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Review

Animal Models for the Calculation of Circulating Tumor Cells for Experimental Demonstration

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Abstract. *Metastasis is a process which is characterized by the existence of tumor cells in the bloodstream. This is a necessary situation in order for the malignant cells to be transported to other organs. Thus, the importance of circulating tumor cells (CTCs) in the study of carcinogenesis is widely accepted. These tumor cells are nowadays a topic of intensive research all over the world. CTCs are expressed from tumor cells and the clinical analysis of this expression may help the recognition of a tumor in an earlier stage and also there is an effort to monitor the tumor burden according to these cells. Although a plethora of clinical studies has been conducted, it is still unclear whether the use in clinical aspect will prove to be beneficial in the near future. Few animal models with neoplasia have been studied concerning the circulating tumor cells and it is likely that CTCs may have a predictive, diagnostic or therapeutic value. Herein, the authors review all studies in which human CTCs were transplanted into animals. Therefore, more clinical studies*

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using standardized methods for measuring CTCs are required to elucidate these issues.

The metastatic dispersion of malignant cells is a characteristic of neoplasms. During metastasis, a portion of malignant cells is separated from the primary tumor, penetrates the surrounding tissues and enters in the bloodstream. Circulating tumor cells (CTCs) abandon the primary site, enter in vascular circulation, avoid the immune detection, exit from the micro-vessels to a specific target tissue in which the metastatic environment is developed (1). CTCs probably exist in two ways inside the blood flow, either as clusters binding to macrophages and platelets or as single cells (Figure 1) (2, 3). Their total number and presence during treatment is an independent factor of worse prognosis (4). This is the reason why they are increasingly studied for their potential use in disease recognition and monitoring although their research is considered challenging, due to their low numbers, plasticity, heterogeneity, short lifetime and fragility (5).

Herein, we discuss the results acquired from the clinical analyses of CTCs, exclusively from animal models, concerning many types of malignancies such as colorectal cancer, melanoma, Merkel cell carcinoma, osteosarcoma, hepatocellular, pancreatic, ovarian and breast carcinoma.

Materials and Methods

A review of the literature was performed using the PubMed database and Cochrane library to find original research articles assessing the calculation of circulating tumor cells and the metastatic potential of different cell lines in animal models. Specifically, articles published between 1984 and 2019 were identified using keywords such as animal models, mice, metastasis, circulating tumor cells and neoplasia. Moreover, the references of all selected articles were reviewed to identify any additional, potentially eligible studies. The initial search identified 291 articles. The results were cross-checked to exclude overlapping series or duplicates. After removal of duplicates, 242 remained. These were screened and 222 were excluded because they were only abstracts; or were irrelevant to the topic. Only publications in the English language were reviewed. The full-text articles assessed for eligibility were 20. None of them was excluded. The inclusion process is shown in PRISMA flow diagram (Figure 2).

Results

Many animal models were used from researchers in order to examine the oncogenic potential of the CTCs. In 1988, Morikawa *et al.*, obtained metastatic cells from patients with colon carcinomas (CCs) which were directly adapted to growth in cell culture (6). The isolated cells were also injected into several organs of different nude mice. A few weeks later, some of the mice developed 1-5 liver tumor colonies in different organs such as spleen or the cecum. Their primitive results showed that the nude mouse can be used to isolate and expand metastatic cells from CCs.

Five years later, Furukawa *et al.*, examined transplanted human pancreatic cancer (PC) cells to the pancreas of nude mice (7). This transplantation led to tumor formation and subsequent liver metastasis. The metastatic model of PC cells using transplantation of histologically intact tumor tissue provides a model of metastatic pancreatic cancer both at the level of local invasion and metastasis in a nude mouse model.

Another study from Kiguchi *et al.*, reported that the orthotopically implanted tumors grew effectively in the ovaries of all the mice implanted, establishing a 100% (22/22) take rate (8). It was remarkable that when tumor tissues of human ovarian cancer were implanted orthotopically into the ovarian capsule of nude mice, tumor growth was observed in the ovary, as well as invasion and metastasis to the lung, to the peritoneum, and other organs. This biological behavior was similar to that of human ovarian cancer, indicating that this animal model of the disease was an appropriate one for therapeutic investigations. Invasion and metastasis observed after orthotopic transplantation appeared not random and not due to leakage, since the pattern was so different than after subcutaneous transplantation. The ovarian tissues are a superior host for this ovarian cancer than in the subcutaneous space as evidenced by a 22 out of 22 take rate in the ovary as opposed to a 2 of 5 take rate in the subcutaneous site. The subcutaneous dissemination or metastasis was limited to regional lymph nodes and on the kidney as opposed to extensive invasion dissemination, and distant metastasis after orthotopic transplantation.

