Circulating tumor cells in breast cancer with orthotopic allograft mouse model

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Directed by Professor Joon Jeong, M.D., Ph.D

The Doctoral dissertation submitted to the department of Medicine, the Graduate School of Yonsei University in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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* Abstract

Circulating tumor cells in breast cancer with orthotopic allograft mouse model

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(Background) Breast cancer causes death not because of the primary tumor in the breast but because of metastases in distant sites that gradually cause organ dysfunction.[1] Circulating tumor cells (CTCs) are cells that have detached from the primary tumor or metastatic tumor sited and entered the peripheral circulation.[2] We established orthotopic breast cancer animal model of breast cancer with CTCs and validated the role of surgical treatment in the subjects with primary breast cancer without CTCs.

(Materials and Methods) We implanted 1 $\times 10^4$ GFP expressing 4T1 cells in 4^{th} or 5^{th} mammary fat pad of 8 week-old BALB/cAnNCrl mice(n=69). For the evaluation of role of surgery in early breast cancer with no CTC, we removed the tumor cell just before CTCs appeared in circulation (n=35). After removal of the primary tumor we evaluated the extent of surgical procedures. If there was any suspected residual tumor tissue in operation bed, the procedure was categorized as R1 resection. If operation bed was not suspected with residual tumor tissue, the resection was categorized as R0 resection.

(Results) All the experimental animal showed CTCs after primary tumor appeared (n=69). Furthermore, tumor volume and the number of CTCs increased over time and tumor volume showed statistically significant correlation with CTCs (p<0.001).Next, we removed primary tumor before a CTC was detectable with flow cytometry assay (n=35). The mice showed no CTCs during follow up if removal of the tumor results in complete resection of the tumor, R0 resection (n=25). However, we could detect CTCs during follow up period if the resection results in simple wide excision, R1 resection (n=10) (p<0.001).

(Conclusions) We successfully developed orthotopic allograft mouse model with breast cancer and circulating tumor cells, which is most similar with human breast cancer evolution. We also showed R0 resection of the primary tumor could result in no CTCs during follow up period if the resection was done when there was no CTCs. We believe this finding could be translated in the clinical field.

Key Words: Breast cancer, Circulating tumor cell, Mouse model

Circulating tumor cells in breast cancer with orthotopic allograft mouse model

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Introduction

Breast cancer causes death not because of the primary tumor in the breast but because of metastases in distant sites that gradually cause organ dysfunction.[1] Treatment of breast cancer, however, has been evolved from the locoregional treatment of breast itself. In 1894, Halsted published the Johns Hopkins Hospital experience with radical mastectomy, reporting a remarkable local regional control rate of 73% with no operative mortality. The actuarial survival rate was double that of untreated patients, with a 5-year survival rate of 40%, despite the advanced stage of many of the tumors and the lack of any adjuvant therapy.[3,4] Considering the advanced stage of many of the tumors in Halstedian era and the lack of any adjuvant therapy at that time, radical mastectomy Halsted suggested had became and remained the

standard of care until the 1970s.[3] The long term results of Halsted′s innovation published 30 years later, however, failed to support this idea that distant metastases occurred late in the disease process.[5] Only 12% of breast cancer patients treated by Halsted and his students survived 10 years despite radical surgery. Moreover, recent surgical studies, completed in an era when patients were diagnosed earlier with smaller tumors, provided better results from radical mastectomy with 50% of patients survival after 10 years, still 20%~30% of patients with negative axillary nodes at diagnosis and 70% of those with nodal metastases experienced disease recurrence and die of their disease within 10 years when treated by local surgery alone.[6] These data led to the seemingly obvious conclusion that distant metastases, although not evident clinically, must be present in many patients at the time of the initial diagnosis.

Breast cancer in Korea shows striking increase during last 10 years, now we have more than 16,000 patients per year in Korea and most of the mortalities come from the metastatic disease, not from the primary breast lesion.[7] Currently, detection of breast cancer metastasis relies on clinical manifestations of the spread to distant organs,[8] but the methods are usually inept at detecting metastasis at the earliest stage and accurately predicting the clinical outcome of the disease.[8]

Circulating tumor cells are cells that have detached from the primary tumor or metastatic tumor sites and entered the peripheral circulation.[2] Evidence for the clinical relevance about the presence of CTCs in patient with breast cancer has been revealed in several researches. As a prognostic factor, the presence of CTCs before or after neoadjuvant or adjuvant chemotherapy is associated with poor prognosis in several clinical studies.

In patients with metastatic breast cancer, the number of CTCs before treatment is an independent predictor of progression-free survival and overall survival and Hayes et al. also reported the prognostic value of CTC counts during therapy.[9, 10] The clinical relevance of CTCs also has been shown in patients with primary breast cancer. Zhang et al. analyzed clinical impact of CTCs in early stage breast cancer and the estimated pooled hazard ratio showed that CTC positivity was associated with a significantly increased risk of death (HR,2.78;95% CI,2.22-3.48;p=0.00).[11] Several clinical studies also clarified the predictive value of CTCs for identifying chemotherapy-resistant patients. [11,12]

CTCs could also serve as monitoring for patients with advanced diseases, because serial monitoring of advanced disease could help to capture spatial and temporal heterogeneity during tumor evolution which is considered one

of the major reasons for the current failure of cancer systemic treatments whether administered early or later in the disease course.[13,14]

Lin et al.[15] suggested specific molecular profile or phenotype that can be utilized to predict the future course for a CTC will be needed because there still exists a subpopulation of patients with the presence of CTCs in blood those never develop overt metastasis. While CTCs guided treatment switching failed to show survival benefit,[16] Broersen et al [17] suggested that CSCs could be one of the promising target for new therapeutic approaches to prevent metastasis in breast cancer. Taken together into accounts, CTCs are moving from a simple tumor burden marker to potential biomarker providing information on tumor heterogeneity and tumor biology and becoming potential target for treatment.

