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Review

Circulating Tumor Cells in Diagnosis and Treatment of Lung Cancer

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Abstract. *Circulating tumor cells (CTCs), detached from the primary tumor or metastases and shed in the patient's bloodstream, represent a relatively easily obtainable sample of the cancer tissue that can indicate the actual state of cancer, and their evaluation can be repeated many times during the course of treatment. As part of liquid biopsy, evaluation of CTCs provides a lot of clinically relevant information, which reflects the actual, real-time conditions of the disease. CTCs can be used in cancer diagnosis or screening, real-time long-term disease monitoring and even therapy guidance. Their analysis can include their number, morphology, and biological features by using immunocytochemistry and all "-omic" technologies. This review describes methods of CTC isolation and potential clinical utilization in lung cancer.*

Despite major advances in the diagnostics and treatment, lung cancer remains the most lethal cancer disease on a global scale. According to a WHO estimate, there were 2.09 million new lung cancer cases and 1.76 million lung cancer-related deaths in 2018 (1). Therefore, similarly to other cancer diagnoses, a significant effort is dedicated on the potential use of circulating tumor cells (CTCs) in prognosis assessment, disease monitoring and even in therapy management.

CTCs, detached from the primary tumor or metastases and shed in patient's bloodstream, represent a relatively easily obtainable sample of cancer tissue. As a liquid biopsy, evaluation of CTCs provides a lot of clinically relevant information, which reflects the actual, real-time conditions of the disease (2).

CTCs are very rare in the bloodstream; therefore, a number of different enrichment and isolation methods have been developed (2). Also, the evaluation of CTCs can be performed on many levels providing different kinds of information. The CTCs enrichment methods as well as their potential clinical use in lung cancer is introduced below.

CTC Isolation and Detection Methods – Basic Principles

Exploration of CTCs can provide a lot of clinically-relevant information; however, CTCs are very rare in the bloodstream. Every single CTC is surrounded by 10^6-10^7 mononuclear white blood cells (2, 3). Therefore, in order to be able to study and assess CTC- number and characteristics, it is necessary to separate them from the ambient blood cells.

Although many methods have been developed to isolate CTCs, there are only two basic approaches to isolate CTCs: a) isolation methods based on detection of specific surface markers of the CTCs (and/or white blood cells), also called "label-dependent systems/methods" and b) methods independent of specific markers, based on physical or biological properties of the CTCs, "label-independent systems/methods" (2).

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Label-dependent methods. The term "label-dependent methods" comprises all CTC isolation methods, which identify CTCs according to the presence or absence of specific markers. The majority of label-dependent methods use primarily the epithelial cell adhesion molecule (Ep-CAM), a specific epithelial tissue marker (2). Other markers additionally used are human epidermal growth factor receptor 2 (HER2) (4), mucin 1 (MUC1), cytokeratins *etc*. (5, 6).

There is a major disadvantage common to label-dependent methods; CTCs must express the specific markers tracked by the particular isolation method. However, CTCs do not necessarily express these markers, therefore they may be missed. It has been shown that the CTC population has a continuum of phenotypes, from strictly epithelial CTCs, hybrid CTCs to mesenchymal CTCs (7), due to their ability to undergo epithelial-to-mesenchymal transition (2). Each phenotype is characterized by the expression of different markers. Epithelial CTCs express EpCAM and cytokeratins, mesenchymal CTCs express vimentin or N-Cadherin, while hybrid CTCs express both epithelial and mesenchymal markers (7). Mesenchymal CTCs would probably be missed by most label-dependent methods. Some studies evaluating efficiency of label-dependent methods have shown that only a small fraction of CTCs is detected when compared to label-independent systems (8, 9). To overcome the CTC-specific marker disadvantage, different label-dependent methods include negative depletion of CD45-positive leucocytes (10).

CELLSEARCH® circulating tumor cell test. Despite the disadvantages of label-dependent methods, the CellSearch® (Menarini Silicon Biosystems) method is still being accepted as the "gold standard" among the CTC-isolation and detection methods. It is the only CTC-isolation system that has been approved by the US Food and Drug Administration (FDA) for clinical use in patients with metastatic breast, colorectal and prostate cancer (11). So far, the lung cancer field is not covered by CellSearch®.

