Experimed 2023; 13(2): 86-92

ORIGINAL ARTICLE

DOI: 10.26650/experimed.1260973

Evaluation of DNA Microarray in Biomarker Detection in Cell-free DNA from Colorectal Cancer Cell Lines: A Proof-of-Concept Study

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Cite this article as: Kazan HH, Karahan CP, Celik E, Ozketen AC, Birgucu Cagil D, Ergun MA. Evaluation of DNA microarray in biomarker detection in cell-free DNA from colorectal cancer cell lines: A proof-of-concept study. Experimed 2023; 13(2): 86-92.

ABSTRACT

Objective: DNA microarray is a powerful method to identify genomic anomalies including small insertions, duplications and/or deletions. This method is widely used in routine genetic screening for explaining the genetic background of certain phenotypes, for example, cancer. Cell-free DNA (cfDNA), which is an approach that may give information about the somatic tissues in peripheral blood, is another popular method used in routine genetic screening to understand the background of particular phenotypes, one of which is cancer. There is limited available research that investigates the involvement of these two approaches to decipher novel cancer biomarkers in the literature. However, detection of cancer biomarkers, especially non-invasive types, has been of great interest to research groups.

Materials and Methods: In the present study, we used colorectal cancer as a model tumor to figure out whether we could determine definite biomarkers from cfDNA using DNA microarray methodology. We isolated cfDNA from the cell-free mediums of the cultures of colorectal cancer cell lines in the presence of the control group which was the healthy epithelial colon cell line.

Results: Our results underlined significant alterations that were deletions and/or duplications in some of the genomic regions in a cell line-specific manner.

Conclusion: We propose that DNA microarray could be used to assess the sub-types of certain cancers in a non-invasive manner using cfDNA approaches.

Keywords: cfDNA, carcinogenesis, colorectal cancer, DNA microarray, biomarker

INTRODUCTION

Colorectal cancer (CRC) is one of the most widespread cancers estimated by the Global Cancer Observatory listed in the 'cancer today' data of 2020 (1). CRC is in third place for the number of incidences; however, it ranks second in terms of mortality rate (2). Considering the estimated number of 1.9 million cases annually, this notorious disease devastates the economy and life expectancy worldwide. The early diagnosis of the tumorigenesis and profiling the mutations may increase the survival rate by improving the chance to fight back. It has been reported that only 30-40% of the cases were diagnosed at early stages (3). Hence,

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Submitted: 07.03.2023 **Revision Requested:** 24.03.2023 **Last Revision Received:** 30.05.2023 **Accepted:** 05.06.2023 **Published Online:** 04.08.2023

there is a continuous need for novel diagnostic processes to detect the disease in the initial phases.

Current screening processes for CRC involve analyzing the presence of blood in stool samples, fecal immunochemical tests and colonoscopies (4). When the result is positive, a biopsy takes place to profile the tumor. Nevertheless, the heterogeneity of cancer remains a big challenge to identify mutations (5). Testing blood samples or other body fluids could decrease the compliance of patients. Cell-free DNA (cfDNA) is one of the most promising macromolecules found in body fluids such as blood, saliva and urine (6). It originates from apoptotic, necrotic, or secretory pathways of both healthy and cancer cells of the body (4). These cleaved small DNA sequences contain biomarkers to identify and characterize heterogeneous tumor cells without blockage by tissue-specific boundaries. Therefore, cfDNA has been targeted and exploited by several studies to detect markers of cancers for early diagnosis. In these reports, next-generation sequencing (NGS), real-time polymerase chain reaction (RT-PCR) or droplet digital PCR (ddPCR) have been employed to identify the markers (7-9).

DNA microarray is a robust strategy for the identification and high-throughput screening of genomic anomalies including point mutations, chromosomal alterations such as deletions, and insertions, and copy number variations (CNVs). Microarray tools provide advantages in cancer diagnosis, classification and determination of treatment options (10, 11). Therefore, combining DNA microarray strategy with cfDNA approach would be a vigorous tactic to identify and analyze the markers embedded in cfDNA without meddling with tumor heterogeneity, tissue type and time points. Large-scale screening of cancer markers at any time interval could be possible with this robust, flexible and effortless method using only body fluids.

The present study aimed to clarify any possible markers in the cfDNAs obtained from different CRC cell lines using DNA microarray approach. Up to now, this is the first study assessing biomarker availability by combining both cfDNA and DNA microarray approaches.