Detection and characterization of CTCs, unfortunately, has several technical limitations. Since the first report on tumor cells in blood stream was attributed to Ashworth in 1869,[18] the technology to capture and characterize CTCs with high analytical validity has only been available over the last decade.[19]Over 40 difference devices have been reported to isolate and characterize CTCs from whole blood and most of them are based on exploiting the differences between epithelial CTCs and normal hematopoietic cells in size, weight, elective charge, or flow characteristics or expression of

epithelial or cancer-specific marker.[20] Currently, the most commonly used strategy to capture CTCs involves coating a solid phase matrix of some sort with an antibody directed against a surface-expressed epithelial marker, usually the epithelial cellular adhesion molecule(EpCAM). The only FDA-approved, commercially available assay based on this strategy has been designated CellSearch®(Veridex, LLC;Raritanm, NJ), and has been shown in several studies to be associated with worse prognosis in breast as well as colorectal and prostate cancer.[21] However, this strategy is not perfect. EpCAM is only expressed by approximately 80% of all breast cancers, and the intra-patients expression of EpCAM by CTCs can be highly variable.[22] In this regard, EpCAM may be lost by cancer cells that are undergoing epithelial to mesenchymal transformation, a recently recognized process that may be a fundamental property of the metastatic phenotype.[23] Similar challenges in detecting and quantifying rare metastatic cells in mouse models of human breast cancer has hindered the ability to quantify early steps in metastasis and to determine the timing and location of metastatic spread of cells.[24]

Experimental investigation of tumor growth and metastasis of human cancer often involves xenotransplantation of human tumor tissue or tumor cells into immunocompromised animals such as athymic nude mice (*nu/nu*), SCID

(severe combined immunodeficiency) mice (*xid*), or beige (*bg*) mice.[25-29] However, CTCs can be explained more perfectly in immune-competent animal model, because CTCs may be present in very small numbers and must be identified and survived against a large background of normal host cells, especially cells engaged in host immune systems.[30]

The goal of the present study was to develop and optimize orthotopic allograft mouse model with breast cancer and circulating tumor cells, which is most similar with human breast cancer evolution. This study can also help to validate the current and future knowledge of CTCs.

Another high priority questions for the breast cancer research field is to identify molecular signature to select patients who could be spared chemotherapy and which low-risk patients require no adjuvant therapy.[30] This question is associated with the fundamental question about the growth mechanisms of breast cancer whether breast cancer is a local disease that spread in an orderly fashion and becomes systemic, or whether breast cancer is a systemic disease at its inception.[3,31] These two opposing theories classically referred to as the Halsted and Fisher paradigms, formed the basis for breast cancer treatment in the 20th century. The increasing acceptance of the Fisher paradigm over Halstead′s theory resulted in a shift away from more radical surgery to increasing use of systemic therapies in recent

decades. The increasing body of evidence, however, demonstrated that local control does impact survival in stage I to III breast cancer and raised the question if we can select patients who could be spared chemotherapy.[32] Earlier diagnosis largely as a result of screening and the major impact of the widespread application of hormone therapies to patients with estrogen receptor-positive disease also resulted in that the number of patients for whom any benefit in outcome as a result of adjuvant chemotherapy become to be so small as to be outweighed by concomitant toxic side effects.[33]

It could be a hypothetical option for sparing chemotherapy for the low risk patients to remove primary tumor before any tumor cells penetrate into and become available to be detected in circulatory system. The hematogenous dissemination of cancer and development of distant metastases is the cause of nearly all breast cancer deaths and the lymphatic system does also eventually drain into the systemic venous system via the azygous vein.[34] Therefore if these metastatic cells can be removed before spread through the haematogenous route, the patients could be cured with surgical removal only.[34, 35]

In this study, we developed orthotopic allograft mouse model with CTCs and investigated the role of surgical resection of the primary tumor with no evidence of CTCs.

Materials and methods

1. Experimental breast cancer cell lines

A. 4T1 breast adenocarcinoma mouse cell line

We used the 4T1 breast adenocarcinoma mouse cell line for a model system to understand characteristics of circulating tumor cells, because tumor growth and metastatic spread of 4T1 cells has the advantage of being able to be transplanted into immune-competent recipients.[36] 4T1 mouse mammary tumor cell lines were cultured in RPMI 1640 (Gibco, Grand Island, NY) supplemented 10% fetal bovine serum (FBS; Cansera, Canada), antibiotic-antimycotics (Gibco) at 37℃ in a humidified atmosphere containing 5% CO_2.

B. Establishing Green Fluorescent protein (GFP)-expressing tumor cell lines.

For the expression of GFP, hMGFP was cloned to the pCl neo vector in pDNA through PCR. 4T1 cell line was transfected with GFP-cloned plasmid using FuGENE HD Transfecion Reagent/Optimem (Promega/Gibco) to achieve individual tumor cell visibility in vivo. Efficiency of transfection was checked 2 days after transfection. Tumor cell populations with stable

transfection with hMGFP were cultured in the same medium described above supplemented with 10mg/ml of puromycinin in 1:20 ratio. Single clone was separated with cloning cylinders (PolyLabo, Strasbourg, France) after 2-week subculture with every 3-day changes of media and GFP positive cells were isolated by Flow cytometry (fluorescence microscope) to establish transfected with 4T1/GFP multi-clone cell lines, which then were cultured in 0.025mg/ml puromycin enriched medium.

2. Orthotopic mouse model

A. Experimental animal and Orthotopic Implantation of 4T1/GFP cells

We used 8 week-old female mice, BALB/cAnNCrlmice (Charles-River,Quebec, Canada). Using animals for experiments, protocol for this research was reviewed by the ethics committee and conformed to the guidelines for Care and Use of Laboratory Animals. We also followed standard procedures during all of the following procedures.