The CellSearch® method is based on the anti-EpCAM immunomagnetic enrichment of blood sample and downstream CTC detection and evaluation by immunofluorescence. Whole blood is supplemented with ferrofluid nanoparticles conjugated with anti-EpCAM antibodies in order to capture CTCs. After further processing, cells are stained with DAPI (unspecific nuclear stain) and fluorescence-labelled antibodies against cytokeratins CK8, CK18 and/or CK19 and against the CD45 marker. The sample is analysed by automated fluorescence microscopy and evaluated by trained pathologists. CTCs are defined as DAPI+, CK8/18/19+ and CD45- cells, and leucocytes are defined as $DAPI^+$ and $CD45^+$ cells (11, 12). To improve CellSearch® method performance, an additional fluorescence channel has been added to enable detection of another marker of interest (*e.g*. HER2) (13).

CTC count obtained with the CellSearch® method can be used as a prognostic factor. The number of CTCs per 7.5 ml of blood ≥5 in metastatic breast and prostate cancer and ≥3 in metastatic colorectal cancer are associated with worse disease-free and overall survival rates (14-16). CellSearch® system is often used as a reference method while developing new isolation methods.

Adna test. AdnaTest (Qiagen) is a method combining specific separation of CTCs and a relatively easy way of CTC transcriptome evaluation. CTCs are immuno-magnetically separated from other blood components using magnetic beads conjugated with anti-EpCAM and anti-MUC1 antibodies (5). Obtained cells are lysed and mRNA is isolated and further evaluated. AdnaTest kits are produced in several variants, enabling the user to perform reverse transcription PCR on the retrieved mRNA after isolation and determine the expression of chosen genes (*e.g*. AdnaTest LungCancer variant offers assessment of EpCAM, ALDH1 and additionally TWIST1, Akt-2 and PI3Kα expression) (5, 17).

MagSweeper (non-commercial). MagSweeper is another method using anti-EpCAM immunomagnetic separation. The blood sample is diluted with buffer solution and ferrofluid-labelled anti-EpCAM antibodies are added to the solution. Cells are collected with rotating magnetic rods, which are put into the sample. Washing steps are performed and the obtained cells are subsequently released from the rods using external magnets (11, 18). Enrichment rates of CTCs are reported to be very high (18). Enriched cells can be cultivated and/or further analysed.

Magnetic-activated Cell Sorting (MACS). MACS (Miltenyi Biotec) is another modification of anti-EpCAM immunomagnetic separation. CTCs are captured on immunomagnetic beads and the sample is passed through a column, which is placed in a strong magnetic field. Non-captured cells pass the column, while CTCs on magnetic beads stay inside the column. Magnetic field is then removed and CTCs are eluted. Captured cells can be cultivated and/or further analysed (19).

Microfluidic chip methods. Microfluidic chip technologies use anti-EpCAM separation under the condition of regulated flow inside the separation device. Antibodies are conjugated with magnetic beads and the cells captured on the beads stay inside the chip due to magnetic field (*Isoflux*) (2, 20). Alternatively, the antibodies are directly attached to the wall of the microfluidic device (*CTC-Chip, HB-Chip*) (21, 22). Captured cells are eluted and cultivated/analysed.

EPISPOT Assay (non-commercial). EPISPOT assay represents a $CD45^+$ cell depletion method. CTCs are

enriched by leucocyte depletion and cultured in wells where antibodies against MUC-1, CK19 and other proteins according to the cancer type have been attached. Selected proteins are then marked with adequate fluorochrome-labelled antibodies and visualised with fluorescence microscopy (5, 10).

CellCollector®. CellCollector® (GILUPI) is a modification of anti-EpCAM isolation method for use *in vivo*. CellCollector uses a wire with anti-EpCAM antibodies attached on its surface. This wire is inserted through a cannula directly into patient's bloodstream and is exposed to a high amount of patient's blood (units of litres compared to 7.5 ml used in other systems) (2).

Label-independent methods. Contrary to the label-dependent methods, which are based on specific surface markers to separate CTCs, label-independent methods do not rely on surface markers. These methods use specific physical or biological properties of CTCs to isolate them, and include size-based separation, density-based separation, systems using specific electrical properties of the CTCs (dielectrophoresis) and systems using the invasive capacity of CTCs (2, 3, 5, 23).