MATERIALS AND METHODS

Cell Lines and Cell Culture

In the present study, sporadic colorectal cancer cell lines, LIM1863; colorectal carcinoma cell lines, HCT116 and CACO-2; and colorectal adenocarcinoma cell lines with epithelial morphology HT-29 were used in the presence of normal colon fibroblast cell lines CCD-18Co. All cells were grown in RPMI 1640 medium (Thermo Fischer, USA) in the presence of 1% penicillin/streptomycin (Thermo Fischer, USA) and 10% fetal bovine serum (FBS; Thermo Fischer, USA) in an incubator under 37^{0}C and 5% CO_2 conditions. Cells were routinely passaged when they reached 80% confluency. All experiments were performed when the confluency was 80%.

Isolation of cfDNA

cfDNAs were isolated from the mediums in which the cells were cultured. When the cells reached 80% confluency, the medium on the cells were carefully obtained. Next, medium was slightly centrifuged at 200 g for 5 min to pellet the remaining cells. The supernatants were used for cfDNA isolation using a ZipPrime cfDNA isolation kit (ZipPrime Ltd., Turkiye). In short, 1 ml of the medium was lysed by pre-heated lysis buffer containing proteinase K (Thermo Scientific, USA). Then, the lysate was mixed by a binding solution including binding beads, and shaken for 10 min at room temperature. After mixing, the supernatants were removed with the help of a magnetic rack, and the beads were washed with washing solutions twice. Finally, the beads were dried and re-suspended with 20 µl of the elution buffer, and the supernatants containing cfDNA were obtained. The concentrations and purities of the isolated cfDNAs were determined by both NanoDrop (NanoDrop ND-1000; Thermo Scientific, USA) via optical density ratios (OD260/280 and OD260/230), and Qubit 4 Fluorometer (Thermo Scientific, USA).

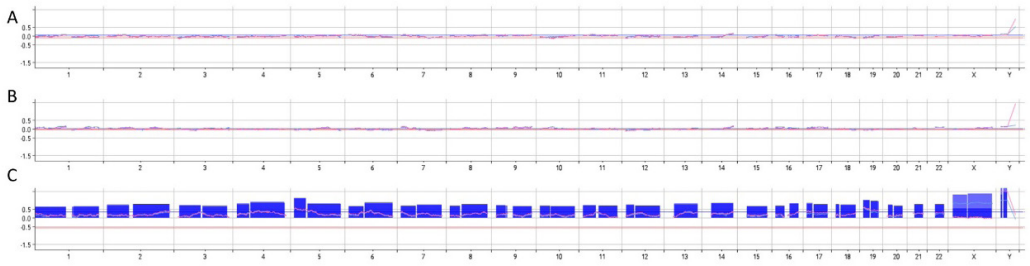

Figure 1. Comparison of the microarray results obtained from CCD-18Co cfDNA with the reference and assessment of genomic DNA (gDNA) contamination. The cfDNAs from CCD-18Co cells were isolated at three different culturing processes. One of them was used as the reference for all cell groups while the others (A and B) were assessed as a study group. As a further control, one of the cfDNAs was deliberately contaminated by commercial human reference DNA and a microarray was performed after whole genome amplification (WGA) step (C). Blue bars correspond to the amplifications of the regions.