Two days prior to orthotopic injections, 4T1/GFP cells were placed in non-selective fresh media and prepared for implantation. Target number of tumor cells was 1×10^4.

On the day of tumor cell implantation, the anesthetized mice were placed in supine position. We carefully dissected around the breast tissue and implanted

4T1/GFP tumor cells in 0.1ml phosphate-buffered saline (PBS) orthotopically in 4^{th} or 5^{th} mammary fat pad of 8 week-old virgin female mice. Each experiment included a control animal with vehicle injection. We used these control groups for quality control of each experiment and also for determination of the lowest level of detection.

Animals were housed in groups of 3~5 animals after tumor cell implantation in conventional pathogen free condition on shaved pine bedding.

The animals were maintained on a 12 hours light/dark cycle, with food and water proceeded *ad labium.* Euthanasia was performed by cervical dislocation after Avertin anesthesia after 6 weeks and 20 weeks observation in case of experiments for animal model establishment and removal of primary tumor, respectively. The whole process of this experiment is schematically described in Figure 1.

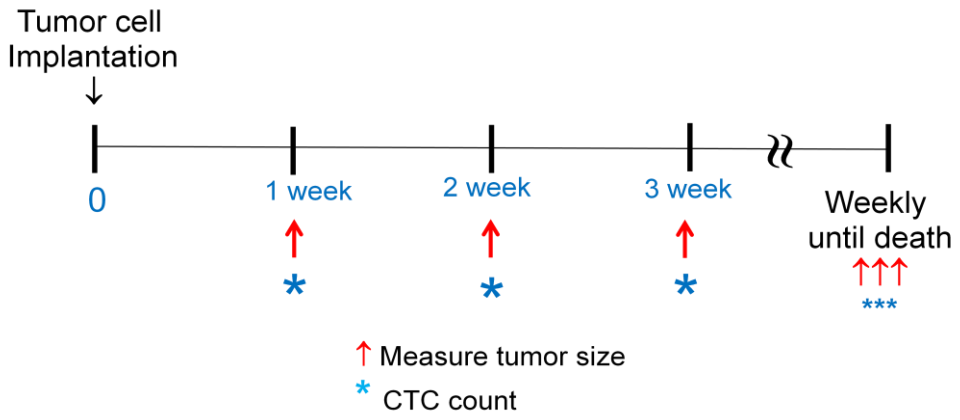


Figure 1. Schematic schedule of experiment. 4T1/ Green Fluorescent Protein (GFP) tumor cells in 50 ㎕ phosphate-buffered saline (PBS) orthotopically implanted in 4th or 5th mammary fat pad of 8 week-old virgin female mice. After implantation of the tumor cell, weekly blood draw was done and examined for CTCs with flow cytometry. Without any intervention, mouse usually died around 6~8 weeks after tumor cell implantation.

B. Tumor size measurement by Digital caliper

To assess tumor growth, we used Digital caliper and acquired the width and length of tumor once a week. We calculated tumor volume by the formula, π (3.14)´L (tumor length)´W(width)^2/6, using average of width and height of the tumor.

3. Enumeration of CTCs

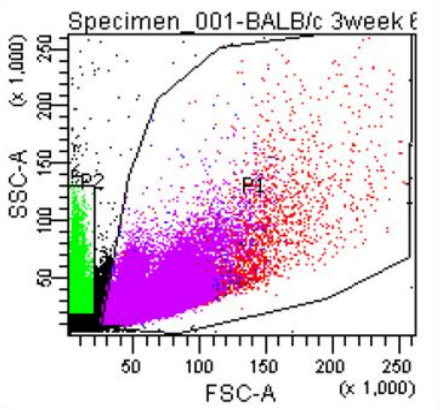
We drew 15 ㎕ of blood from tail vein of the mouse every once a week to detect and enumerate CTCs. Red blood cells were lysed using an RBC Lysis Solution (BioLegend, San Diego) and incubated for 30 min at 4 ℃. Fixed number of cell counting beads in 10 μl (Life technologies, Seoul, Korea) was added for absolute count of 4T1/GFP tumor cells and flow cytometry was performed using a FACS Calibur instrument (Becton, Dickinson and Company, Cowley, U.K.) and analyzed using CellQuest software (Becton Dickinson). At least 15,000 events were acquired for each sample.

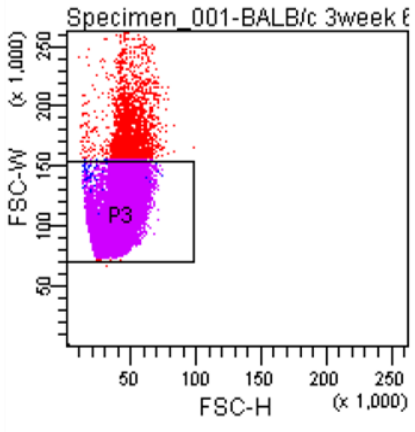
First, to confirm the utility of this method, 50, 200, 500, 1000 4T1/GFP tumor cells were spiked into PBS and human blood. Fixed number of cell

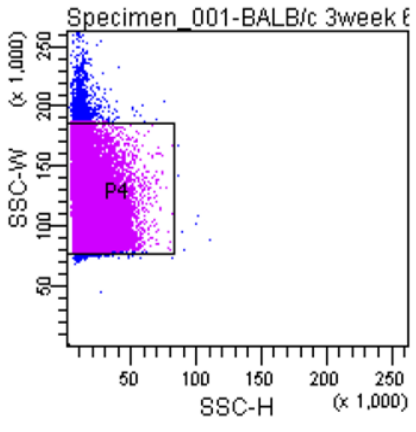
counting beads in 10 μl (Life technologies, Seoul, Korea) added each sample for enumeration of the tumor cell number with flow cytometry.

