Single-Cell Analysis of CTCs and Biomarker Detections

Subjects: Oncology

Contributor: Tanzila Khan , Therese M. Becker , Joseph W. Po , Wei Chua , Yafeng Ma

The field of single-cell analysis has advanced rapidly in the last decade and is providing new insights into the characterization of intercellular genetic heterogeneity and complexity, especially in human cancer. Circulating and disseminated tumor cells (CTCs and DTCs) are cancer cells that dissociate from primary and metastatic cancer sites and enter the circulation with potential to seed distant metastases. CTCs can be enriched or isolated from a simple blood liquid biopsy. Analysis of multiple single CTCs has the potential to allow the identification and characterization of cancer heterogeneity to guide best therapy and predict therapeutic response.

whole genome amplification | circulating tumor cell (CTC) | single-cell analysis | biomarkers

1. Breast Cancer

Breast cancer (BC) is the most common female cancer and CTC is a predictive marker of poor survival and metastatic relapse [1]. The detection rate of CTCs correlates with the number of metastatic sites, and BC patients with brain metastasis may have the highest CTC counts [2].

The hormone status of BC, such as expression of the estrogen receptor (ER) or progesterone receptor (PR), indicates the feasibility of ER-targeted endocrine therapy [3]. However, no correlation was found between total CTC number and/or ER expression status as determined by immunocytostaining and the intensity of ER staining in primary tumors [4]. Only 81.3% of patients were positive for ER expression in CTCs, while ER-negative CTCs were also found in ER-positive patients, delineating the genetic inconsistencies between CTC counts. ER status in CTCs might have predictive power with regard to response and resistance to endocrine therapy and may thus help in the choice of better treatment options [4]. One study performed Sanger sequencing on CTC WGAs (MALBAC), which resulted in the identification of the *ESR1*-Y537S variant known to produce a constitutively active receptor and *ESR1*-T570I (a novel mutation) in exon 8 [5]. This study found *ESR1*-Y537S heterozygously and homozygously in single CTCs and confirmed mutations in matched cell-free DNA (cfDNA) in one patient. Interestingly, in another patient, heterozygote *ESR1*-T570I and homozygote *ESR1*-Y537S were found in a single CTC, but *ESR1*-T570I could not be detected in matched cfDNA [5]. Thus, using two entities extractable from a blood biopsy, CTCs and cfDNA biomarkers may complement each other and enhance the chance of finding disease-related variants. However, in another study that screened for exon 4, 6 and 8 *ESR1* mutations after WGA (Picoplex, MALBAC), none was found in individual CTCs [4].

The PI3K/AKT/mTOR pathway (Phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin) regulates cell growth, survival, and angiogenesis. Upregulated activity has been linked to oncogenesis and is a major therapeutic target [6]. In BC, mutations in PIK3CA are found in about 40% of ER-positive cancers and have been implicated in resistance to HER2-based therapies [7]. Pharmacologic targeting of PIK3Ca in HR (hormone receptor) +/HER2-metastatic BC offers significant benefits to patients with endocrine therapy resistance [8]. Several single CTC-based studies [8][9][10][11][12] were conducted to study mutations in the PIK3CA gene. Heterogenous expression of PIK3CA mutations among CTCs and matched primary tumors, and even among CTCs from the same patient, was observed. Individual PIK3CA mutations found in Ampli1-amplified CTCs included E542K and H1047R [8], as well as E542K, E545K and H1047R, as was determined in a second study [10]. Another study found PIK3CA mutations (E542K, E545K, H1047R, H1047L and M1043V) in exon 9 and 20 in at least one CTC in 36.4% of the patients tested [13]; similar data were reported in other studies [11][12] (**Table 1**).

Table 1. The application of WGA and biomarker detection of single CTCs in various cancer types.