The primary advantage of label-independent methods is that they are marker-independent. Label-dependent methods isolate only CTCs that express the targeted markers, while label-independent methods should be able to isolate a wider continuum of potential CTCs. A very precise subsequent characterization is needed as many other cells (*e.g.* leucocytes) are isolated during the label-independent separation process.

Size-based separation. Size-based separation methods use special porous membranes, with a pore size of approximately ±8 μm. Erythrocytes pass the membrane through the pores; CTCs are captured in a mixture with leucocytes that should be discarded. Cells captured on the membrane can usually be cultivated, stained and evaluated using both bright-field and/or fluorescence microscopy. Both mRNA and DNA can be isolated from the CTCs and further examined (24, 25). Size-based separation systems are represented for example by the ISET® system (Rarecells), Screencell® system (Screencell) (5) or MetaCell® system (MetaCell Ltd.) (25), and CellSieve® (Creatv MicroTech Inc.). Microcavity Array system (MCA) also uses porous membrane, but the pores are regularly arranged, thus allowing single CTC tracking (8). Parsortix™ technology (Angle plc) is a representative of microfluidic technology using separation by size and cell deformability (26).

Density-based separation (density gradient centrifugation). Density gradient centrifugation (using Ficoll-Paque® solution) is a method routinely used to separate mononuclear cells from a blood sample. Upon centrifugation of whole blood and in the presence of suitable separation medium, layers containing particular cell types are formed. The same method can be applied for CTCs isolation. They have similar density as mononuclear cells, so they are collected in the same layer. This method is relatively inexpensive, but the contamination by white blood cells is very high (27, 28). OncoQuick® system (Greiner Bio One) uses improved density-based separation method with special porous barrier above the separation medium. The presence of this barrier increases the efficiency of the separation process (28, 29).

Separation using the invasive capacity of CTCs. VitaAssay™ (Vitatex) is a representative of the separation methods based on the invasive capacity of CTCs. This method is based on the property of CTCs that when placed on a collagenous cell adhesion matrix (CAM) they penetrate this matrix and ingest it. CAM is fluorescently labelled and thus cells ingesting CAM are visualised (30).

Methods of combined approaches. Some platforms combine both label-dependent and label-independent approaches to increase efficiency of CTC capture. As an example, CTC-iChip is a microfluidic device that combines size-based filtration and immunomagnetic separation. There are two modes of CTC-iChip: positive selection mode, which uses size-based separation and then captures the $EpCAM^+$ fraction of the cells; and negative selection mode, which uses size-based separation and then depletes $CD45^+$ cells (31).

Levels of CTC Evaluation in NSCLC

Information potential carried by CTCs can be studied at many levels: simple presence of CTCs, CTC count, morphology and immuno-cytochemistry, genomic, transcriptomic, and proteomic analysis.

Presence of CTCs. The most basic level of CTC evaluation is a qualitative statement of their presence. Devriese *et al*. have shown, using a positive immunomagnetic enrichment method, that CTCs were present in 21 out of 46 (46%) stage IV NSCLC patients (32). Epithelial circulating cells were also found in 3 out of 46 (7%) healthy controls. In another study, Hofman *et al*. have shown, using size-based filtration method, that the presence of CTCs is independent of the stage of the tumor (CTCs found in 49% stage I, 48% stage II, 48% stage III and 52% stage IV patients) or tumor histology (*e.g.* 47% samples of patients with adenocarcinoma or 40% samples of patients with squamous cell carcinoma were CTC-positive) (33). Therefore, the presence of CTCs in the patient's bloodstream provides an uncomplete information that is very limited and not sufficient for clinical utilization.

CTC count in standardised blood sample. To obtain clinically-relevant information using CTCs, not only qualitative but quantitative assessments of CTCs in blood samples should be performed. Several studies have shown that in breast, prostate and colorectal cancer (14-16), the CTC count at baseline could be used as a prognostic factor. Krebs *et al*. have divided patients into favourable (<5 CTCs at a baseline/7.5 ml blood) and unfavourable (≥5 CTCs at a baseline/7.5 ml blood) groups. CTCs were isolated using the CellSearch® system. Patients in the favourable group had better progression-free survival (PFS) and overall survival (OS) rates than patients in the unfavourable group (PFS median 6.8 *vs*. 2.8 months and OS median 8.1 *vs*. 4.3 months) (34). A similar study performed by Zhang *et al*., has shown that CTC count ≥8 before chemotherapy was a strong and independent predictor of worse PFS (35). However, another study by Coco *et al*. showed a non-significant or even inverse relationship between CTC counts and PFS and OS. (36) A comparison of these and other similar studies using CellSearch® system pointed that they differ significantly in the percentage of CTC-positive patients (22% to 76%) at baseline (37-39). The reproducibility is low probably due to the CellSearch® system disadvantage as a label-dependent isolation method.