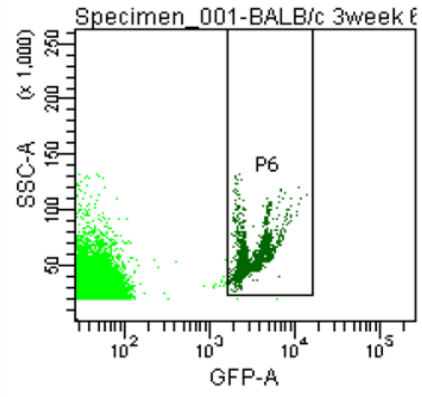
After we confirmed the utility of this method, we detected and enumerated CTCs according to the above procedure and the gating strategy for detection of 4T1/GFP tumor cells is presented in Figure 2

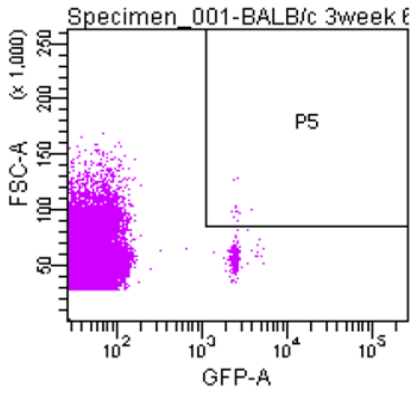
We considered positive CTC, if the number of counted cells is more than 5. Specificity has both technical and biological distinctions and technically it is essential to distinguish identified cells from both normal constituents of the surrounding blood environment.[37] Even with careful attention to separation of epithelial from hematopoietic cells, on occasion, normal subjects are found to have circulating tumor cells.[10] We also found some CTCs in control group and the lowest level of detection was determined according to data from normal control. The lowest level of detection was defined as more than 5 cells because the maximum false positive CTCs number was less than 5 cells.

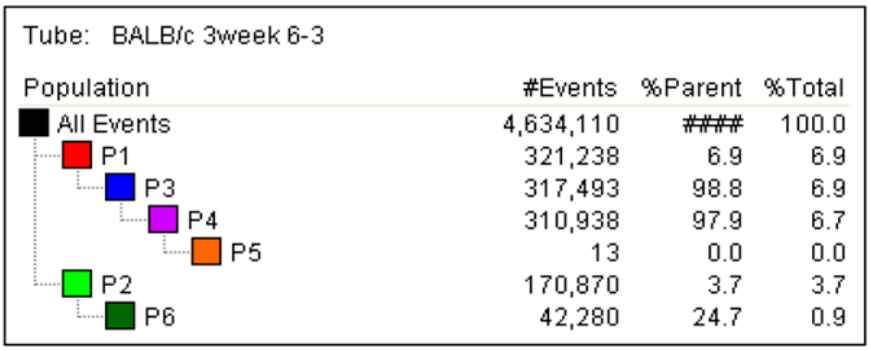

Specimen_001-BALB/c 3week
SSC-A
(x 1,000)
FSC-A
(x 1,000)
P1
P2


Specimen_001-BALB/c 3week
FSC-W
(x 1,000)
FSC-H
(x 1,000)
P3


Specimen_001-BALB/c 3week
SSC-W
(x 1,000)
SSC-H
(x 1,000)
P4


Specimen_001-BALB/c 3week
SSC-A
(x 1,000)
GFP-A
P6


Specimen_001-BALB/c 3week
FSC-A
(x 1,000)
GFP-A
P5



Tube: BALB/c 3week 6-3

Population	#Events	%Parent	%Total
All Events	4,634,110	####	100.0
P1	321,238	6.9	6.9
P3	317,493	98.8	6.9
P4	310,938	97.9	6.7
P5	13	0.0	0.0
P2	170,870	3.7	3.7
P6	42,280	24.7	0.9

Figure 2.Detection of tumor cells by flow cytometry. GFP expressiong 4T1 tumor cells were implanted in 8 week-old female BALB/c mice and evaluated 3 weeks after tumor implantation with flow cytometry. These figures illustrate the gating strategy for detection and enumeration of circulating 4T1/GFP cells. (A) Size gating for detection CTCs. CTCs are gated in P1 and beads in P2. (B&C) Gating out other than single cells. Events with higher FSC-A relative to FSC-H (B) and larger SSC-H relative to SSC-H(C) are gated out. (D & E) Enumeration of the CTCs gated in P5 (E) and beads gated in P6 (D). (F) Results from flow cytometry assay. With known beads count of 48809, CTCs was enumerated as 16.07 in this case.

4. Tumor resection

To define the role of surgical treatment in early breast cancer with no CTCs, we removed tumor just before CTCs appeared. For this study, we first defined the smallest tumor size with CTCs. With this time point, we tried to prove whether complete surgical resection of the primary tumor before any CTCs appear could results in no CTCs during further follow up. On the day of tumor resection, the anesthetized mice were placed in supine position. We carefully dissected and removed the breast tumor and cauterized the surgical bed with animal surgical knife (Aaron RAM™ cauteries, Stoelting, Wood Dale, IL) to confirm R0 resection. Animals were housed in groups of 3~5 animals after tumor cell implantation in conventional pathogen free condition on shaved pine bedding.

The animals were maintained on a 12 hours light/dark cycle, with food and water proceeded *ad labium.* We observed the disease course in each mouse, time to local recurrence, overall survival, CTCs in each set-up time according to the time table of disease progression.

