Single-Cell Analysis of CTCs and Biomarker Detections

Subjects: Oncology

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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.

Keywords: 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 M
				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**

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic M
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 ir including somatic mutatic and *PDGFRA* (3 mutation patient heterogeneity, di status between CTCs
Gasch, C. et al., 2016 [13]	CellSearch	Micromanipulator TransferMan NK2	GenomiPhi, Ampli1	Sanger sequencing, PCR	33	114 single CTCs	*PIK3CA* mutations
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 sig Low VAFs for somatic vari metastasis, complex rear observed, high discordanc marked heterogeneity
Li, S. et al., 2020 [2]	CellCollector	CellCollector	REPLI-g	NGS (HiSeq X-Ten Illumina)	17	0–15 CTCs	Different metastatic corresponding high-fre
Neumann, M. H. et al., 2016 [16]	CellSearch	CellCelector	Ampli1	For library preparation, the multiplex PCR-based Ion Torrent AmpliSeqTM technology with Ampli1 CHPCustom Beta panel	2	7 single CTCs	Functional *PIK3CA* SNP (G in CTCs of patient 1 but
Neves, R. P. et al., 2014 [12]	CellSearch	FACS	Ampli1	aCGH (CNAs), qPCR	30	192 single CTCs	72.9% WGA success rate show *CCND1* amplification, 20 in c.3140 were found patients), *TP53* mutations ir fou
Paolillo, C. et al., 2017 [5]	CellSearch	DEPArray	MALBAC	Sanger sequencing	3	40 single CTCs and 12 WBCs	*ESR1* mutations (Y537S a
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 ide Six different mutations in t c.3140A>G, c.1633G>A, c.1 were ide
Polzer, B. et al., 2014 [11]	CellSearch	DEPArray	Ampli1	ERBB2 qPCR (CNV), PIK3CA Sequencing, aCGH	66	510 single CTCs and 189 leukocytes	*PIK3CA* mutations Analysis of ERB
Schneck, H. et al., 2013 [8]	CellSearch	NA	Ampli1	Multiplex PCR, SNaPshot	44	NA	*PIK3CA* mutations in exor and H1047R, were detecte E545A were
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 spora matched primary tumors, a SNVs, SNV mutations in *A* occurred in CTC-share behaviour-related S
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 o diagnosed and recurrent liv patterns among isolated CT recurrent liver metastasis; 2 CNV signatures of BCLM, defer
				PC or mCRPC			
Faugeroux, V. et al., 2018 [19]	ISET filtration, CellSearch, Rosettesep	Self-seeding microwell chips, FACS, laser microdissection	Ampli1	WES (10x depth coverage)	11	179 WGA samples or 34 WES	Shared *GRM8*, *TP53* and *PT* CTC samples and other

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic M
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* amplificatior
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* amplificatio samples, *ERG* genomic patients, *PTEN* loss, genor reading and prolif
Magbanua, M. J. et al., 2012 [22]	CellSearch, IE/FACS	IE/FACS	WGA4	aCGH	12	9 patient bulk CTCs	Gains in 8q and loss *AR* region of chr X of CT 78% of
Rangel-Pozzo, A. et al., 2020 [23]	ScreenCell filtration	Laser microdissection	Ampli1	WES	9	21 single CTCs and 4 lymphocytes	Genetic variations in nin pathways, including telome (TRF2), SNVs and indels maintenance genes and kn presence of CNAs in 11 dif the DNA damage re
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 16q21. *AR* gain, *TMPR* and *MYC* and other gain dele
				Lung Cancer			
He, Y. et al., 2017 [25]	CellCollector	CellCollector	REPLI-g	NGS (hotspot panel v2)	5	6 CTCs	44 cancer-related genes e analyzed CTCs and some were identified in *KIT*, *SM*
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, MALB WGS is a robust workflow f of the WGA methods achiev specificity
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 p PI3K/AKT, ERK pathways. *NOTCH1*, *PTP4A* further validated by RT-q cohort of NS
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 w samples from case 1, d *ALK* fusion in case 2, ale of *ALK* (p.G1202R) in cas mutation and an *RAF1* o ident
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* mutation INDEL p.K746_A750d p.E545K), *RB1* (p.R320*) an p.T155I) were only shared b tumor and CTCs; gain re contains the c-Myc gene; which contains the telome (*TERT*) gene; chromosom 17q22, 17q25.3 and 20p13, 19 CTCs o
				Colorectal Cancer			