In 2006, Schülter *et al.*, reported a study, in which the unprecedented correlation between the formation of colorectal metastases in specific organs and the relations between circulating tumor cells and the endothelium of the vessels of specific target organs is described (9). According to their findings CTCs were observed within the pulmonary microcirculation and cell adhesions were found regardless of size. All cell lines presented increased adhesion rates, no matter their metastatic ability, mainly in the liver and the lungs and scarcely in other organs.

In 2008, Havens *et al.*, demonstrated that tumors started by LNCaPC4-2B and PC-3 cells generate metastatic cells to several intra-abdominal organs and bone tissue (10). One unexpected part of their findings was the fact that different levels of tumor diffusion were observed in a variety of tissues, even though essentially equal levels of LNCaP C4-2B and PC-3 cells were detected in the bloodstream. The different levels of tumor cells in the tissues can be explained by the combinations of local growth factors and integral autonomous proliferation differences. As such, this model could identify mechanisms that control inactivity and detect prostate cancer stem cells.

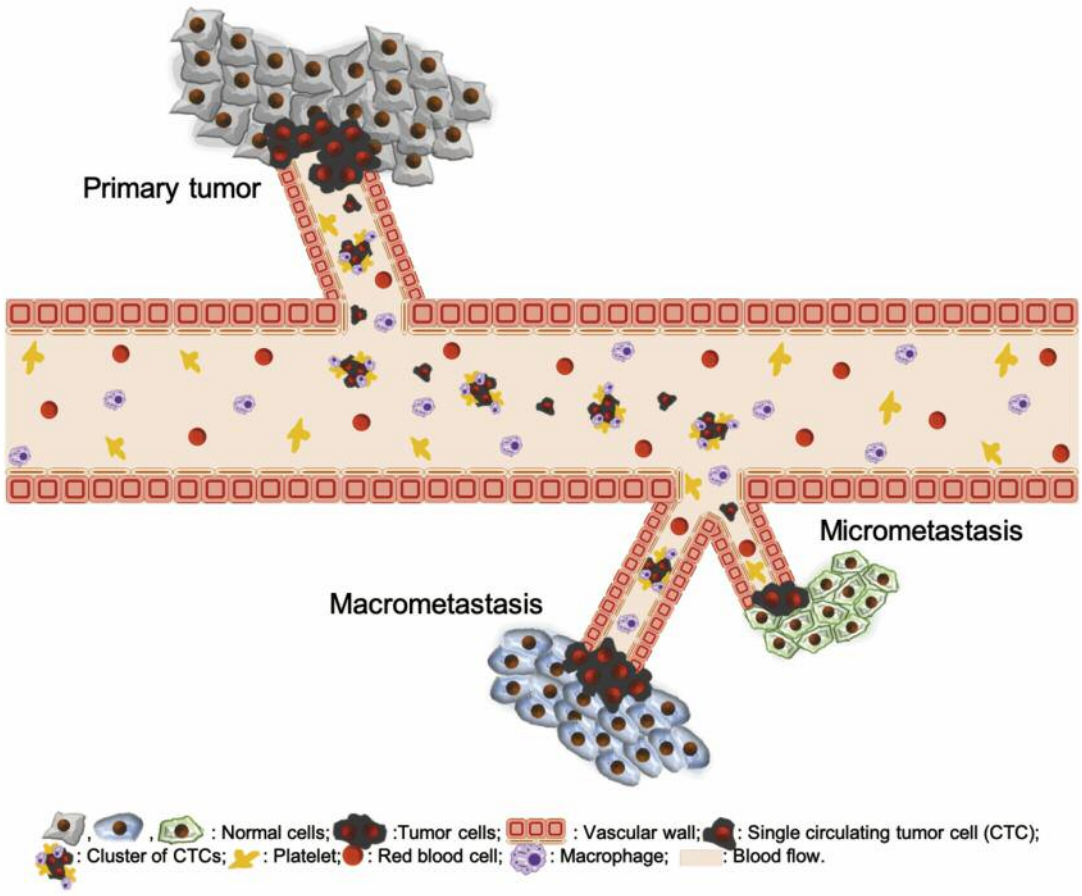

Figure 1. *Circulating tumor cells (CTCs) abandon the primary site, enter the bloodstream, avoid the immune detection, exit from the micro-vessels to a specific target tissue in which the metastatic environment is developed. CTCs probably exist in two ways inside the blood flow, either as clusters binding to macrophages and platelets or as single cells.*

The same year, Munoz *et al*., developed an aggressive model of advanced metastatic melanoma (11). They created a highly invasive human melanoma cell line using the human cell line WM239 and they named it 113/6-4L. This specific cell line was selected from spontaneous lung metastatic cells arising from the WM239 line, which were then adjusted to tissue culture. The initial line WM239 required 4 to 6 months for the formation of visible metastatic nodules in lungs, but surprisingly the 113/6-4L cell line only required 6 weeks for the formation of metastatic tissue in the lungs. This model used a single clone of a murine melanoma cell line (B16), which metastasized only to meninges and, thus, did not recapitulate clinical disease presented with metastases in the brain parenchyma. Last but not least, it allowed the examination of alterations which occur in the transition from poorly metastatic (WM239A cell line) to highly visceral metastatic variant (113/6-4L) and then to brain-metastatic phenotypes.

Some years later, Francia *et al*., described the *in vivo* development of metastatic melanoma models (12). They examined the origin of variants of the human MeWo melanoma cell line. This cell line was proved capable of spontaneous metastatic spread. Furthermore, they also used highly metastatic variants, such as the 113/6-4l subline originated from the WM-239 human melanoma. They engineered these highly metastatic variants through two rounds of *in vivo* selection involving orthotopic primary tumor cell embedding, subsequent tumor resection and separation of metastatic cells from visceral metastasis which appeared several months later.

The same year, Li *et al*., investigated the correlation between the metastatic potential and depletion kinetics of CTCs *via* "*in vivo* flow cytometer" (13). In particular, PC-3 prostate cancer cells are depleted in a quicker rate from the circulation compared to LNCaP cells. Moreover, it is worth mentioning that the low-metastatic HepG2 cells present