5. Statistical analysis

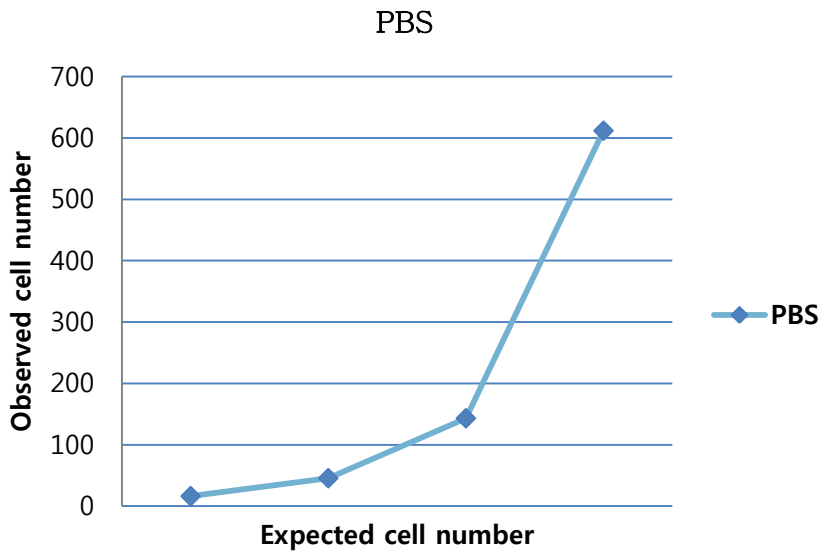
Data analysis was performed using SPSS version 20.0(IBM-SPSS, Chicago, USA). Correlation and regression analysis were performed to validate the CTCs and determine factors influencing CTCs. *p* values were two-tailed, and we judged valued of less than 0.05 to be statistically significant.

Results

1. Animal model with CTCs

First we validated whether we can detect CTCs with flow cytometry. We spiked 50, 200, 500, 1000 4T1/GFP tumor cells into PBS and human blood and the results are in Figure 3. Observed cell numbers are well correlated with expected cell numbers and each experiment showed statistically significant correlation. The correlation coefficients with Spearman′s rho test were 0.93(p=0.036) for CTCs in PBS, 0.97(p=0.012) for CTCs in human blood, respectively.

A


PBS
700
600
500
400
300
200
100
0
Observed cell number
Expected cell number
PBS

B

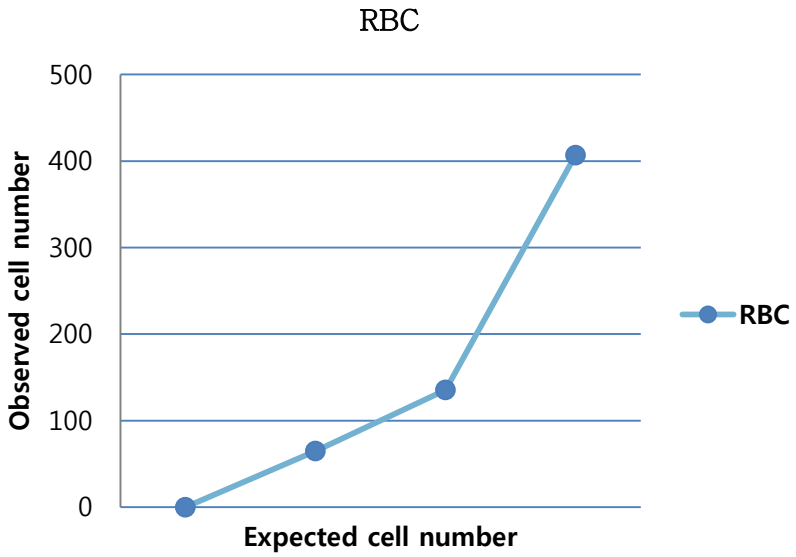

RBC
500
400
300
200
100
0
Observed cell number
Expected cell number
RBC

Figure 3. Linearity of tumor cell detection in spiking experiments. Green Fluorescent protein expressing cells were spiked into the PBS(A) and Human Blood(B) and counted with flow cytometry to validate enumeration of the circulating tumor cells (CTCs) with the flow cytometry. Expected cell numbers are in X axis and observed cell numbers are in Y Axis in each graph. Counted cell number showed statistically significant correlation with spiked cell number and correlation coefficient were 0.93(p=0.036) for CTCs in PBS (A), 0.97 (p=0.012) for CTCs in RBS (B) by Spearman′s Rho test, respectively.

Next, we tested if CTCs are detectable and countable in orthotopic allograft models of mouse with breast cancer (n=69) and control animal (n=11). The entire experimental animal (n=69) showed CTC after implantation of 4T1 tumor cells and lived for less than 6~8 weeks if there was no intervention.

We found this animal model with CTCs is useful in the future research, because once tumor was established, all the experimental animal showed CTCs and most CTCs in experimental mouse were started to be detected during 2 weeks ~ 4 weeks after injection of tumor cell. 24/69(34.8%) and 23/69(33.3%) of first detected CTCs were detected on 2nd week and 3rd

week, respectively and 17/69(24.6%) of first detected CTCs were detected on 4th week. Furthermore, size of the tumor and the number of CTCs showed linear correlation with the passage of time (Figure 4).

