Circulating DNA as a Biomarker for Early Detection of Cancer: A Brief Update with an Emphasis on Lung Cancer

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Abstract: In most cases, early detection and treatment of cancer are prerequisites for full recovery. However, its early detection continues to be extremely difficult since its characteristic symptoms rarely manifest at the onset of disease. The development of highly sensitive, specific biomarkers also capable of reflecting pathological changes is, therefore, of the upmost importance. Lung cancer, for example, is most frequently diagnosed during the late metastatic stage so that, as a result, malignant neoplasms in the lung are among the leading causes of cancer-related deaths worldwide. Circulating DNA is a non-invasive diagnostic assay that has been greatly improved for the purposes of diagnosis, prognosis, and treatment of cancer. In the current review, we discuss recent data concerning circulating tumor-derived DNA as a tool for early detection, diagnosis, and follow-up. Given the scope of this review, the focus will be on lung cancer, one of the most prolific and lethal cancers affecting humanity today.

Keywords: Genetic polymorphism, circulating DNA, lung cancer, risk factor, biomarkers.

INTRODUCTION

When cells first undergo mutations in key target genes and become cancerous, the symptoms do not typically present for months, or even years, imposing serious difficulties for researchers and clinicians to effectively ensure both early and accurate diagnoses. It is acknowledged that effective cancer therapy is often the result of early detection. For this reason and others, the development of reliable methods primarily utilizing non-invasive biomarkers for early-stage cancer detection is of such overriding importance.

Thanks to Mandel and Metais, the existence of circulating extracellular DNA in the bloodstream was reported as early as 1948 [1]. And the correlation between cell-free nucleic acid levels in plasma and cancer was initially researched in 1977 by Leon *et al.* [2], who demonstrated for the first time that the plasma levels of free circulating DNA were much higher in cancer patients than in healthy controls.

Nevertheless, the mechanisms by which cell-free DNA is released into the bloodstream remain unknown. It has been suggested that lysis of tumor may be the source of the DNA found in plasma/serum of cancer patients. However, the vast majority of reports in the cell-free DNA field indicate that cell death by apoptosis or necrosis could play a role in this phenomenon. But, although we cannot discard an apoptotic-mediated mechanism, we must keep in mind that efficient clearance of apoptotic cells is critical for normal tissue homeostasis, which, in turn, may make it more difficult to explain the liberation of cell-free DNA in the bloodstream of normal individuals due to apoptosis. Contrariwise, the acquisition of apoptotic resistance leading to aberrant cell accumulation is, indeed, an important event in carcinogenesis despite its non-correlation with the hypothesis of apoptotic-mediated, cell-free DNA release in cancer patients.

Concerning necrosis and notwithstanding eventual negative results [3], increased amounts of DNA in the bloodstream of cancer patients with metastasis have been described [4-6]. Even so, after treatment with radiotherapy, assumed to intensify necrosis, the amount of circulating DNA in these patients has been found to remain stable. There are additional data in the literature showing that the circulating DNA in cancer patients mimics cancer-cell DNA. In fact, circulating tumor-derived nucleic acids in cancer patients may harbor tumor-specific genetic alterations and, in this way, could become an interesting target for the non-invasive examination of tumor DNA and ultimately might prove to be a suitable target for the development of diagnosis, prognosis, and follow-up cancer testing [7-12].

FREE CIRCULATING DNA AS A BIOMARKER FOR DETECTING GENETIC ALTERATIONS

Mutations are heritable changes in DNA, ranging from a single DNA base to a large chromosomal segment. Cancer progression is thought to be driven by the accumulation of a wide spectrum of genetic and epigenetic alterations, including point mutations, chromosomal aberrations, high-frequency microsatellite instability, structural and numerical chromosome abnormalities, and hypermethylation in the promoter region of tumor suppressor genes. The unavoidable rate of endogenous and exogenous DNA damage might account for the accumulation of these abnormalities over several rounds of cell replication. On the other hand, abnormally-elevated levels of genetic instability are mitigated by DNA repair enzymes, responsible for