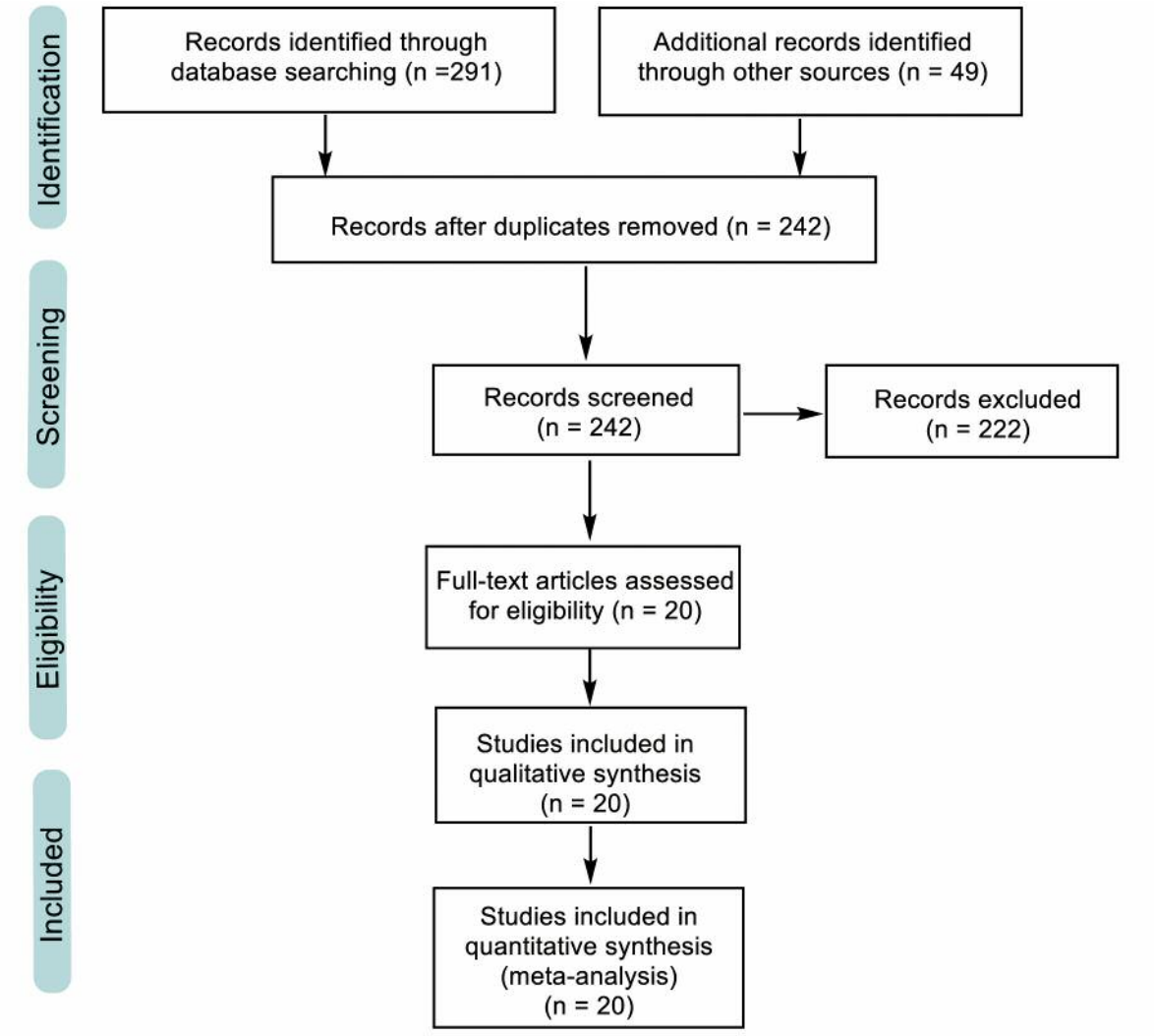

Figure 2. *PRISMA flow diagram for the current study.*

significantly slower depletion kinetics. The differences in depletion kinetics that were observed can be considered as possible biomarkers in circulation for discovering malignancy and predicting the patients' response to treatments.

One year later, Gil-Bernabe *et al*., developed a premetastatic model (14). After an intravenous injection of tumor cells in animals bearing a primary tumor in comparison to malignant-free animals, this model provoked an increase in the number of pulmonary metastases. The existence of a primary tumor caused an increase in the population of CD11b cells and in MMP-9 in the lung. Then, the team injected intravenously B16F10 cells into mice bearing B16F10 subcutaneous tumors. As the premetastatic model had predicted, the presence of a primary tumor led to an increase in survival and in the number of pulmonary metastases. Finally, they investigated and proved that coagulation contributes to formation of the premetastatic status.

The same year, Kang *et al*., conducted a study, in which the isolation, detection and cultivation of circulating tumor cells from blood samples of cancer-bearing mice was investigated (15). For that purpose, a microfluidic-micromagnetic device that separates cells was used. Significantly, according to their findings, a rise in the number of circulating tumor cells in mice blood was observed, that was depended on time and was linked to a notable increase of the metastatic tumor cells. These findings are useful and can be used in drug screening, in order to choose the suitable treatment for patients on an individualized basis.

Another study of Kang *et al*., proposed as alternatives to human cancer xenografts the genetically engineered mouse

models (GEMMs) (16). These mouse models were used to test the efficacy of experimental drugs such as the inhibitors of angiogenesis and inhibitors of matrix metalloproteinases. The metastatic process in these models could be monitored if the cells were tagged with molecular markers. These animal models as it is well referred mimic in many aspects, the complex situation of neoplasia in patients.

The same year, Nanduri *et al*., created an orthotopic and highly metastatic mouse model of colorectal cancer (CRC) (17). They introduced human CRC cell lines orthotopically into the caecum of immunodeficient mice to generate local tumors, which generated distant metastases and CTCs with high reproducibility. In their mouse model, CTCs did not overexpress CD47or down-regulate calreticulin. As they state in their study, the resulting tumors closely resembled their human counterparts and grow in immunocompetent hosts. Therefore, this mouse model mimicked the corresponding human malignancy.