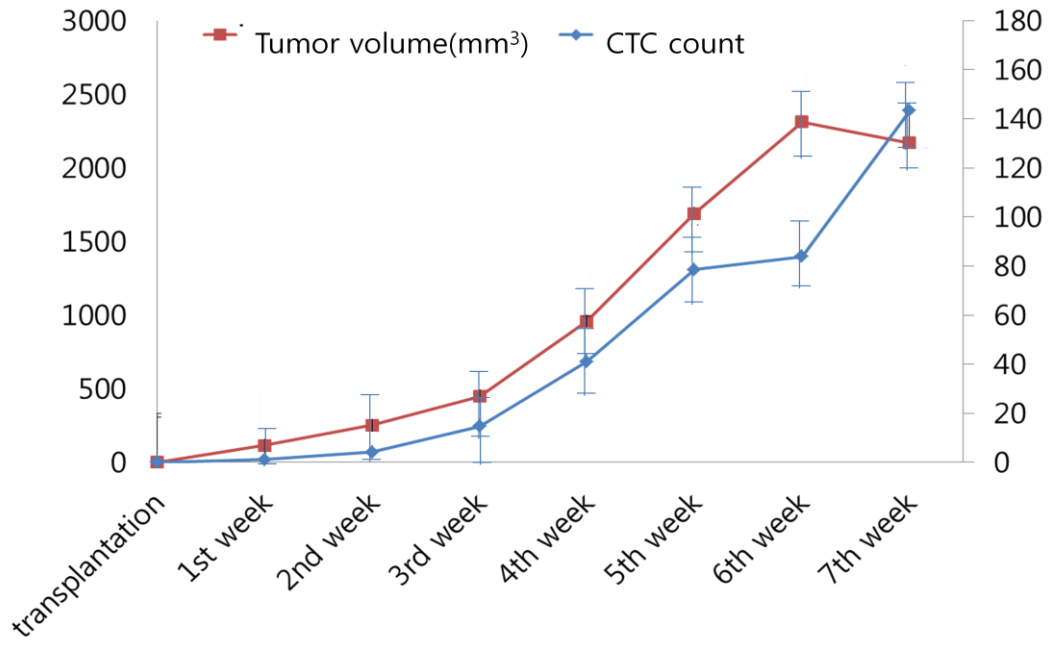


Figure 4. Tumor volume (mm^3) and CTCs counts over time. Tumor size (red line) and CTCs count (blue line) were increased over time.

We analyzed what could be the factors related with CTCs. Tumor size showed statistically significant correlation with CTCs with $p<0.001$ by Pearson′s test. (Figure 5).

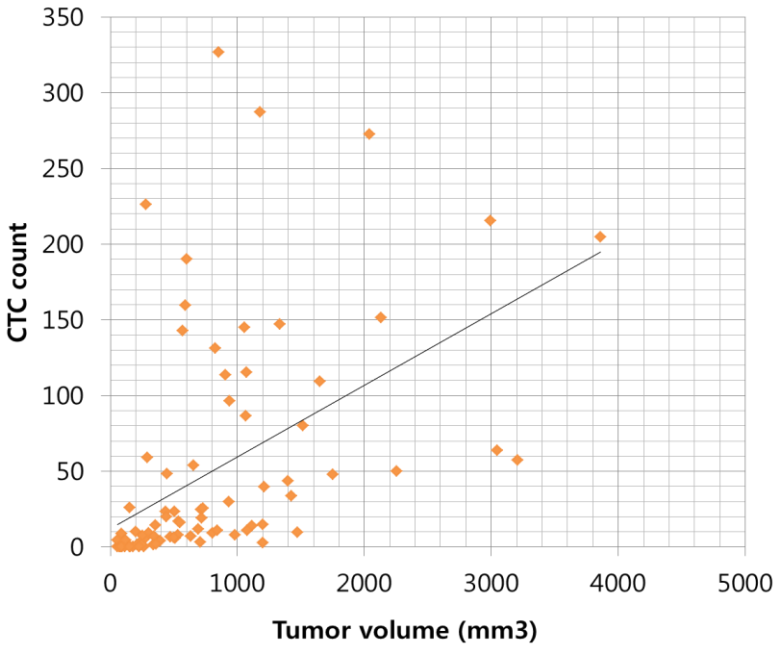


Figure 5. Correlations between CTCs and tumor volume(㎣). The CTCs showed statistically significant correlation with tumor volume with R=0.731(p<001) by Pearson′s test

2. Tumor resection

Given the data that all of the experimental animal showed CTCs after they were implanted with 4T1 tumor cells and lived only for 6~8weeks, we next evaluated the role of surgical intervention when there is only primary tumor with no CTC.

First, we evaluated what is the smallest size of the tumor of when CTCs were first detected was 47.28 mm^3 (1.3percentile) and the range of size was between 47.28 mm^3 to 1327.34 mm^3. Next, we removed the tumor before CTCs are detectable in blood stream. Practically, we removed the primary tumor before the tumor became 47.28 mm^3, the smallest size of tumor with observable CTCs, determined in the above process.

We found that if there were local recurrence around the tumor bed, that number of CTCs during follow up time showed statistically significant difference(($p<0.001$). We observed local recurrence in 10 mice, defined as R1 resection group and no local recurrence in 25 mice, defined as R0 resection group and the enumeration data of CTCs are in Figure 6.

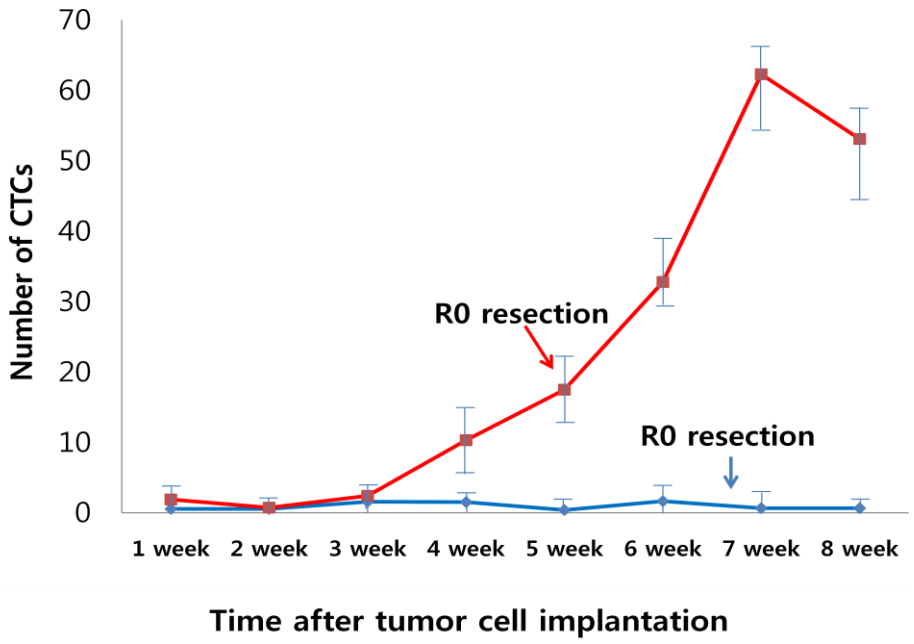


Figure 6. Enumeration data of CTCs after primary tumor removal. Study animals were stratified according to the tumor recurrence around the tumor bed. If there was any local recurrence the mice was stratified into R1 resection, and if there was no local recurrence, the mice was stratified into R0 resection. In R0 resection group (blue line), there was no CTCs (less than 5 CTCs), but R1 resection group resulted in reappearance of the CTCs.