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic Mutations and Alterations
mBC or HER2- mBC							
Babayan, A. et al., 2013 [4]	Density gradient	Micromanipulator TransferMan NK2	PicoPlex	Multiplex PCR	4	8 single CTCs	*ESR1* mutations in exons 4, 6 and 8 were not found
De Luca, F. et al., 2016 [14]	CellSearch	DEPArray	Ampli1	NGS (Ion AmpliSeq Cancer Hotspot panel v2)	4	3–5 single CTCs per patient	51 sequence variants in 25 genes were found, including somatic mutations in *TP53* (8 mutations) and *PDGFRA* (3 mutations). High intra- and inter-patient heterogeneity, discordance in mutational status between CTCs and primary tissue
Gasch, C. et al., 2016 [13]	CellSearch	Micromanipulator TransferMan NK2	GenomiPhi, Ampli1	Sanger sequencing, PCR	33	114 single CTCs	*PIK3CA* mutations in exon 9 and 20
Kaur, P. et al., 2020 [15]	Microfluidic ANGLE Parsortix	NA	REPLI-g	WES (SNVs, CNAs and SVs)	5	5 CTCs and 5 WBCs	Elevated C>T mutational signature in patient samples. Low VAFs for somatic variants in CTCs compared to metastasis, complex rearrangement patterns were observed, high discordance between paired samples, marked heterogeneity of somatic landscape
Li, S. et al., 2020 [2]	CellCollector	CellCollector	REPLI-g	NGS (HiSeq X-Ten Illumina)	17	0–15 CTCs	Different metastatic sites have their own corresponding high-frequency mutation genes
Neumann, M. H. et al., 2016 [16]	CellSearch	CellCelector	Ampli1	For library preparation, the multiplex PCR-based Ion Torrent AmpliSeqTM	2	7 single CTCs	Functional *PIK3CA* SNP (G to A, E545K) was detected in CTCs of patient 1 but not in CTCs of patient 2

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic Mutations and Alterations
				technology with Ampli1 CHPCustom Beta panel			
Neves, R. P. et al., 2014 [12]	CellSearch	FACS	Ampli1	aCGH (CNAs), qPCR	30	192 single CTCs	72.9% WGA success rate, 46.2% of WGA products show *CCND1* amplification, mutations in *PIK3CA* exon 20 in c.3140 were found in CTCs (2/12 analyzed patients), *TP53* mutations in exons 5, 7 and 8 were not found
Paolillo, C. et al., 2017 [5]	CellSearch	DEPArray	MALBAC	Sanger sequencing	3	40 single CTCs and 12 WBCs	*ESR1* mutations (Y537S and T570I) were identified
Pestrin, M. et al., 2014 [10]	CellSearch	DEPArray	Ampli1	Sanger sequencing (hotspot regions in PIK3CA exon 9, 20)	18	115 single CTCs	33% of patients had an identified *PI3KCA* mutation. Six different mutations in the *PI3KCA* gene, such as c.3140A>G, c.1633G>A, c.1624G>A, c.1624G>A, etc., were identified
Polzer, B. et al., 2014 [11]	CellSearch	DEPArray	Ampli1	ERBB2 qPCR (CNV), PIK3CA Sequencing, aCGH	66	510 single CTCs and 189 leukocytes	*PIK3CA* mutations in exon 9 and 20. Analysis of ERBB2 alterations
Schneck, H. et al., 2013 [8]	CellSearch	NA	Ampli1	Multiplex PCR, SNaPshot	44	NA	*PIK3CA* mutations in exon 9 and 20, such as E545K and H1047R, were detected, but E542K, E545G and E545A were not found
Wang, Y. et al., 2018 [17]	FACS combined with oHSV1-hTERT-GFP viral infection	FACS	MALBAC	WGS for CTC, WGS and WES for matched primary and metastatic tissue	8	11 single CTCs	SNVs accumulated sporadically among CTCs and matched primary tumors, at least 2 CTCs shared 394 SNVs, SNV mutations in *APC* and *LRP1B* genes co-occurred in CTC-shared and bulk tissue, CTC behaviour-related SNVs were verified
Zou, L. et al., 2020 [18]	CellSearch	Micropipetting	MALBAC	WGS (CNV and gene set enrichment analysis)	2	Single CTCs, but number is unknown	Different frequencies of CNVs between newly diagnosed and recurrent liver metastasis; similar CNV patterns among isolated CTCs of recurrent BCLM and recurrent liver metastasis; 25 genes were identified as CNV signatures of BCLM, including β-defensins and defensins
				PC or mCRPC			
Faugeroux, V. et al.,	ISET filtration, CellSearch,	Self-seeding microwell chips,	Ampli1	WES (10x depth	11	179 WGA samples or	Shared *GRM8*, *TP53* and *PTEN* mutations in epithelial CTC samples and other CTC-exclusive variants