An analogous study used ISET® size-based separation system. Cut-off for the sorting of the patients into favourable and unfavourable groups was 50 circulating cells of nonhaematologic origin. Patients in the favourable group had again better PFS and OS rates (40).

CTC count at baseline can be used as a predictor of PFS and OS, but, as a single number, the obtained information is limited. Monitoring CTC counts in patient blood samples during therapy can offer novel information. With baseline CTC count as a reference, diminution of CTC counts after surgery and/or chemotherapy may indicate remission. Subsequent CTC counts can serve as an indicator of minimal residual disease; persistent low or zero CTC counts suggest establishment of remission. Conversely, increasing CTC numbers probably indicate a relapse of the disease, even prior to evident clinical symptoms. Therefore, increasing CTC counts during therapy could serve as an indication to change the chemotherapeutic drug (41). Utilization of CTCs in monitoring therapy is further discussed below. In lung cancer, Tong *et al*. have shown that the change of CTC counts during chemotherapy can act as an indicator of a chemotherapeutic response and as a strong PFS and OS predictor (42).

Morphology and biological features. Morphological evaluation is a necessary step to identify and discriminate CTCs from the surrounding cells while using label-independent CTC isolation methods. Basic morphological malignant features include: increased nucleus size and area; irregular nucleus shape; specific chromatin structure (chromatin granules); increased number and size and/or changed shape of nucleoli; increased cell size and area; increased N:C ratio; presence of mitotic figures, formation of 3D sheets of cells during culture and more. Although these parameters usually serve for CTC identification only, they are measurable and could potentially be used as additional markers.

Table I. *Example of a panel of genes for CTCs transcriptomic analysis (54).*

Tumor-associated genes					
1	ACTB	11	KRT19	21	CD57
2	CD45	12	KRT20	22	SYP
3	CD68	13	EGFR	23	HER2
4	EpCAM	14	CHGA	24	ER
5	MUC1	15	MLANA	25	PR
6	TTF1	16	S100B	26	MUC16
7	KRT5	17	VIM	27	SCGB
8	KRT6	18	VEGF	28	CD24
9	KRT7	19	WT1	29	CD44
10	KRT18	20	CD56	30	ALDH
Chemoresistance-associated genes					
1	MRP1	4	MRP5	6	MDR1
2	MRP2	5	MRP7	7	ERCC1
3	MRP4				

Del Ben *et al*. have shown that CTCs secrete lactate and acidify their microenvironment. They measured pH of aqueous droplets containing trapped CTCs, in oil emulsion, using pH sensitive fluorescent dye (43).

The metabolism of CTCs is supposed to be intensified. To visualise these hypermetabolic CTCs, methods based on glucose uptake can be used. Cells can be supplemented with fluorescent glucose analogue (*e.g*. 2-NBDG) for a short time and then be evaluated using fluorescence microscopy. Turetta *et al*. have shown that glucose uptake values of CTCs are a median of 10 times higher than those of white blood cells (44).

Immunocytochemistry. Irrespective of using immunocytochemistry in label-dependent isolation methods, some specific surface or intracellular molecules of CTCs are used as targets for immunocytochemistry or immunostaining in lung cancer. Ilie *et al*. evaluated the expression of MET protein using immunocytochemistry on CTCs isolated by the ISET® system from NSCLC patients' blood samples. They showed that expression of MET in CTCs correlates with expression of MET in primary tumor (45).