Discussion

Circulating tumor cells (CTCs) are important for the metastatic process of carcinoma.[38,39] Currently CTCs is known as prognostic markers in metastatic breast cancer and predictive marker showing treatment response.[9,10]Detection of the CTCs in Breast cancer is, however, challenging since these cells are rare, occurring at a frequency of one tumor cell per 10^6 to 10^7 mononuclear cells.[8] Thus enrichment for CTCs is essential before further analysis.[17]Available cell enrichment techniques are either morphology or immunology-based.[39] Morphology-based techniques can be subdivided into density gradient centrifugation and filtration by size of the cells.[17] Immunology-based techniques use immunomagnetic isolation, often in conjunction with antiepithelial cell adhesion molecule(EpCAM) or anticytokeratin(CK) antibodies for positive selection of CTCs, or anti-CD-45 for negative depletion of mononuclear cell.[34,38] Since none of the enrichment methods yields a pure population of tumor cells, for all separation techniques needs a detection method to distinguish CTCs from other captured cells. These cell detection method can be cytometric or nucleic acid-based. Currently we have a limited number of markers and several test including CellSearch$^{\grave{O}}$ CTCs test (Veridex LLC, San Diego,CA) used for the isolation

or detection of the CTCs, none of them are standardized for clinical application.[2]

While CTCs are exceedingly rare compared with normal blood cells, the vast majority CTCs die in the blood stream, with only a minor fraction representing viable metastatic precursors.[41] Clinical data also reflect this finding and not all the patients will develop metastatic disease during their follow up, even though 9% to 38% of the patients with early breast cancer are reported to have CTCs in initial presentation.[35,42] While a direct involvement of CTCs in the metastatic cascade is unquestionable, probably not all CTC subpopulations have the same metastatic potential.[43] Yu et al.[44] demonstrated the occurrence of dynamic changes in epithelial and mesenchymal composition of CTCs in patients with metastatic breast cancer and found an association between treatment resistance and presence of CTCs with mesenchymal features in patients who were serially monitored through disease progression.

Such a study provides proof of concept that variation of specific CTC subpopulations can serve as an indicator of the rate of cancer adaptation during treatment and represents, therefore, an opportunity to identify new therapeutic targets and resistance markers possibly anticipating clinical progression.[43] To fully exploit the opportunity offered by CTC molecular

characterization in the clinical setting, it therefore is mandatory to have an appropriate animal model with CTCs.

Circulating tumor cells are also known as purported intermediates of metastatic dissemination and are likely to contain cellular clones responsible for disease progression. Clinical data also reflect this finding and not all the patients will develop metastatic disease during their follow up, even though 9% to 38% of the patients with early breast cancer are reported to have CTCs in initial presentation.[35,42] CTCs therefore represent a preferred source for the identification of drug targets and these cells should be targeted by new therapeutic approaches to prevent metastasis in breast cancer.[43] Evidence supporting this suggestion also has been revealed in recent clinical trials reporting earlier detection of CTCs can lead to improve patient′s outcomes only after effective treatment is available.[16]

A prospective randomized clinical trial has been conducted in the Southwest Oncology Group(SWOG 0500) to test whether women with metastatic breast cancer who have elevated CTCs after one cycle of first line chemotherapy have improved outcomes as a result of switching early to an alternate therapy and this trial was based on the hypothesis that these patients will have improved outcomes by minimizing the time and toxicity spent on ineffective

therapies and by spending more time on effective therapy.[16] The results, however, failed to show benefit from early switching to an alternate cytotoxic therapy in terms of overall survival, and authors suggested it might be preferable to prepare that patient for participation in prospective trials of novel therapies, rather than expose a patient whose CTCs have not been reduced after one cycle of first-line chemotherapy to the toxicities of second- or later-line chemotherapy. Factors leading to the generation and evolution of CTCs from a primary tumor are, however, largely unknown.[41]

With the goal of providing a reliable research model allowing the acquisition of valuable information on CTC features in the clinical setting, we established the orthotopic mouse model with CTCs with success and confirmed that we can enumerate CTCs, which is relevant and useful to monitor disease progress.

We next defined the role of surgical resection of primary tumor. The significance of CTCs in patients with operable breast cancer is still a matter of research.[35] In theory, because metastasis should result from a cell migrating from the primary tumor through the blood to a distant organ, the detection of CTCs could play a role in identifying patients at higher risk of relapse. One of the aims of the adjuvant chemotherapy would be to eliminate these cells from the circulation, thus preventing their invasion of the distant

organs. Some studies have investigated the importance of CTCs in primary breast cancer, but discordant results have been reported.[35] One reason for this resides in the difference between the methods used in each studies and procedures usually performed after an in-house procedure without standardization of reagents or cutoffs, and without a clear definition of the criteria used to define a CTC, resulted in a great difficulty in comparing the various studies.[35] Moreover, a high degree of variability in terms of stage of disease, timing and number of blood samplings, clinical end point, statistical analysis, and insufficient follow-up make the interpretation of clinical findings difficult.[35] These findings also urge to establish appropriate animal model with CTCs and evaluate the role of surgical treatment for primary breast cancer according to whether CTCs exist or not.