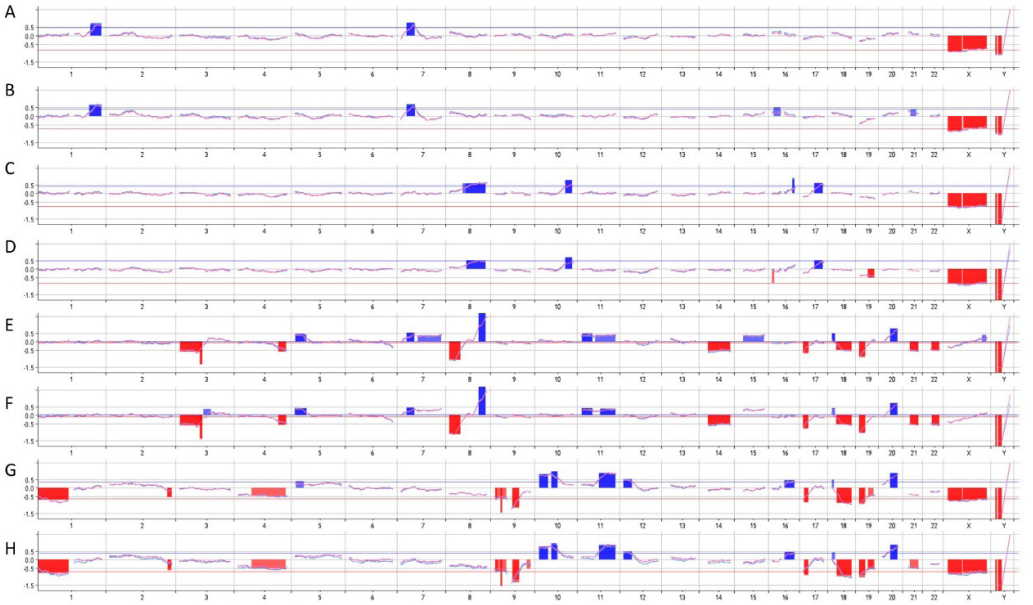

Figure 2. DNA microarray results from cfDNAs obtained from LIM1863 (A-B), HCT116 (C-D), HT-29 (E-F) and CACO-2 (G-H) cells. Red bars point to the losses while blue ones underline the gains.

Whole Genome Amplification (WGA) and DNA Microarray

The isolated cfDNAs were whole-genome amplified using REPLI-g WGA kit (Qiagen, Germany) according to the supplier's instructions. The amplification was checked with 1% agarose gel. WGA products were labeled with Cyanine3 (Cy3) or Cyanine5 (Cy5) fluorophores at 37^{0}C for 2 h. After the purification step, the labeled DNAs were hybridized using GenetiSure Pre-Screen 8x60K slides (Agilent Technologies, Inc., USA) at 67^{0}C for 16 h. After the washing and drying steps, the slides were scanned by a Sure Scan Microarray Scanner (Agilent Technologies, Inc., USA). The data were analyzed by CytoGenomics Single Cell Analysis software (Agilent Technologies, Inc., USA). All annotations were according to hg19.

Statistical Analyses

The obtaining mediums and cfDNA isolations were duplicated at different periods and thus each sample was run twice. For aberration detection, the log2 ratio was set to default as 0.35 for amplification/gain and -0.45 for deletion/loss as proposed by the application note of the system (12).

RESULTS

Characterization of Isolated cfDNAs

The cfDNAs isolated from the culture mediums of the cells were fluorescently quantified and the concentrations were determined between 1.51-25.3 ng/µl. The gDNA contamination was followed by deliberately addition of gDNA into one of isolated cfDNAs and performing DNA microarray by this sample. According to the results (Figure 1), possible availability of gDNA bring about remarkable copy gains compared to non-contaminated samples, underlying there were not any gDNA contamination in the study group.

Comparison of CNVs between the cfDNAs

After the DNA microarray step, the chromosomal segments were compared to detect the common and differentiated CNVs between the cfDNAs isolated from the cell lines (Figure 2). The results underlined that some CNVs listed in the Table 1 were specific to each cell line while others were common between the cell lines as a combination of LIM1863& HT-29, HCT116& HT-29 and HT-29& CACO-2 by same loss or gain patterns (Table 2).

DISCUSSION

CRC is one of the deadliest cancer types worldwide and the number of patients with CRC were predicted to approximately be doubled by 2040. CRC subtypes are traditionally classified according to the segment of cancer location; nonetheless, they have more complicated molecular classifications. The molecularly classified subtypes have been shown to associate with clinical outcomes. However, these subtype definitions are limited to a picture of the clinical outcome and further approaches are still needed (13).

cfDNA consists of the small DNA segments that are released by the cells into the circulatory system. In cancer, cfDNA may consist of tumor-derived DNA sequences, which could serve as a platform for biomarkers for the detection and monitoring of cancer and therapy. Numerous studies have frequently focused on cfDNA-based biomarker detection using several

Table 1. The differentially affected regions by gains or losses for each cell line.

Cell line	Chr	Start-Stop	Cytoband	Size (kb)	Gain/Loss
LIM1863	1	206075579-248563836	q32.1 - q44	42,488.258	Gain
	7	23194747-23822183	p15.3	627,436	
HCT116	8	67352994-142205781	q13.1 - q24.3	74,852.788	Gain
	10	104003117-131291788	q24.32 - q26.3	27,288.672	
	17	43120016-77220974	q21.31 - q25.3	34,100.959	
HT-29	5	1130129-45142098	p15.33 - p12	44,011.97	Gain
	11	2909874-44075140	p15.4 - p11.2	41,165.267	
	3	1464291-90273445	p26.3 - p11.1	88,809.155	Loss
	8	215827-43407979	p23.3 - p11.1	43,192.153	
	14	19849591-107152122	q11.2 - q32.33	87,302.532	
	21	14420615-48022981	q11.2 - q22.3	33,602.367	
	22	21240223-51170223	q11.21 - q13.33	29,930.001	
CACO-2	1	746608-121150012	p36.33 - p11.2	120,403.405	Loss
	2	226945229-242938241	q36.3 - q37.3	15,993.013	
	4	53656120-157605164	q12-q32.1	103,949.045	
	9	3022488-12155545	p24.2 - p23	9,133.058	
		23915540-30045342	p21.3 - p21.1	6,129.803	
		69430459-97151185	q21.11 - q22.32	27,720.727	
	10	3314825-38219750	p15.2 - p11.1	34,904.926	Gain
		49954828-72912411	q11.23 - q22.1	22,957.584	
	12	792951-34285770	p13.33 - p11.1	33,492.82	
	16	51171823-87138780	q12.1 - q24.2	35,966.958	

