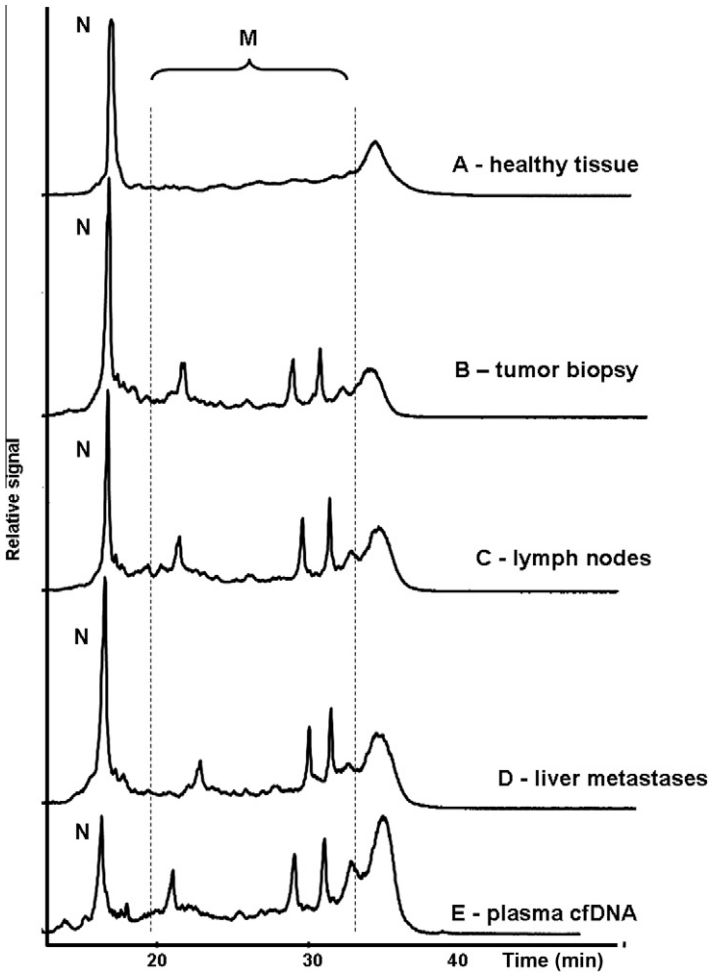

Fig.2. Use of denaturing capillary electrophoresis (DCE) technique to detect somatic mutations (KRAS and TP53) in multiple sample types on a patient with advanced colon adenocarcinoma. A normal tissue with only the wild-type peak and no mutant peaks (A), tissue from tumor biopsy (B), lymph nodes (C), liver metastases and plasma containing cell-free DNA (E) all showing wild-type peak followed by mutant homoduplex peak and two mutant heteroduplex peaks.

desired target sequence). A traditional mutant enrichment is achieved by restriction of the wild-type sequence prior to or during the amplification. In a typical experiment, after initial PCR cycles, a specific restriction enzyme is applied to cut the wild-type fragments, resulting in enrichment of the mutated amplicons in the reaction mixture. The PCR then proceeds, alternatively, with additional subsequent restriction steps. From 2004 through 2008, such an approach was shown to enable detection of KRAS mutated cfDNA in plasma, serum, and urine of colorectal cancer patients [27,39,61–65]. A *Bst*NI restriction enzyme targeting wild-type KRAS sequence was used in the process. The aim of the study was to compare the ideal amount of input volume of plasma, serum, or urine for cfDNA isolation. Better results were evaluated from a greater volume of isolated material. However, positive findings in plasma in patients did not correspond in any way with the patients' stage of the disease, and the conclusion focused only on a comparison of the DNA yields.

Recently an alternative technique was introduced in which the mutant fragments are preferably amplified by lowering the temperature of the PCR annealing step [66]. At a lower annealing temperature, nonmutated fragments remain as DNA double strands inaccessible for annealing of primers. At the same time, mutant fragments, randomly forming imperfect mismatch duplexes, will referred to as COLD–PCR (coamplification at lower denaturation temperature PCR), is enrichment of the mutated fragments over nonmutated wild-type fragments. The technique has been shown to detect low levels of KRAS and BRAF mutations in colorectal cancer, suggesting it as a suitable tool for cfDNA [67]. From a practical point of application in routine diagnostics and cfDNA monitoring, the above mutant enrichment methods are relatively simple and attainable to a standard molecular diagnostic laboratory [68,67,69].

BEAMing

In 2005, Diehl and coworkers introduced a dedicated approach for detection of mutations in plasma of colorectal cancer patients [21]. PCR products formed by amplification of target DNA sequence containing a specified mutation were mixed with magnetic beads, and the mixture was dispersed into the trillions of microparticles in water/oil emulsion. Then, a second PCR was performed using the primers bound to magnetic beads, followed by hybridization of resulting PCR products with two types of specific fluorescently labeled probes: mutated and nonmutated sequences, each labeled with a different fluorescent dye. Finally, the beads were analyzed using flow cytometry. The technique, referred to as BEAMing,

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