Studies (Author, Year)	CTC Isolation	CTC Recovery	WGA Kits	Downstream Molecular Analysis	CTCs+ Patients Analyzed	CTC Nr Analyzed for WGA	Main Findings in Genetic M
Fabbri, F. et al., 2013 [30]	OncoQuick	DEPArray	Ampli1	Sequencing and pyrosequencing	21	16 samples or cases	*KRAS* gene mutations in 5 and G13D-KRAS mutatio different gro
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 7/2 (G12V) in 33% of CTCs, *PIK* E542K) in 39% of CTCs, dete
Li, R. et al., 2019 [31]	Microfluidic chip (SCIGA-chip)	Microfluidic chip (SCIGA-chip)	MDA	Illumina sequencing (SNPs/SVs)	1	2 single CTCs and 1 WBC	A novel method involving blood collection to WGA somatic mutations (e.g., C etc.) and 153 structure v
				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 single CTCs sequence detection rate in single CTC found in a
				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 ddF V600E/K mutatio
Ruiz, C. et al., 2016 [34]	RBC lysis	Micromanipulator	GenomePlex	CNV analysis	40	Single CTCs and WBCs	Deletions of *CDKN2A* ar of *BRAF*, *TERT*, *MDM2* a amplifications in
				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, CTCs, some mutations in tl
Ferrarini, A. et al., 2018 [36]	CellSearch	DEPArray	Ampli1	WGS (CNAs), aCGH	3	15 single CTCs and 7 WBCs	A large amplification (100 the *c-MYC* gene, copy nur the *BRC*
Gao, Y. et al., 2017 [37]	CellSearch	Micropipetting	MALBAC	WGS and WES for SNV/indels, SVs, CNs	23	97 single CTCs	Homozygous deletion o the *MYC* gene; 11 focal including well-known tur oncogenes, which wer

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.

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) and *PI3K/AKT* signaling pathway genes. *AR* alterations in CTCs, especially *AR* amplification and expression of splice variant AR-V7, predict poor treatment outcomes for ADT [20][21][38][39]. *ERG* amplification of CTCs is also informative for treatment selection and might contribute to resistance to taxane therapy [21].

WGA-based single-CTC analysis found significant numbers of shared mutations in *PTEN*, *GRM8* and *TP53* among PC CTCs, particularly if they were of epithelial phenotype. Some recurrent mutations found in CTCs correlated with matched metastatic tissue. Interestingly, sequencing multiple CTCs did not significantly change the number of mutations found [19]. This may indicate that heterogeneity is less of an issue, as these mutations may be shared by most CTCs and are likely early events in cancer formation. Both epithelial and non-epithelial CTCs showed CTC-exclusive alterations affecting invasion, DNA repair mechanism, cancer-driver, and cytoskeleton genes [19]. The shared mutations between matched tissue and CTCs might provide insights into the metastatic spread of cancer and the origins of CTCs, as it is assumed that more mutations are acquired during cancer progression and spread.

aCGH analysis of CTC WGA products from CRPC patients demonstrated genomic gains in >25% of CTCs. Such genomic gains were observed in *AR*, *FOXA1*, *ABL1*, *MET*, *ERG*, *CDK12*, *BRD4* and *ZFHX3*, while common genomic losses involved *PTEN*, *ZFHX3*, *PDE4DIP*, *RAF1* and *GATA2*. *AR* and *NCOA2* amplification were found in 50% and 43.75% of CTC WGAs, respectively, while *ERG* amplification was found in 40% of patient CTCs. Loss of *KDM6A* was found in 6.25%, while *KDM6A* gain was found in 50% of mCRPC CTC samples. *MYCN* gene amplification was observed after the development of enzalutamide resistance. Similarly, *PTEN* gain was observed before starting enzalutamide, and *PTEN* loss appeared after enzalutamide treatment [21]. Another aCGH analysis of WGA CTCs found *AR* gain in 78% of nine patient bulk CTC samples (that is, samples combining more than a single CTC). However, *AR* gain in CTC WGA samples is not always found in matched tissues and may be due to previous archival tissues failing to represent tumor evolution; nevertheless, some copy number alterations, including gains and losses of chromosome 8p and 8q, are concordant between CTCs and primary tumors [22].

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 loci, and in chromosome 5p, the *TERT* gene (telomerase reverse transcriptase) loci, 17q22, 17q25.3 and 20p13. The CNV patterns of individual CTCs from the same patient were reproducible. It was also found that CNV patterns were not changed upon different drug treatments [29].

Floating tumor cells (FTCs) from the pleural fluid of lung adenocarcinoma patients were enriched and amplified. *EGFR* exon 19 deletion (del L747_A750), an *EGFR* activating mutation that makes patients eligible for EGFR inhibitor therapy, was detected in 63.2% of FTCs in one patient. In a second patient, the *EML4-ALK* (echinoderm microtubule associated protein-like 4–anaplastic lymphoma kinase) fusion variant, which is a novel target in a subset of non-small cell lung cancer cases, was detected in 85% of isolated FTCs. The *ALK G1202R* mutation, a known Alectinib-resistance mutation, was the only mutation identified throughout multiple FTC samples from another patient [28].

4. Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer and second most common death-causing cancer in Australia. It is a lethal cancer with a high mortality rate due to distant metastasis. A number of driver genes are commonly identified in CRC, including mutated *BRAF*, *KRAS*, *EGFR* and *PIK3Ca* [9][30][40]. EGFR is the 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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