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preventing unacceptably high mutation rates so as to ensure high-fidelity DNA replication. Elevated levels of genetic instability are also modulated *via* xenobiotic enzyme activity by catalyzing reactions that lead up to a number of carcinogens. Besides the application of circulating DNA technology in clinical trials based on the amount of cell-free DNA in the bloodstream, its analysis in the plasma/serum of cancer patients allows for the identification of a myriad of inherited, pre-cancerous mutations such as point and epigenetic events affecting the expression of caretaker and gatekeeper genes clearly implicated in the multistep nature of cancer development. In addition, this technology equally identifies microsatellite instability and chromosomal rearrangements. Therefore, it can be reasonably postulated that the concentration of free circulating plasma DNA and the genetic profile of patients suffering from various types of cancerous diseases can be easily investigated by means of a simple, non-invasive blood test, highlighting genetic factors involved in predisposing an individual to cancer. From this perspective, circulating DNA would appear to be an excellent tool for early detection, diagnosis, and follow-up procedures in addition to large-scale prospective clinical trials in high-risk individuals.

ALTERED EPIGENETIC REGULATION ASSESSED BY THE CELL-FREE CIRCULATING DNA TECHNIQUE

It is widely agreed that changes in DNA methylation may be involved in numerous cases of cancer. DNA methylation occurs spontaneously in cells as a physiological process, acting as a regulatory mechanism for gene expression. Within the context of the CpG island, it is possible that DNA methylation is the source of the transcription silencing of genes. It has been demonstrated that DNA methylation in the promoter region of tumor suppressor genes may be the underlying molecular mechanism responsible for this early event in carcinogenesis. Detection of epigenetic DNA methylation is promptly assessed by the circulating DNA technique, thereby opening up the possibility of using the methylated-based analysis of circulating DNA as a tool for early cancer detection [11, 13-18].

Liggett *et al.* [19] reported a differential methylation status of cell-free circulating DNA among patients with pancreatic cancer and those with inflammatory disease alone. Moreover, a methylation profile of circulating plasma DNA in pancreatic cancer patients was reported by Melnikov *et al*. These authors found that at least five genes were consistently predictive for plasma-based pancreatic cancer with reasonably high sensitivity and specificity outcomes in the developed composite biomarker [20]. *Via* a microarray-based technique, they [21] were also able to analyze the differential methylation profile of ovarian cancer tissues and plasma from women belonging to high-risk groups. The results concerning this investigation into cell-free circulating DNA unveiled novel genes that could be informative for cancer detection, suggesting that differential methylation profiling can be achieved in heterogenous samples with reasonable accuracy.

Furthermore, Goessl *et al*. [22] investigated the GSTP1 promoter hypermethylation in DNA isolated from plasma, serum, semen, and urine taken from the prostate carcinoma tissues of 33 prostate cancer patients and 26 control patients with benign prostatic hyperplasia (BPH). It was found that 94% of the prostate cancer tissues and 72% of the plasma and/or serum samples exhibited GSTP1 promoter hypermethylation. Similar lines of evidence were found by Wong *et al*. [23] when working with tumor-associated, aberrant p16 methylation in the bloodstream of hepatocellular carcinoma patients. In this study, p16 methylation was shown in 73% of HCC tissues using methylation-specific PCR. Among the 16 cases found to present aberrant methylation in tumor tissues, similar changes were also detected in the plasma/serum samples of 81%. Such studies have indicated a good correlation between restricted expression at the tissue level and the occurrence of detectable levels of candidate biomarkers in serum/plasma DNA. In this connection, the circulating methylated DNA approach has been applied as a biomarker in various forms of cancer, including pancreatic [19, 20, 24, 25], ovarian [26-29], prostate carcinoma [30-34], hepatocellular carcinoma [35-38], esophageal adenocarcinoma [39, 40], colorectal carcinoma [8, 41, 42], breast [3, 43-45], head and neck squamous cell carcinoma [46-48], non-Hodgkin lymphoma [49], and lung cancer [10, 16, 18, 50-52].

LOSS OF GENETIC STABILITY DETECTED BY THE CELL-FREE PLASMA/SERUM DNA TECHNIQUE

It has been well established in the literature that proto-oncogenes (e.g., ras, EGFR) and tumor suppressor genes (e.g., p53) play a key role in carcinogenesis. Proto-oncogenes normally control the frequency of cell division. When a proto-oncogene mutates, it becomes a permanently-activated oncogene, and, in this capacity, induces successive rounds of cell growth. Tumor suppressor genes have remarkable properties that slow down cell division in response to DNA damage by either triggering apoptosis under certain conditions or by allowing DNA repair processes to take place, both of which are anticarcinogenic processes.