We hypothesized if we could remove primary tumor completely without any detectable tumor cells before CTCs appeared, the mice would have no CTCs and no detectable tumor recurrence during follow up time. We defined appropriate time for surgery as primary tumor became the maximum size without CTCs, retrieved from the previous data, 47.28mm^3. We removed the tumor when tumor size hit the limit, 50mm^3. If R0 resection was successfully done, then the mice lived for more than 14 weeks, the longest duration of 20 weeks, which is longer than 5~6 weeks, the expected life span of mice

injected with 4T1 mammary carcinoma cell line. Surgical treatment of breast cancer has been transformed through change in both the biological understanding and the clinical presentation of the disease.[4] Radical mastectomy was replaced eventually by breast conserving therapy since randomized clinical trials reported that breast conserving surgery with radiation results in equivalent survival to mastectomy.[4,45] In current clinical practice for treatment of breast cancer, however, include not only surgical treatment, but also multimodal approach such as chemotherapy, targeted therapy, and anti-hormonal therapy, not all of them are available approaches for other cancers, such as stomach cancer, liver cancer, gall bladder cancer, etc.[4,46] Another caution comes from the clinical trials supporting the minimal surgical approach themselves. All of the clinical trials supporting breast conserving surgery or minimal axillary approach deal with early breast cancer such as tumor size less than 2~4cm or no clinical nodal metastasis.[47, 48] In this study we confirmed that if surgical removal could be done with no residual tumor cells including CTCs, surgical treatment could be powerful approach for the solid tumor even without adjuvant treatment, such as radiotherapy or chemotherapy.

Conclusion

We successfully established orthotopic allograft mouse model with CTCs. CTCs appearance was statistically correlated with tumor size. We defined the role of surgical removal of primary tumor without residual tumor cells nor CTCs and found surgical treatment itself could cure the patients without any other adjuvant treatment in early stage disease without CTCs.

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국문 요약

동종이식 마우스모델을 이용한 혈중 암세포 연구

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한애리

(배경)유방암 환자는 유방 내 원발암의 국소 진행에 의한 사망은 거의 없으며 전신전이에 의한 다장기 기능부전으로 사망하게 된다.[1] 혈중암세포는 원발암 또는 전이성 병변으로부터 유래되어 순환계로 유입된 세포를 말한다.[2] 혈중 암세포는 유방암의 전신전이를 설명하는 가설 중 하나이다. 따라서 혈중 암세포를 구현한 마우스 모델은 혈중암세포에서 제기되는 여러 가지 의문을 설명할 수 있는 좋은 기반이 될 것으로 생각된다. 이에 본 연구에서는 유방암의 혈중암세포를 발현하는 동종이식 마우스 모델을 확립하고자 하였다. 또한 원발암의 수술적 제거와 혈중암세포와의 관계를 규명함으로서 전통적 암치료인 수술적 치료의 의의에 대해 살펴보고자 하였다.

(방법) 1 ´10^4 GFP expressing 4T1 cells 을 8주령

BALB/cAnNCrl 마우스의 4번째 또는 5번째 유방 조직에 이식하였다. 이후 마우스를 주기적으로 관찰하고 채혈하여 전체적인 상태와 함께 혈중 암세포를 Flow tyrometry 기법으로 확인하였다. 동종이식 마우스 모델을 확인한 후 다음의 연구를 시행하였다. 혈중암세포가 없는 상태에서의 원발암 제거술이 갖는 의미를 확인하고자 하였으며 이를 위해 우선 종양세포가 안정적으로 이식되어 종괴를 형성하면서도 혈중암세포는 없는 상태를 종양의 크기로 수치화하였으며 이후 해당기간에 종괴를 절제하였다. 종괴 절제 정도를 확인하여 육안적으로 암세포가 확인되지 않고 절제연을 충분히 확보한 경우 R0로, 그렇지 않은 경우 R1, 두 개의 그룹으로 마우스를 나누어 관찰하였으며 주기적으로 상태관찰과 함께 혈중암세포 출현여부를 확인하였다.

(결과) 혈중 암세포 연구를 위한 동종이식 마우스모델이 69 마리의 마우스에서 모두 성공적으로 확립되었다. 1×10^4 GFP expressing 4T1 cells 이식 후 채혈된 혈액 내에서 혈중암세포가 확인되었고 이는 시간이 지남에 따라 증가하는 것을 관찰할 수 있었다. 원발암의 수술적 절제와 혈중암세포의 출현 여부와의 관계를 알아보기 위한 다음 실험에서 모두 35 마리의 마우스에서

수술적 치료와 혈중암세포의 출현 여부와의 관계를 확인하였으며 출현을 결정하는 것은 원발암의 수술적 정도였다. 즉 원발암을 충분한 절제연을 확보하여 잔존암이 없게 제거하였던 경우 혈중암세포는 출현하지 않았지만(n=25) 그렇지 못한 경우 추적관찰 도중 혈중암세포가 출현하는 것을 확인할 수 있었다. (n=10)(p<0.001)

(결론) 동종이식 마우스모델은 혈중암세포연구를 위한 성공적인 동물모델이 될 수 있음을 확인할 수 있었다. 아울러 확립된 동물모델로 원발암의 수술적 치료와 혈중암세포의 출현과의 관계를 확인할 수 있었던 바 혈중암세포가 없는 상태에서 원발암을 제거할 때, 육안적으로 잔존암이 없고 충분한 절제연을 확보하는 R0 절제술을 한다면 이후 추적관찰에서 혈중암세포가 출현하지 않는 것을 확인하였다.

핵심되는 말 : 유방암, 혈중암세포, 원격전이, 수술적 치료