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic Mutations and Alterations
2018 [19]	Rosettesep	FACS, laser microdissection		coverage)		34 WES	
Greene, S. B. et al., 2016 [20]	Epic Sciences	Eppendorf TransferMan NK4 micromanipulator	SeqPlex Enhanced	Sequencing with Illumina NextSeq500 using a High Output kit in a Paired-End 2x150 format (PE 2x150) (CNV)	7	67 single CTCs	*AR* amplification and *PTEN* loss
Gupta, S. et al., 2016 [21]	CellSearch, RBC lysis and CD45 depletion	IE/FACS	RepliGene, WGA4	aCGH (CNV)	16	16 CTCs and matched leukocytes	*AR* amplification in 50% of CTC samples, *ERG* genomic amplification in 40% of patients, *PTEN* loss, genomic alteration in chromatin reading and proliferative pathways
Magbanua, M. J. et al., 2012 [22]	CellSearch, IE/FACS	IE/FACS	WGA4	aCGH	12	9 patient bulk CTCs	Gains in 8q and loss in 8p; gains in the *AR* region of chr X of CTCs, including *AR* gains in 78% of cases
Rangel-Pozzo, A. et al., 2020 [23]	ScreenCell filtration	Laser microdissection	Ampli1	WES	9	21 single CTCs and 4 lymphocytes	Genetic variations in nine telomere maintenance pathways, including telomeric repeat-binding factor 2 (TRF2), SNVs and indels associated with telomere maintenance genes and known cancer drug response; presence of CNAs in 11 different pathways, including the DNA damage repair (DDR) pathway
Wu, Y. et al., 2016 [24]	Density gradient, negative and positive selection with magnetic beads	Laser microdissection	PicoPLEX (<40 cells), WGA2 kit (GenomePlex for microdissected tissues)	SNP array profiling (CytoSNP-12 and omni1-Quad bead chips, NspI 250k, SNP6.0, and CytoScanHD arrays), Nanostring (nCounter Cancer CN panel)	8	8 disseminated tumor cells (bulk cells)	Gain of Ch 7 and 8q, loss in 8p, 12q23, 10q26, 13q and 16q21. *AR* gain, *TMPRSS2/ERG* alterations and *MYC* and other gained regions, *FOXO1* gene deletion
				Lung Cancer			
He, Y. et al., 2017 [25]	CellCollector	CellCollector	REPLI-g	NGS (hotspot panel v2)	5	6 CTCs	44 cancer-related genes existed in mutations in the analyzed CTCs and some cancer-related mutations were identified in *KIT*, *SMARCB1* and *TP53* genes

2. Prostate Cancer

Prostate cancer (PC) is the most common cancer type diagnosed in men; eventually, it develops into castrate-resistant prostate cancer (CRPC) following standard of care androgen deprivation therapy (ADT). Commonly altered genes during CRPC progression include *AR* (androgen receptor), *ERG* (ETS-related gene), *c-MET* (tyrosine-protein kinase MET), *PTEN* (phosphatase and tension homology deleted on chromosome 10)