Table 2. The same regions affected by gains or losses for the cell lines.

Cell lines	Chr	Start-Stop	Cytoband	Size (kb)	Gain/Loss
LIM1863& HT-29	7	23822183-52965122	p15.3 - p12.1	29,142.94	Gain
HCT116& HT-29	8	115281701-142205810	q23.3 - q24.3	26,924.11	Gain
HT-29& CACO-2	4	157605164-187638862	q32.1 - q35.2	30,033.699	Loss
	11	73712308-134790190	q13.4 - q25	61,077.883	Gain
	17	44684-21180656	p13.3 - p11.2	21,135.973	Loss
	18	861334-10532357	p11.32 - p11.22	9,671.024	Gain
		20663689-77954136	q11.2 - q23	57,290.448	Loss
	19	277373-23035449	p13.3 - p12	22,758.077	Loss
	20	31257259-60346875	q11.21 - q13.33	29,089.617	Gain

high-throughput methods such as NGS (14-17). However, the drawbacks of the NGS systems, such as high cost and time requirements, restrict the wide applicability of the approach (18). Therefore, other high-throughput methods including the DNA microarray approach should be widespread. Huge chromosomal structural alterations and CNVs have extensively been linked to carcinogenesis, pointing out the importance of DNA microarray method in the detection and monitoring of cancer (19).

Although two phenomena, cfDNA, and DNA microarray, have been separately evaluated in cancer studies, recent efforts have focused on the combination of these approaches. For instance, Azad et al. showed the androgen receptor gene anomalies using cfDNA and DNA microarray in patients with prostate cancer (20). Similarly, the product notes of the Agilent Company presented a detailed protocol for the assessment of the power of the DNA microarray on the single-cell level (12). However, none of the studies focused on the sub-typing of specific cancer by the combination of DNA microarray and cfDNA. As a part of an ongoing study, we aimed to evaluate the efficacy of the DNA microarray method on cfDNAs isolated from the medium of the CRC cell lines; suspension sporadic colorectal cancer cell lines; LIM1863; adherent colorectal carcinoma cell lines HCT116 and CACO-2; and adherent colorectal adenocarcinoma cell lines with epithelial morphology HT-29, in the presence of adherent normal colon fibroblast cell line CCD-18Co. The properties of these cell lines, especially secretome profiles have been proven to be distinguishable at the molecular level (21, 22), underlying diversity in the genetic background. Hence, it may be possible to sub-type these cells according to differences in the cfDNA profiles.

In this study, we isolated cfDNAs from the mediums where the cells were grown. Accordingly, we removed the cells by centrifugation and used cell-free medium to obtain cfDNAs. Although we did not perform the size analyses to clearly ensure the isolation of cfDNAs, whose size was about 160 bp (16), we confirmed the lack of genomic DNA (gDNA) owing to the centrifugation step and the concentrations of isolated DNA, which were in a range of 1.51-25.3 ng/µL. Moreover, a recent study underlined that the mediums on the cells could contain mitochondria and intact mitochondrial DNA (23); therefore, a size analysis of cfDNA would not be a practical approach to prove the purity of the cfDNA. To further ensure the lack of the gDNA, additionally, we analyzed the cfDNA from the reference cell line, CCD-18Co, whose cfDNAs were separately isolated from the reference one. The involvement of gDNA in the cfDNA was proved to alter the DNA microarray results (12). However, we did not realize any differences in the CCD-18Co duplicates compared to the reference well (Figures 1A and B). This result may demonstrate the lack of gDNA contamination in the cfDNA samples. Importantly, when gDNA was deliberately contaminated into the isolated cfDNAs, the array results were completely problematic showing significant amplification of all chromosomes (Figure 1C). This result also proved that the cfDNAs were gDNA-free.