The allele-specific PCR technique has been successfully applied to detected tumor-associated mutations in proto-oncogene as well as tumor suppressor genes from plasma/serum. Previous research by Salbe *et al*. [53] has shown that point mutations of the *K-ras* gene at codon 12 are found in about 40% of cases with colorectal cancer. In another study, Gormally *et al*. [54] reported interesting results in a prospective study demonstrating that the TP53 and KRAS2 mutations can be properly detected in plasma DNA of healthy subjects. Their results suggest that the KRAS2 mutation is detectable ahead of a bladder cancer diagnosis and that the TP53 mutation may be associated with environmental exposure. More recently and in the same vein, He *et al*. [55] reported the detection of epidermal-growth-factor-receptor mutations in plasma by mutant-enriched PCR assay to predict the response to gefitinib in patients with non-small-cell lung cancer, suggesting that this approach has the ability to provide a reliable guidance for clinical decision making in the treatment of advanced NSCLC patients.

CELL-FREE CIRCULATING DNA AS A DIAGNOSTIC MARKER IN LUNG CANCER

Lung cancer is a global public health care problem and a leading cause of death in both men and women. Nonetheless,

effective screening recommendations for early-stage lung cancer detection have not yet been established for any of the high-risk groups, including asymptomatic high-risk populations like older smokers [56-58]. Inherited predisposition to lung cancer can be characterized by a complex genetic trait. Although familial clustering of lung cancer is rare, particular mutations in the genes associated with the clinical responsiveness of lung cancer have been reported. The first known mutation to occur in nonsmoking lung cancer patients was described by Lynch *et al*. [59]. These authors revealed a significant association between somatic mutations in the tyrosine kinase domain of the epidermal-growth-factor receptor (EGFR) and the responsiveness of non-small-cell lung cancer to gefitinib. Since then, several groups have confirmed these findings [60-66].

Furthering this line of research, many authors have investigated the EGFR pathway genes in lung cancer. Among the oncogenes integrating this pathway, RAS and EGFR itself appear to harbor the most frequently-detected activating mutations reported, with the majority of these mutations being located in non-small-cell lung cancer tumors. In a recent study by Soh *et al.* [67], the mutational status, copy-number gain, and relative ratio between mutant and wild-type alleles in the EGFR pathway, including KRAS, BRAF, PIK3CA, and EGFR, were also examined. It was found that mutant allele-specific imbalance is frequently present in mutant EGFR and KRAS. These alterations can be correlated with increased mutant-allele transcription and gene activity. Interestingly, with regard to adenocarcinoma lung tumors, all reports have indicated that KRAS mutations and copy-number gains correlated with shortened survival periods.

Thus, it has been well established that EGFR plays a key role in lung cancer therapy. Even so, the role of this oncogene in the development of lung caner remains nebulous. In this regard, the findings reported by Li and Hemminki [68] may shed some light on this issue in that they describe an inherited predisposition to early-onset lung cancer due to mutations in the EGFR oncogene in a familial clustering with several cases of NSCL. KRAS belonging to the EGFR pathway genes is known to have mutated into various kinds of human tumors. Frequently detected in lung cancer cases [69], almost all mutations in this gene are clustered into three hot spots (namely, codons 12, 13, and 61). Investigations into cancer susceptibility, prevention, risk assessment, management, and therapeutic approaches concerning the KRAS oncogene are currently underway.

Another gene deserving of attention that may possibly be involved with an increased susceptibility to lung cancer is p53. This tumor-suppressor gene is involved in multiple processes implicated in the maintenance of genomic stability, including DNA damage checkpoints, DNA repair, and apoptosis. As a consequence, mutations in p53 have been reported in a wide variety of tumor cells. A lung-cancer risk in germline p53 mutation carriers has been reported by Hwang *et al.* [70], who also observed that mutation carriers who smoked were significantly more susceptible to the development of lung cancer than their nonsmoking counterparts. In addition to p53, mutations in $p16^{INK4a}$ have also been investigated as early events and as potential biomarkers for early lung cancer diagnosis [71-74].