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic Mutations and Alterations
Lu, S. et al., 2020 [26]	CellSearch	DEPArray	MALBAC, REPLI-g, WGA4, Ampli1	Targeted sequencing, WES, WGS	4	80 single CTCs and 11 WBCs	Comparative study, MALBAC WGA coupled with LP-WGS is a robust workflow for CNV profiling, but none of the WGA methods achieve sufficient sensitivity and specificity by WES
Mariscal, J. et al., 2016 [27]	CELLection Epithelial Enrich Dynabeads	NA	WTA2	Gene expression profiling (Agilent 4x44k gene expression arrays), qPCR	42 NSCLC patients and 16 controls	NA	CTC-specific expression profile associates with the PI3K/AKT, ERK1/2 and NF-kB pathways. *NOTCH1*, *PTP4A3*, *LGALS3* and *ITGB3* were further validated by RT-qPCR in an independent cohort of NSCLC patients
Nakamura, I. T. et al., 2021 [28]	AutoMACS	DEPArray	SMARTer PicoPLEX	NGS (Todai OncoPanel, AmpliSeq for Illumina comprehensive cancer panel, WGS) and Sanger sequencing	2	40 single floating tumor cells in pleural effusion	*EGFR* exon 19 deletion was confirmed in 63.2% of samples from case 1, detection of 85% *EML4-ALK* fusion in case 2, alectinib- resistant mutation of *ALK* (p.G1202R) in case 2. A *BRCA1* truncating mutation and an *RAF1* oncogenic mutation were identified
Ni, X. et al., 2013 [29]	CellSearch	Micropipetting	MALBAC	WGS at ~0.1× sequencing depth and WES for SNV/indel	11	72 single CTCs (including 4 leucocytes)	*EGFR* mutations (such as one INDEL p.K746_A750del), *PIK3CA* (such as p.E545K), *RB1* (p.R320*) and *TP53* mutations (such as p.T155I) were only shared between the liver metastatic tumor and CTCs; gain region in chromosome 8q contains the c-Myc gene; gain in chromosome 5p, which contains the telomerase reverse transcriptase (*TERT*) gene; chromosomal regions, including 3q29, 17q22, 17q25.3 and 20p13, had significant gain in all 19 CTCs of patients
Colorectal Cancer							
Fabbri, F. et al., 2013 [30]	OncoQuick	DEPArray	Ampli1	Sequencing and pyrosequencing	21	16 samples or cases	*KRAS* gene mutations in 50% of cases. G12C, G12D and G13D-KRAS mutations in one patient in three different groups of CTCs
Gasch, C. et al., 2013 [9]	CellSearch	Micromanipulator TransferMan NK2	GenomePlex, GenomiPhi	Targeted sequencing for KRAS, BRAF and PIK3CA gene, qPCR for EGFR	5	69 single CTCs	*EGFR* amplification in 7/26 CTCs, *KRAS* mutations (G12V) in 33% of CTCs, *PIK3CA* mutations (E545A and E542K) in 39% of CTCs, no *BRAF* locus change detected
Li, R. et al., 2019 [31]	Microfluidic chip (SCIGA-chip)	Microfluidic chip (SCIGA-chip)	MDA	Illumina sequencing	1	2 single CTCs and 1	A novel method involving all processing steps from blood collection to WGA preparation, 11 shared somatic

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3. Lung Cancer

The detection of certain driver mutations, such as in *EGFR* and *ALK* fusion, is associated with the early stages of lung cancer, its development and drug resistance [25]. Genetic analysis of CTCs from the same patient can give overall information about deletions, fusions, insertions and SNVs in the metastatic tumor and such changes can be monitored during treatment, even in the presence of cell-to-cell heterogeneity; however, a large number of CTCs needs to be sequenced [29].

Ni. et al. observed number of mutations in different genes, such as *EGFR, PIK3CA, RB1* and *TP53*, after exome sequencing of single-CTC WGA products. Amongst these alterations, one INDEL in the *EGFR* gene (K746_A750del), which is a target for tyrosine kinase inhibitors (TKIs), was found in CTCs as well as in the primary and metastatic tumors of the patients, while other mutations in *PIK3CA* (E545K), *TP53* (T155I) and *RB1* (R320*) genes were only observed in CTCs and metastatic tumors in the liver. This study also found some common CNV regions that have important roles in cancer development, such as cell proliferation, differentiation and protecting chromosomal ends from degradation. These regions include regions of gain in chromosome 8q, the *c-Myc* gene