Another study by Guibert *et al*. has focused on PD-L1 expression in CTCs from patients with NSCLC. They used immunofluorescent staining to visualize CTCs expressing

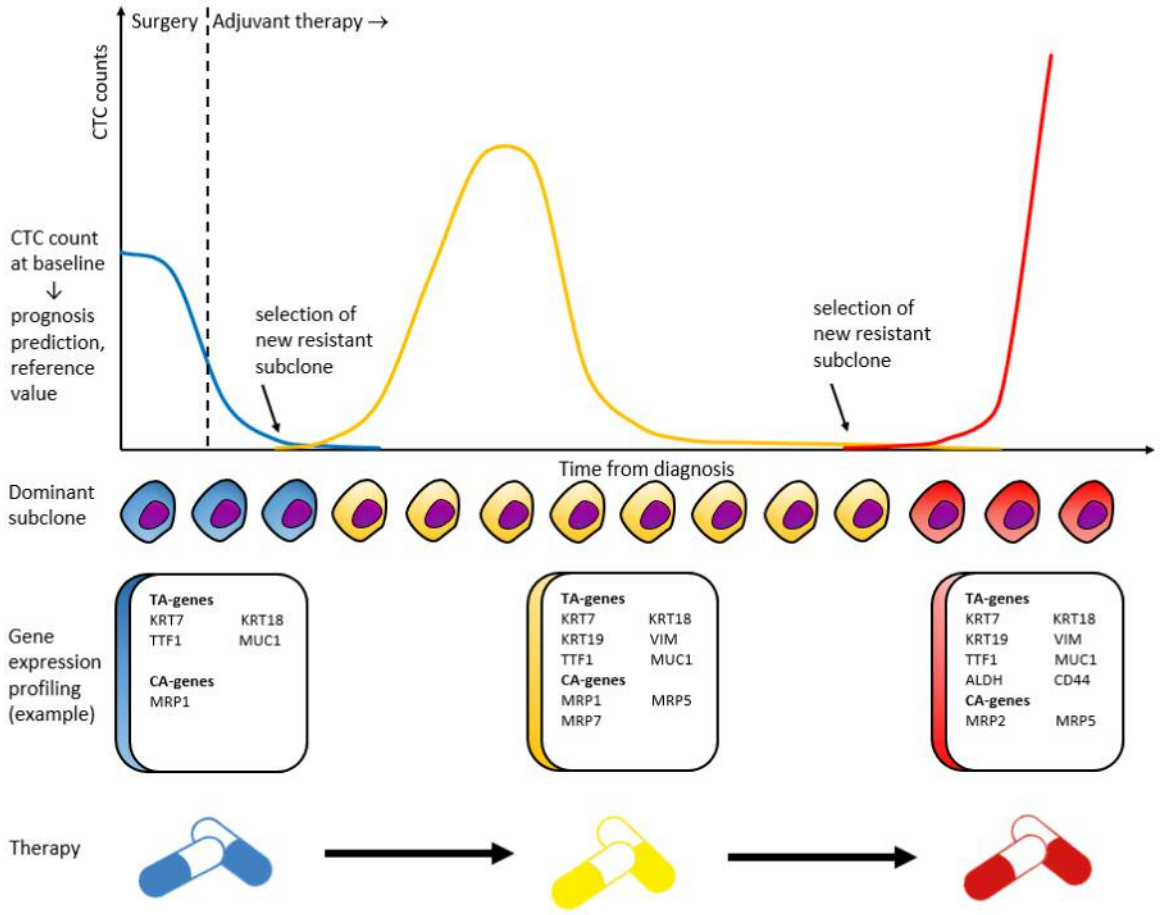

Figure 1. *CTC count monitoring including gene expression profiling of tumor associated (TA) and chemotherapy associated (CA) genes to guide therapy in time (41, 62).*

PD-L1 and to determine the percentage of PD-L1 positive CTCs. They also compared PD-L1 expression in CTCs and in the primary tumor and reported that CTCs were more often PD-L1 positive than the tissue. According to this finding, they suggested that PD-L1 expression in CTCs could be used as an indication for treatment using PD-1/PD-L1 immune checkpoint inhibitors (46).

Genomic analysis. DNA analysis and mutation assessment of the CTCs can provide a lot of clinically-relevant information. Although CTCs represent only a subpopulation of all tumor cells, it has been shown that they can reflect quite well the primary tumor heterogeneity. Turetta *et al*. have compared *EGFR* and *KRAS* mutations in primary tumors and in CTCs and reported that the same mutations were present in 16 out of 23 cases (70%) (44). Marchetti *et al*. have detected the same *EGFR* mutation in CTCs and in primary tumors in 29 out of 31 cases (94%) (47).