In 2015, Giuliano *et al*., isolated circulating tumor cells and introduced tumor cells in bone marrow (BM-DTCs) from cancer-bearing mice and were investigated for human pan-cytokeratin and nuclear counterstaining of red blood cell-lysed blood, respectively (18). According to the results, lung metastases were significantly related to the detection of circulating tumor cells. No correlation was detected with disseminated tumor cells in bone marrow. At the basis of these data, CTCs and BM-DTCs observed in cancer-bearing mice can be considered as a valuable model in tumor metastases investigation.

In 2016, Schölch *et al*., demonstrated an orthotopic mouse model of CRC which correlated metastases and CTCs (19). They were able to collect and culture the resulting CTCs *in vitro*, and proved their tumor-forming ability when re-injected into mice. Genes associated with cell–cell adhesion (claudin-7, CD166) were significantly down-regulated, indicating a more metastatic behavior of CTCs compared to bulk tumor cells originated from hepatic metastases. The stem cell markers DLG7 and BMI1 were significantly up-regulated in CTC, indicating a stem cell-like phenotype and increased the capacity of tumor formation and self-renewal. In concert with their *in vitro* and *in vivo* tumorigenicity, these findings indicated stem cell properties of mouse-derived CTCs. As a matter of fact, they developed an orthotopic mouse model of CRC recapitulating the process of CRC dissemination a predictable and reproducible manner. CTCs derived from this model exhibited stem cell like characteristics and were able to form colonies *in vitro* and tumors *in vivo*. Finally, they were able to isolate CTCs from these mice, demonstrated their *in vitro* and *in vivo* tumorigenicity and performed a qPCR expression study on the CTCs, proving the stem cell properties as long as the reduced cell to cell adhesion in CRC derived CTCs (19).

The same year, Gorczynski *et al*., breast cancer-bearing mice were employed in order the metastatic spread to lung and liver to be investigated, measuring the appearance of circulating tumor cells in peripheral blood of the subjects (20). Significantly, the role of cytokines was clarified. According to the findings of this study, cytokines enhance the metastatic potential, by operating on regions in the host or directly on tumor cells.

Powell *et al*., reported a method, in which paired isogenic patient-derived xenograft models of triple-negative breast cancer were developed and implanted into mouse glands (21). The aim of this study was to monitor the tumor growth and metastasis, utilizing bioluminescence imaging. Flow cytometry was also used to estimate the circulating tumor cells. More specifically, the role of p53 in metastatic potential was tested. According to the clinical results, p53 loss increased the late-stage tumor progression rate. Moreover, absence of B cell translocation gene 2 (BTG2) resulted in increasing of tumor growth and reduced survival. Therefore, BTG2 could be considered as a valuable tool predicting for triple-negative breast cancer (TNBC).

In 2017, Knips *et al*., examined the metastatic abilities of two different cell lines of human Merkel cell carcinoma, WaGa and MKL-1 respectively, through transplantation to immunodeficient mice (22). The transplantation led to the formation of metastasis and development of CTCs. In addition, worse prognosis was correlated to the WaGa cell line, the up-regulation of small T-antigen, Wnt signaling pathway and greater inflammatory response.

Next year, Johan *et al*., implanted osteosarcoma LM8 cells to immunocompetent mice (23). Furthermore, they cultured a vesicular stomatitis virus which demonstrated both an anti-metastatic potential against osteosarcoma cells and a detection tool against CTCs. Thus, this model highlights the need of further research concerning this viral-based therapeutic approach on human osteosarcoma.

The same year, Zhang *et al*., conducted a study, in which the metastatic potential of adherent and suspension BCCs were investigated *in vitro* and *in vivo* (24). For the purpose of the study, BCCs were developed in order to mimic the suspension state. According to the findings, he suspension state resulted in increase of the metastatic potential of BCCs. However, it gently suppressed tumor growth. Moreover, when the catalytic activity of COX-2 was inhibited, and the migration and invasion of BCCs was suppressed. As a result, this study demonstrates that suspension state affects the metastatic potential of CTCs. Additionally, COX-2 inhibitor can be considered as a factor leading to anti-metastasis.