Mutations in EGFR, KRAS, P53, and $p16^{INK4a}$, good examples of genes implicated in genomic stability, may actually increase the risk of cancer. Since cell-free circulating DNA does commonly exhibit tumor-related alterations, the detection of abnormalities in caretaker and gatekeeper genes in the bloodstream may be used as a non-invasive DNA-based test for early detection. With regard to lung cancer, multiple articles in recent years have reported a putative association between molecular genetic and epigenetic changes in EGFR pathway genes [75-81], p53 [82], p16 [83-88] and treatment outcomes. Giovannetti *et al*. [89], have likewise presented interesting results for the prognostic and treatment optimization of NSCLC patients by comparing the connections between EGFR and AKT1 polymorphisms and outcome/toxicity. It was determined that EGFR-activating mutations significantly correlated with response time, longer periods of progression, and overall survival. Conversely, the polymorphic genotype (AKT1-SNP4 A/A) seems to be implicated in the primary chemotherapeutic resistance to EGFR tyrosine kinase inhibitors.

Mutations in the KRAS oncogene were also screened by Wang *et al*. [90] on plasma-extracted DNA and matched tumor tissues of 273 lung cancer patients. It was concluded that the KRAS mutation in plasma DNA correlates with the mutation status of the matched tumor tissues of NSCLC patients and that the plasma KRAS mutation status may be associated with a poor tumor response to EGFR-TKIs in these patients. Altogether, these studies appear to highlight a potential value for cell-free circulating DNA in the early detection, prognosis, and clinical management of lung cancer.

POLYMORPHISMS OF THE XENOBIOTIC-METABOLIZING GENES DETECTED IN CIRCULATING DNA

Xenobiotic metabolism occurs through a multistage process involving several detoxification enzymes working in a simultaneous or alternate fashion in classical two- phase steps: phase I, characterized by chemical modifications leading to the creation or release of functional groups; and phase II, in which other functional groups or metabolites are attached to the functional groups originated in phase I. Glutathione S-transferase (GSTs), for example, catalyzes the reaction that attaches glutathione to the xenobiotic compound or its metabolites, thereby promoting the neutralization of their electrophilic center. These events lead to the detoxification of a wide range of carcinogens like the polycyclic aromatic hydrocarbons (PAHs), ethylene oxide, and styrene [91]. Inherited deletion of the GST genes, such as the GSTM1 null polymorphism, is thought to abolish enzymatic activity and, by implication, can be used as a cancer-susceptibility biomarker. Moreover, there is substantial evidence in the literature suggesting that higher levels of polycyclic aromatic hydrocarbon–dGMP adducts are capable of inducing DNA lesions and genomic instabilities in lung tissues. In this connection, our group recently [18] observed a close relationship between smoking status and lung cancer. In this study, the most significant risk

for tobacco smoking and lung cancer was found in the association of null genotypes for GSTM1 and GSTT1.

CORRECTIONS OF TECHNICAL BIAS IN THE CELL-FREE CIRCULATING NUCLEIC-ACID TECHNIQUE: ASSESSMENT OF LIMITATIONS AND POTENTIAL IMPROVEMENTS

The utilization of cell-free circulating nucleic acids has been introduced as an easy-to-use, non-invasive technique for cancer diagnosis. In addition to being valuable sources of biomarkers, nucleic acids are biomarkers themselves. As such, they can effectively be used as tools for early detection, diagnosis, and follow-up of the disease. Their major technical challenge, however, is the limitation exerted by the low quantity and poor quality of tumor-specific DNA and its contamination by normal DNA. To overcome these shortcomings, some published articles have focused on the development of alternative protocols.

It is assumed that human blood contains a pool of tumor-specific DNA derived from focal areas of a heterogeneous primary tumor containing diverse genetic abnormalities, If true, plasma DNA would probably be an appropriate source for detecting the loss of heterozygosity (LOH) [92]. Detection of LOH in cell-free circulating DNA may be constrained by the restrictions mentioned above. Muller *et al.* [93] have attempted to overcome this dilemma by taking advantage of a PCR-based fluorescence microsatellite assay for 12 polymorphic markers for the purpose of investigating LOH in the cell-free plasma DNA of 59 prostate cancer patients. To enrich the DNA portion containing shorter fragments [94], fractionation of plasma DNA into high and low molecular weights was performed. To enhance assay sensitivity for LOH detection in circulating DNA and avoid artificial discoveries, the authors proposed increasing the yield and specificity of PCR by adding tetramethl-ammonium chloride to each PCR reaction. In an effort to improve assay sensitivity for quantitative analysis of DNA methylation in circulating DNA, on the other hand, Vaissiere *et al.* [95] proposed a novel combination of methods based on genome-wide amplification of bissulphite-modified DNA template followed by quantitative methylation detection using pyrosequencing. These authors successfully analyzed multiple genes from a small amount of starting DNA. Furthermore, there is evidence in the literature suggesting that the integrity of serum-circulating DNA may play a biomarker role in detecting cancer-tumor progression and regional metastasis [6]. In order to improve analyzing the size of circulating DNA and quantifying circulating nucleic-acid fragments in human serum, Liu *et al.* [96], have developed a PCR-free, single-molecule assay for CAN analysis based on microfluidic, cylindrical illumination, confocal spectroscopy. Thus, the authors have put forward an alternative method, which, if accompanied by an appropriate assay design and probe specificity, may be able to accurately analyze DNA integrity in cell-free circulating DNA.