The genes often tested in NSCLC include: *EGFR, ALK, RET, ROS-1, BRAF, ERBB2, PIK3CA, KRAS, AKT1* and many others. DNA methylation analysis has also been performed (48-50). However, conventional methods, including cytogenetical and molecular-cytogenetical techniques can be also applied in CTCs. The standard technology used for gene and chromosomal rearrangements is fluorescent *in situ* hybridization (FISH) (*e.g*. in *ALK* rearrangements) (51). Nowadays, single-cell genotyping has become a very popular method, allowing determination of cancer "phylogenesis" and evolution. Demeulemeester *et al*. have constructed cancer "phylogenetic" trees using single-cell sequencing (52).

Clinical utility of genomic analyses has been demonstrated using the *EGFR* T790M variant, which is related to an acquired resistance to gefitinib or erlotinib. The discovery of this mutation even in a small portion of cells should lead to reassessment of eventually incompatible treatment (53).

Transcriptomic analysis. Another information-containing molecule that could be evaluated in CTCs is RNA, primarily mRNA. The amounts of specific mRNA transcripts inside

Table II. *Overview of chosen studies regarding CTCs in lung cancer.*

	Research group	Isolation platform	Number of samples	Main results	Reference
2010	Hofman *et al.*	ISET®	208 P + 39 HC	Circulating non-haematologic cells were found in 102 out of 208 patients (49%), in 76 patients (37%) were these cells evaluated as "cells showing malignant features" by at least 5 of 10 cytopathologists. No non-haematological circulating elements were found in healthy controls.	(40)
2011	Hofman *et al.*	ISET®	250 P + 59 HC	Circulating non-haematologic cells were found in 123 out of 250 preoperative patients (49%), in 102 patients (41%) were these cells evaluated as CTCs by at least 5 of 10 cytopathologists. No non-haematological circulating elements were found in healthy controls.	(33)
	Krebs *et al.*	CellSearch™	101 P (14 stg. IIIa, 27 stg. IIIb, 60 stg. IV)	No CTCs were detected in patients with stage IIIa. In 2 out of 27 patients (7%) with stage IIIb were found ≥2 CTCs per 7.5 ml blood (maximal count: 3 CTCs). In stage IV patients, ≥2 CTCs per 7.5 ml blood were found in 19 out of 60 patients (32%), 9 patients had ≥5 CTCs per 7.5 ml blood. CTCs count ≥5 CTCs per 7.5 ml blood was strong predictor of reduced FPS and OS.	(34)
2012	Devriese *et al.*	MACS®+ qF PCR	46 P + 46 HC	Sample was labelled as CTC-positive when sample was found to have positive expression of at least one of these genes: CK7, CK19, EGP, FN1. CTC-positive were 21 (46%) samples from patients with NSCLC and 3 samples (7%) from healthy controls.	(32)
	Punnoose *et al.*	CellSearch™	37 P	CTCs were detected in 28 of 37 samples (76%). Decrease in CTCs counts during therapy was correlated with radiographic response and also associated with longer PFS.	(39)
2013	Pirozzi *et al.*	Ficoll-Hypaque + ICC (pan-CK)	45 P	11 blood samples (24.4%) obtained from tumor-draining pulmonary vein during operation were CTC-positive	(27)
2014	Ilie *et al.*	ISET®	168 COPD P + 77 HC	CTCs were found in 5 of 168 patients (3%). All of these five patients developed lung cancer within 4 years. No CTCs were found in other COPD patients and in healthy controls. None of them developed lung cancer during follow-up.	(69)
	Faugeroux *et al.*	FA-FISH	32 P	CTCs were detected in all blood samples (100%) of patients with metastatic lung cancer. Presence of ALK-rearranged cells in primary tumor matched the presence of ALK-rearranged CTCs.	(51)
	Marchetti *et al.*	CellSearch™	37 P + 12 HC	CTCs were detected in 15 patients (41%). EGFR mutations were described in CTCs in 13 of 15 CTC-positive patients (87%) and in 16 of 22 (73%) samples containing only elements marked by CellSearch as potential neoplastic elements (cellular "suspicious objects" not fulfilling all the CellSearch™ criteria and large naked nuclei)	(47)
2015	Zhang *et al.*	Cyttel (negative immuno-magnetic selection)	46 P	CTCs were detected in 40 of 46 patients (87%), CTCs count ≥8 CTCs per 3.2 ml blood was associated with reduced PFS and OS	(35)
2016	Crosbie *et al.*	CellSearch™	30 P	CTCs were more frequently detected in tumor-draining pulmonary vein (13 of 30 samples, 43%) than in peripheral vein (6 of 27 patients, 22%). Presence of ≥18 CTCs and ≥1 CTM in 7.5 ml blood from tumor-draining vein was associated with significantly shorter FPS and OS.	(38)
2017	Coco *et al.*	ScreenCell	73 P	No significant influence of CTC count at baseline in determining PFS and OS was observed.	(36)
	Tong *et al.*	Cyttel	127 P	In 107 of 127 samples (84%) were detected ≥2 CTCs per 3.2 ml blood. CTC count at baseline ≥8 CTCs per 3.2 ml blood was related to shorter PFS and OS. Rise in CTC count during follow-up was also associated with poorer prognosis.	(42)
	Ilie *et al.*	CellSearch™, ISET®	256 P (CellSearch™), 106 P (ISET®)	In ISET®-processed samples, ≥1 CTCs were found in 80 of 106 samples (75.5%) and ≥5 CTCs were detected in 79 samples (74.5%). Using CellSearch™ system, ≥1 CTCs were found in 83 of 256 samples (32%) and ≥5 CTCs were detected in 30 samples (12%). Level of MET expression correlated between tumor tissue and CTCs isolated with ISET®.	(45)
2018	Turetta *et al.*	Flow cytometry (2-NBDG)	30 P	A new CTC isolation method based on sorting CTCs and blood cells according to their metabolic activity (uptake of fluorescent glucose analogue) was introduced and verified by mutational analysis of EGFR and KRAS genes of enriched CTCs fraction.	(44)