In 2019, Vishnoi *et al*., transplanted CTCs from patients suffering from TNBC with liver metastasis to immunodeficient mice (25). Bone-marrow and liver tissue cells were obtained from the animals and the results showed that CTCs derived from animals and women had the same transcriptomic signature. Thus, the identification of a gene signature through

Table I. *Studies for circulating tumor cells in animal models.*

	Author	Year	Study characteristics
1	Morikawa *et al.* (6)	1988	• Metastatic cells from HCC patients. • Transplantation in nude mice. • Development of 1-5 liver tumors.
2	Furukawa *et al.* (7)	1993	• Human pancreatic cancer cells. • Transplantation in nude mice. • Tumor formation and liver metastasis.
3	Kiguchi *et al.* (8)	1998	• Orthotopically implanted tumors in mice. • Metastasis to the lung, to the peritoneum, and other organs.
4	Schlüter *et al.* (9)	2006	• Migration into target organs correlated with their metastatic potential. • Correlation between the metastatic potential of colon carcinoma cells and their ability for cell adhesion within potential target organs.
5	Havens *et al.* (10)	2008	• Primary tumors established by LNCaPC4-2B and PC-3 cells. • Different levels of tumor dissemination. • Different levels of tumor cells in the tissues.
6	Munoz *et al.* (11)	2008	• Model of advanced metastatic melanoma. • Human cell line WM239. • Required 6 weeks for the formation of macroscopic nodules in the lungs and pleural cavity.
7	Francia *et al.* (12)	2011	• Metastatic melanoma models. • Orthotopic primary tumor cell implantation. • Isolation of metastatic cells from visceral metastasis.
8	Li *et al.* (13)	2011	• *In vivo* flow cytometer. • Potential biomarkers in circulation for detecting cancer.
9	Gil-Bernabe *et al.* (14)	2012	• Intravenous introduction of tumor cells. • Increase in the population of CD11b cells and in MMP-9. • Increase in the number of metastatic lung nodules.
10	Kang *et al.* (15)	2012	• Microfluidic-micromagnetic cell separation device. • A rise in the number of CTCs in blood of female transgenic mice linked to a dramatic increase in the numbers of metastatic tumor cells.
11	Kang *et al.* (16)	2013	• Genetically engineered mouse models (GEMMs) as alternatives to human cancer xenografts.
12	Nanduri *et al.* (17)	2013	• Metastatic mouse model of CRC. • Injection of human CRC cell lines. • Local tumors with distant metastases.
13	Giuliano *et al.* (18)	2015	• CTCs and BM-DTCs, isolated from BC PDX-bearing mice. • CTCs and BM-DTCs represent a valuable preclinical model for investigating the role of these cells in tumor metastases.
14	Schölch *et al.* (19)	2016	• Orthotopic mouse model of CRC. • Tumor-forming capacity of CTCs when re-injected into mice.
15	Gorczynski *et al.* (20)	2016	• Investigation of frequency of circulating tumor cells in peripheral blood of tumor bearers. • Cytokines are associated with increased metastatic potential.
16	Powell *et al.* (21)	2016	• Bioluminescence imaging. • Flow cytometry. • Loss of BTG2, a p53 effector protein, contributed to the enhanced tumor growth.
17	Knips *et al.* (22)	2017	• Human Merkel cell carcinomas. • Transplantation to immunodeficient mice. • Formation of metastasis and appearance of CTCs.
18	Johan *et al.* (23)	2018	• Osteosarcoma LM8 cells. • Transplantation to immunocompetent mice. • Formation of tumor. • The injection of a vesicular stomatitis virus demonstrated an anti-metastatic effect and contributed to a more effective detection of CTCs.
19	Zhang *et al.* (24)	2018	• The metastatic potential of adherent and suspension BCCs *in vitro* and *in vivo* was examined. • COX-2 acts as an inhibitor for anti-metastasis.
20	Vishnoi *et al.* (25)	2019	• TNBC cells from liver metastasis. • Transplantation to immunodeficient mice. • Development of TNBC lesions to mice.

HCC: Hepatocellular carcinoma; CTCs: circulating tumor cells; GEMMs: genetically engineered mouse models; CRC: colorectal cancer, MMP-9: metalloproteinase-9; TNBC: triple-negative breast cancer.

the use of animals CTCs could be an important tool for the prognosis and treatment of patients with TNBC.