CIRCULATING DNA VERSUS CIRCULATING TUMOR CELLS TECHNIQUE

In addition to cell free-circulating DNA, detection and quantification of circulating cancer cells in peripheral blood may also improve cancer staging and monitoring. We have already outlined in a previous paragraph specific concerns that are relevant for accurate detection of cell-free tumor DNA in the blood of cancer patients harboring tumor-specific aberration. In the case of circulating tumor cells (CTCs) from blood specimens of patients with cancer the major technical challenge we have to face to detect a very selected few CTCs among so many leukocytes, erythrocytes and epithelial non-tumor cells which are also supposed to be found in blood stream. In this context, when non-tumor cells are inappropriately detected as real circulating tumor cells It can have strong negative therapeutic effects. Nonetheless, technical advances have facilitated the detection of rare circulating tumor cells in the bloodstream. Gabri *et al.* [97], for instance, reported interesting results in an experimental study designed to evaluate molecular detection of circulating melanoma cells by reverse transcription-polymerase chain reaction (RT-PCR). Their results suggest that very small amounts of tyrosinase mRNA which is an enzyme specifically expressed by melanocytes and melanoma cells can be properly detected through nested PCR needing an amount of total RNA comparable to less than a single melanoma cell. More recently, Katz *et al.* [98] proposed a novel methodology to detect circulating abnormal cells in peripheral blood of patients with NSCLC. In an attempt to improve assay sensitivity for quantitative analysis of circulating genetically abnormal cells (CACs) they have developed a technique to determine if a fluorescence in situ hybridization (FISH)-based assay using isolated peripheral blood mononuclear cells with DNA probes targeting specific sites on chromosomes known to have abnormalities in non-small cell lung cancer cases could detect circulating genetically abnormal cells. These authors successfully achieved a significant correlation between the numbers of CACs and the presence of cancer. In order to improve efficient detection of tumor cells from peripheral blood of patients with cancer, Hosokawa *et al.* [99] have developed a microfluidic device equipped with a size-selective microcavity array which can specifically separate tumor cells from in the bloodstream on the basis of differences in the size and deformability between tumor and hematologic cells. The cells recovered on the microcavity array were further processed for image-based immunophenotypic analysis using a fluorescence microscope. These authors successfully recovered lung carcinoma, breast, gastric and colon tumor cells on the microcavity array with high efficiency, suggesting that their microfluidic technique has some potential as a tool for the detection of circulating tumor cells.

CONCLUSIONS

Lung cancer is one of the most frequently-diagnosed cancers worldwide. Lung cancer cells undergo metastasis very soon after carcinogenesis, characterizing the lung neoplasm as a particularly dangerous, life-threatening cancer. Free circulating DNA has been used as a non-invasive source of biomarker candidates, having been deemed particularly useful in cancer staging, prognosis, and treatment selection. Our knowledge regarding the molecular features of lung malignancies, and the ability to accurately measure an abnormal amount of free circulating DNA has the potential to greatly advance our current skill levels in making the clinical management of the disease more efficient. Despite remarkable progress in this type of research, however, many controversial results and technical challenges continue to afflict professionals in the field. Consequently, there is no doubt that the development of

either a specific single- or multiple-step diagnostic procedure is urgently needed.

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ABREVIATIONS

NSCLC	=	Non-small-cell lung cancer
EGFR	=	Epidermal growth factor receptor
EGFR TKIs	=	Epidermal growth factor receptor tyrosine kinase inhibitors
HCC	=	Hepatocellular carcinoma
LC	=	Lung cancer
CAN	=	Circulating nucleic acid
CTCs	=	Circulating tumor cells

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