Table II. *Continued*

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Research group	Isolation platform	Number of samples	Main results	Reference
Guibert *et al*.	ISET®	96 P	CTCs were detected in 89 of 96 patients (93%). PD-L1-positive CTCs were found in 83% patients, CTCs were more often PD-L1-positive than tumor tissue (PD-L1-positive cells were present in 41% tissue samples). High baseline CTC counts were associated with poorer prognosis.	(46)

P: Patient; HC: healthy control; CK7: cytokeratin 7; CK19: cytokeratin 19; EGP: human epithelial glycoprotein; FN1: fibronectin-1; ICC: immunocytochemistry; pan-CK: pan-cytokeratin, COPD: chronic obstructive pulmonary disease; FA-FISH: filter-adapted fluorescent in situ hybridisation; ALK: anaplastic lymphoma kinase; EGFR: epithelial growth factor receptor; CTM: circulating tumor microembolus; MET: hepatocyte growth factor receptor, 2-NBDG: fluorescent glucose analogue; KRAS: Kirsten rat sarcoma viral oncogene homolog; PD-L1: programmed death-ligand 1.

the cell are in concordance with expression levels of corresponding genes. Therefore, reverse transcription followed by quantitative PCR (qPCR) are used to measure gene expression (54). An example of panel of genes of interest is presented in Table I.

In lung cancer CTCs, KRT7 and TTF-1 levels have been found to be elevated (55). TTF-1 is considered as highly specific marker for lung adenocarcinomas (56). The utilization of gene expression profiling is well-established in breast cancer; expression levels of ER, PR and HER2 in the primary tumor, but also possibly in CTCs, are used to choose appropriate hormone treatment (57).

When elevated, chemoresistance genes provide CTC immunity against specific drugs (*e.g.* MRP5 is linked with resistance to 5-fluorouracil, platinum derivatives and methotrexate) (41). The finding of elevated expression of some chemoresistance-associated genes should lead to reassessment of eventually incompatible treatment.

RNA sequencing is another method used for assessing precisely the whole transcriptome, especially when assessed at a single-cell level (58, 59).

Proteomic analysis. Protein profiling of CTCs is not a popular analysis of CTCs. However, methods for proteomic analysis of single cells using mass spectrometry or western blotting have been developed (60, 61).

Potential Clinical CTC Utilization

Current clinical applications of CTC – testing can be revised in Table II, where important results of CTC- clinical studies in lung cancer are summarized.