Table I summarizes the aforementioned results of our search.

Discussion

CTCs play a key role to the process of metastasis. They can be assessed through a single blood draw (26). Their evaluation in number and characteristics can be related to cancer aggressiveness and survival rates of patients (27). CTCs also contribute to the detection of the progression of cancer and the possible resistance to treatment. As Vishnoi *et al*., proved, CTCs can offer valuable information in a non-invasive way and in real time concerning possible biomarkers of metastatic potential.

It should be mentioned that some specific and not all types CTCs are related to prognosis, depending on their own biological properties (28). In the study of Knips *et al*., they were two different types of Merkel cell carcinomas with different prognosis and different types of CTCs and biological characteristics. The WaGa cell line was associated with worse prognosis and greater inflammatory response. In 2017, Ahn *et al*., showed that the inflammatory response is positively correlated to cancer progression through the circulating tumor cell process (29). Thus, a plethora of techniques is currently being used in order to characterize biologically the CTCs, such as size, density and immunoaffinity markers rendering these cells as a more reliable tool against cancer (30).

Another important issue which should be mentioned is the isolation methods which are used to detect the CTCs. As far as the animal models are concerned, their detection, through biopsies and invasive techniques, is easier. On the contrary, in the bloodstream, their characterization and isolation is a very challenging task (31). Furthermore, the variety of the isolation techniques and the absence of a gold-standard technique demonstrates the progress which can be made into this field.

Last but not least important, it should be mentioned that the formation of tumor and the appearance of CTCs in the animal models is an effective way in order to examine the pathophysiological mechanisms of certain types of malignancies, and especially the procedure of metastasis. Havens *et al*., reported that the expression of vascular adhesion molecule 1 (VCAM-1), in response to tumor-derived tumor necrosis factor (TNF), through the activation of the endothelial cells is a possible mechanism leading to a premetastatic status (10).

At the basis of these data, circulating tumor cells (CTCs) are considered as potential disease monitors. However, their use as therapeutic targets necessitates a deeper understanding of the biological mechanisms, concerning tumor propagation and their activation against the aggressive growth of tumors. Therefore, further investigation is required, in order thorough biological insights to be achieved, leading to the development of improved therapeutics.

Moreover, given that the majority of CTCs loses their activity against the tumor dissemination, or never be activated, it is essential to investigate distinguishing features between CTCs that manage to act as biomarkers and those that never escape dormancy. By identifying these features, we will be able to develop improved CTCs aiming to an enhanced and simplified clinical trial design. Such improved clinical trials may result in the approval of therapies that are significantly effective in preventing metastasis.

Additionally, whereas animal model trials are conducted in order to identify potential biomarker for the activation, and expansion of metastasis from CTCs, notable improvements can be achieved *via* single-cell genomic analysis of CTCs isolated from cancer patients. The detection of crucial functional regulators *via* such CTCs, will allow the better understanding of the activation of metastasis genes in the genetic basis. As a result, valuable insights concerning the correlation of metastatic relapse to pathological conditions will be achieved.

Taking all these under consideration, a deeper investigation should be done, including larger and improved clinical trials, resulting in observations that will lead to the design of suitable animal model experiments.

Conclusion

CTCs have been investigated as diagnostic, predictive biomarkers and therapeutic targets in many types of cancers. Although CTCs are not commonly used in clinical practice, CTC studies have accumulated a high level of knowledge, especially in lung, prostate, pancreatic, skin tissue, breast and colorectal cancers. Animal models with malignancy demonstrated higher levels of circulating tumor cells but the clinical validity of CTC detection has been questioned. As a result, further clinical research should take place in both animals and human in order to examine their clinical utility against malignancies.

Conflicts of Interest

The Authors declare that there are no conflicts of interest.

Authors' Contributions

NG, CD, AA and DD designed the study. NG, CD, AA and AG wrote the article. NG, CD, AA, AG, VEG, EV, AS, ED, PF, GK, AP and NT collected the data. SV, DS, EAA, KK and DD offered scientific advice. NG, CD, AG and MP revised the manuscript. DD critically revised the manuscript and was the supervisor.

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