Next, we conducted a DNA microarray from cfDNAs obtained from different CRC cell lines compared to that of the reference cell line, CCD-18Co in the presence of biological controls for each cell line. According to the results, the cfDNAs had losses and gains distinguishable between the cell lines (Figure 2), which could be used to *in vitro* determine the subtypes of the CRC phenotypes. Although the amplified or lost regions were highly similar to each other, some regions (for example chromosome 16 for the HCT116 cell line); (Figures 2C and D) was drastically different between the replicas, clarifying that these regions should not be evaluated for subtyping purposes. Notably, there was a huge diversity of the sex chromosome for all cell lines. The system aims to determine the sex of the sample using sex chromosome-dependent probes. According to the American Type Culture Collection (ATCC) and The Cellosaurus databases, LIM1863 and HT-29 originate from female patients while HCT116 and CACO-2 from male ones. Nonetheless, our results did not correspond to the relative sex phenotypes. The success rate of the DNA microarray systems in properly detecting the sex chromosome has been reported to be low (24). Still, the problem with the sex chromosomes would be the result of DNA microarray application on cfDNA and requires further studies. Our propose was not about to examine the sex chromosomes in the present study.

When the common variations between the replicas were evaluated, specific affected chromosome regions were distinguishable between the cell lines. The regions were evaluated comparatively and the differentially altered regions are listed in Table 1, which could be used as a biomarker for subtyping of the CRC. Despite not being for all cell lines, the regions listed in Table 2 were common between the cell lines. Accordingly, the chromosomes of the LIM1863 and HCT116 were shown to be minimally affected while those of HT-29 and CACO-2 cells had dramatic alterations by the cfDNA screening. Chromosomal characterization by gDNA of the CRC cell lines including HCT116, HT-29, and CACO-2 has previously been carried out, and cellular and chromosomal differences were well-reported and showed the remarkable diversity between the cell lines (25), which may confirm the results of the present study. Moreover, these cell lines could be classified according to their differentiation properties and HT-29, and CACO-2 cells compared to LIM1863 and HCT116 have higher differentiation capabilities (26). For particular cell types, differentiation was shown to induce cell death (27) and the association between differentiation and cell death is well documented (28).

When the differentially affected regions (Table 1) were examined by the gene information, 656 genes were seen to have higher copies for the cfDNAs from the LIM1863 cell line. When the pathways were analyzed for these genes by Reactome Database (29), most of the gene-encoded proteins had a role in the olfactory signaling pathway that was already linked to carcinogenesis (30). For the HCT116 cell line, 1720 genes were amplified and the products of these genes mainly had a role in fibroblast growth factor receptor 2 (FGFR2) mutant receptor activation corresponding to colorectal cancer in the literature

(31). In the approximately 5.5 thousand affected genes for the HT-29 cell line, 993 genes whose products acted normally in the olfactory signaling pathway were amplified while 4654 genes whose products were involved predominantly in cellular inflammation, Fc gamma receptor (FCGR) activation and phagocytosis associating with systemic inflammatory clearance lost copies. For CACO-2 cells, similar to HT-29, about 5.5 thousand genes were affected. Of those genes, 1710 of them gained copies and the products of these genes had a role primarily in response to metal ions triggering the carcinogenesis (32), 3792 genes whose products participated in the inhibition of signaling by overexpressed epidermal growth factor receptor (EGFR) that lost copies, putatively to overcome EGFR-targeted strategies. Overall, the products of all affected genes were actualized to directly or indirectly associate with carcinogenesis as expected.

CONCLUSION

The importance of the cfDNA has been well-documented in CRC patients (33-35). However, cfDNAs from patients and/or cell lines should be re-evaluated to diagnose, follow up or treat the CRC by combining diverse approaches. Accordingly, the present study points to the combination of the cfDNA and DNA microarray approaches to find biomarkers specific to subtypes of the CRC cell lines for the first time. Still, further studies are needed to totally explore the potential of these approaches.

Ethics Committee Approval: Ethical approval is not required as the study was performed on cell lines.

Authors' Contributions: Conception/Design of Study – H.H.K., E.C., M.A.E.; Data Acquisition – D.B.C.; Performing experiments – C.P.K., D.B.C.; Data Analysis/Interpretation – A.C.O., H.H.K.; Drafting Manuscript – H.H.K., A.C.O., E.C.; Critical Revision of Manuscript – D.B.C., H.H.K., A.C.O., E.C.; Final Approval and Accountability– H.H.K, E.C., A.C.O., M.A.E.

Conflicts of Interest: The authors declare no conflict of interest.

Financial Disclosure: The authors declare that this study has received no financial support.

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