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic Mutations and Alterations
				(SNPs/SVs) [29]		WBC	mutations (e.g., *C18orf25*, *GFM2*, *DDX60L*, etc.) and 153 structure variations were identified
Pancreatic Cancer							
Court, C.M et al., 2016 [32]	Density gradient and NanoVelcro/LCM microchip	Laser microdissection	REPLI-g	Sanger sequencing	12	119 single CTCs and 103 WBCs	*KRAS* mutations in 92% of patients and 33 out of 119 single CTCs sequenced (resulting in a 27.7% detection rate in single CTCs). No *KRAS* mutants were found in any WBCs
Melanoma							
Reid, A. L. et al., 2014 [33]	RBC lysis, immune-magnetic beads	NA	REPLI-g	ddPCR and castPCR	15	30 CTCs	Comparative study of ddPCR and castPCR. *BRAF*-V600E/K mutations were detected
Ruiz, C. et al., 2016 [34]	RBC lysis	Micromanipulator [28]	GenomePlex	CNV analysis	40	Single CTCs and WBCs	Deletions of *CDKN2A* and *PTEN*; amplifications of *BRAF*, *TERT*, *MDM2* and *KRAS*; chromosomal amplifications in chr12, 17 and 19
Mixed patient cohort							
Aljohani, H.M. et al., 2018 [35]	RBC lysis, CD45 depletion and EpCam positive selection	FACS	REPLI-g	Sanger sequencing, ddPCR	10	NA	Mutations (R34G, E79Q, E82G) in *Nrf2* in isolated CTCs, some mutations in the Keap/Nrf2/ARE pathway
Ferrarini, A. et al., 2018 [36]	CellSearch	DEPArray	Ampli1	WGS (CNAs), aCGH	3	15 single CTCs and 7 WBCs	A large amplification (100 Mbp) on chr 8, including the *c-MYC* gene, copy number loss was detected in the *BRCA2* locus
Gao, Y. et al., 2017 [37]	CellSearch	Micropipetting	MALBAC	WGS and WES for SNV/indels, SVs, CNs	23	97 single CTCs	Homozygous deletion of *PTEN*; amplification of the *MYC* gene; 11 focal regions were identified, including well-known tumor suppressors [9][30][40] or oncogenes, which were deleted or amplified

Note: aCGH: array comparative genomic hybridization; chr: chromosome; CNA: copy number alteration; CNV: copy number variant; mCRPC: metastatic castration resistant prostate cancer; ddPCR: droplet digital PCR; FACS: fluorescence activated cell sorting; IE: immunomagnetic enrichment; ddPCR: droplet digital polymerase chain reaction; RBC: red blood cell; SNV: single nucleotide variant; SNP: single nucleotide polymorphism; SV: structural variant; WBC: white blood cell; WES: whole exome sequencing; WGA4 and WGA2: different versions of GenomePlex; WGS: whole genome sequencing; WTA: whole transcriptome amplification; WTS: whole transcriptome sequencing; NA: not available.

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main therapeutic target; however, responses to EGFR inhibition are variable [9]. The key mutations found in single-cell analysis of CRC CTCs so far are *KRAS*, *PIK3CA* and *EGFR* mutations. Significant heterogeneous expression of *KRAS*, *PIK3CA* and *EGFR* was found among CTCs within the same patient and between different individuals [9][30]. A mutational discordance between primary tumor tissue and CTC WGAs was observed for *KRAS*, and remarkably different *KRAS* mutations in different single-CTC WGAs from the same individual patients have been observed [9][30]. CTCs were observed with increased EGFR expression in some patients, and *EGFR* gene amplification was identified in 7 out of 26 CTC WGAs for three patients [9].

5. Other Cancer Types

Pancreatic cancer is a lethal cancer with a less than 10% 5-year survival rate. *KRAS* is the predominant mutated gene in pancreatic cancer, and targeting KRAS may be an attractive therapy, despite many trial failures for anti-KRAS therapies [41]. *KRAS* mutations have been detected in 92% of patients, with a detection rate of 27.7% in total single-CTC WGAs (REPLI-g, MDA), but not in any WGAs of control WBCs. Interestingly, at least 10 single CTCs are required to reliably detect the *KRAS* heterozygous allele [32], which indicates that single-cell amplification bias responsible for ADO can be reduced by sequencing at least 10 cells together. In a study on single-CTC analysis of melanoma [34], *CDKN2A* and *PTEN* deletions and amplifications of *TERT*, *BRAF*, *KRAS* and *MDM2* were found. Moreover, new chromosomal amplifications of chromosomes 12, 17 and 19 were detected [34].

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