Utilization of CTC in monitoring disease development. When compared to a conventional biopsy, CTC evaluation is relatively inexpensive and non-invasive and therefore repeatable many times during therapy. All these facts make CTC analysis a powerful instrument in monitoring cancer development.

CTC count and count changes during therapy are basic but valid indicators of cancer progression. As described above, CTC count has been proven to be a prognosis predictor in many cancer types including lung cancer (34-40). Monitoring CTC counts during therapy is an instrument that allows assessment of disease development in real time, even prior to overt clinical signs of relapse. A decrease in CTC count after surgery and/or chemotherapy is probably a sign of remission. In contrast, an increase in CTC count indicates reactivation of disease, which should lead to revalidation of therapy (41, 62).

Molecular analysis (*e.g.* gene expression profiling) during therapy can complement assessment of CTC count and provide additional valuable information. Molecular analysis can visualise formation of new resistant CTC subclones during therapy (Figure 1) and identify them in terms of mutation analysis, tumor-associated gene expression, chemoresistance-associated genes etc. (41). Therefore, it provides the ability to revalidate actual treatment and eventually replace it with a compatible one.

Cancer screening and exploring cancer origin by CTC-analysis. CTCs can be detected in blood even in early stages of tumor development (33), therefore they are candidates for cancer screening. Cells classified as CTCs are very rare in blood of healthy people or patients with benign tumours (63). However, there are some conditions under which circulating non-haematological cells can be found in circulation, *e.g.* Crohn disease (64), endometriosis (65) or pregnancy (trophoblasts are found in maternal blood) (66). It is necessary to take this into account when evaluating blood samples for CTCs.

There are only few studies regarding CTC-based cancer screening. Castro *et al*. have evaluated blood samples of 3388 individuals aged 45-80 years with no cancer history.

They found circulating non-haematological cells in the blood of 107 (3.2%) individuals (67).

In lung cancer, there were attempts to combine CTC assessment with established screening methods. He *et al*. had focused on improving specificity of low-dose CT screening program by combining it with subsequent CTC evaluation. Patients with identified pulmonary "ground-glass" nodules and healthy controls were evaluated with regard to CTC counts. Only some patients with nodules had CTCs present in their blood. According to subsequent molecular analysis, these CTCs were found to have "malignant tendency" (68). Another study performed by Ilie *et al*. had focused on CTC assessment in patients with chronic obstructive pulmonary disease (COPD). They detected CTCs in 5 out of 168 (3%) patients. Interestingly, all of these five patients developed lung cancer within 4 years. Furthermore, none of the CTC-negative patients developed cancer within the monitored period. Some studies have confirmed these findings (62, 69, 70).

CTC screening complemented with immunohistochemistry or gene expression profiling of detected cells could aid in tracing the primary tumor origin. Lu *et al*. have found that KRT7 and TTF-1 positive cells corresponded to lung cancer. Accordingly, KRT20 and CDX2 positive cells coincided with colorectal cancer, and PSA and PSMA positive cells with prostate cancer (55).

Conclusion

CTCs are a very intensively studied field of cancer biology. Although biological knowledge underlying CTCs is still limited, it has been demonstrated that they can be extensively utilized in lung cancer management. The role of CTCs is already well established in cancer prognosis, offering another factor to complement conventional TNM staging system. Recent findings suggest that CTCs in (not only) lung cancer can offer more information than plain disease prognosis. CTCs can be used in cancer diagnosis or screening, real-time long-term disease monitoring and even in therapy guidance. They represent relatively easily obtainable sample of the tumor, describing its actual state, and their evaluation can be repeated many times during the treatment. Their count, morphology and biological features including expression of certain markers, proteins, genes and RNAs provide important clinically useful information.

As demonstrated above, evaluation of CTCs provides a lot of clinically relevant and valuable information in lung cancer as well as in other cancer diseases. The future of CTCs lies in complex disease monitoring and accurate therapy guidance. Implementation of CTC testing into everyday practice could help to improve cancer treatment efficacy and to get a little step closer to cancer cure.

Conflicts of Interest

The Authors have no conflict of interest regarding this study.

Authors' Contributions

VM, OM prepared the manuscript, KK revised and complemented the methodological part of data and VB revised and complemented the clinical part of the presented data.

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