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ORIGINAL PAPER

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Optimization of detection of circulating tumor cells by flow cytometry and qRT-PCR

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ABSTRACT

Introduction. Treatment and diagnostic process in solid tumors like lung cancer are still based on invasive methods such as bronchoscopy, solid biopsy et cetera. One of the less invasive methods is a proposed "liquid biopsy" which is based on capturing of tumor cells circulating in the blood.

Aim. The aim of the study was to standardize conditions and to assess the sensitivity of the identification of circulating tumor cells (CTCs) with the use of flow cytometry and qRT-PCR.

Material and methods. In the first model of CTCs, cells from the A549 lung cancer cell line were suspended in 1 ml of healthy donors' blood in 5 spikes increasingly: 0, 10, 50, 100 and 200 and the cells were detected in flow cytometer. In the second model, cells from the A549 and H1975 lung cancer cell lines were used. Spikes were prepared as in the first model, but cells were suspended in 400 µl of healthy donors' blood and were detected with the use of qRT-PCR.

Results. An increasing number of detected cytokeratin positive events from the 1st spike "0" to the last one - "200" was observed by flow cytometry. Median value in the negative control was 0 false positive cells. In tubes from "10" to "200" the median was 5, 43.5, 58 and 78, respectively. Mean sensitivity of flow cytometry was 63.79%. In qRT-PCR, correlation between increasing number of sorted cells in several spikes and the level of mRNA expression for KRT19 gene was not observed.

Conclusion. Commonly available methods like flow cytometry and qRT-PCR seem to be attractive solutions for CTCs detection, but they need pre-enrichment procedures and standardization.

Keywords. circulating tumor cells, flow cytometry, PCR

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Participation of co-authors: A – Author of the concept and objectives of paper; B – collection of data; C – implementation of research; D – elaborate, analysis and interpretation of data; E – statistical analysis; F – preparation of a manuscript; G – working out the literature; H – obtaining funds

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Introduction

Circulating tumor cells – liquid biopsy

Circulating tumor cells (CTCs) were observed for the first time by Thomas Ashworth, a pathologist from Australia, in 1869. He identified cells similar to cancer cells during an analysis of a postmortem blood sample.[1] Subsequently, in 1954, Watanabe observed that clusters of tumor cells injected into murine circulatory systems have great metastatic potential.[2] From this time on, we obtained wide knowledge about properties and functions of circulating tumor cells. A sparse population of circulating tumor cells has been estimated in the circulatory system of patients with advanced cancer at one CTC per billion normal blood cells.[3] CTCs are bigger than white and red blood cells; they have a diameter of ~ 12-25 μm vs 7-15 μm and 8 μm diameter for WBCs and RBCs, respectively.[4] Today, we know that this sparse cell population could be found in the blood because tumor cells are able to migrate into the bloodstream. They spread in whole circulatory system by angiogenesis promotion and intravasation. Tumor cells produce some factors, like vascular endothelial growth factor (VEGF), IL-8, TNF, which stimulate neovascularization; endothelial cells promote the growth of tumor cells. CTCs are able to adhere to endothelium and to form metastasis in distal organs.[5]

CTCs can occur as a single cell and also as a clustered (CTM – circulating tumor microemboli) group of more than three tumor cells that travel together in the bloodstream. The life span of CTM in the bloodstream is shorter than a single cell, which is able to exist in the circulatory system for only several hours. However, it was shown that clusters have a greater predisposition of forming distal metastases than single tumor cells. CTM can be derived from a primary tumor and also can be formed by aggregation or proliferation of single CTCs. They were identified in several cancers such as lung, breast, colorectal, prostate cancer, and also in melanoma or glioblastoma.[6]

During investigation of CTCs phenotype, there was increasing evidence that they are a highly heterogeneous population. At the beginning, they were described as CD45-/EpCAM+/CK+ cells. However, today we know that CTCs partially undergo epithelial-to-mesenchymal transition (EMT). Moreover, part of them are described as circulating tumor stem cells and they cannot be detected with methods based only on epithelial markers.[7] Because EMT+ CTCs were identified in cancer patients, who were classified as CTCs negative with the use of the EpCAM based method, scientists are still trying to find proper markers to catch CTCs.

Why are researchers trying to detect, isolate and analyze circulating tumor cells? What do we want to know from such a sparse population? The main reason for interest in CTCs is the idea of a non-invasive cancer diagnostic strategy named "liquid biopsy".[8] It is based on the assumption that it would be possible to diagnose, set a treatment strategy, and monitor the patient using only a few milliliters of whole blood instead of traditional imaging and solid biopsy. This idea has been developed and the Food and Drug Administration has approved clinical use of the CellSearch System for CTCs isolation in metastatic breast, colorectal and prostate cancer.[9] However, because of the heterogeneity of CTCs, proper isolation of this population is still unavailable, because we do not have a technique which would be able to identify EpCAM+ CTCs, CTCs undergoing EMT process, circulating tumor stem cells, and CTCs clusters simultaneously.

Techniques of CTCs detection

Today, the capturing of CTCs is a widely explored issue. Reviews describing development of methods created for CTCs detection mention dozens of techniques.[3,10–13] They are based on immunoaffinity or biophysical properties of tumor cells. The first aforementioned technology is used in two forms – as positive or negative enrichment.

Positive enrichment means that cells are captured only if they show expression of several surface markers. Positive enrichment is used in many devices designed for CTCs detection, eg. CellSearch System, AdnaTest, MACS, MagSweeper, Isoflux or GILUPI CellCollector.[10] The main surface marker that positive enrichment is based on is EpCAM. After capturing CTCs with the anti-EpCAM antibody, evaluated cytokeratin (CK) expression is evaluated and DAPI staining is prepared. Prognostic value of EpCAM was approved during validation of the CellSearch system in breast and prostate cancer. However, as mentioned above, CTCs are a heterogeneous population and by using EpCAM, we can identify only epithelial cells, and we lose CTCs undergoing EMT and the others.[14]

Although, there is another branch of techniques of CTCs detection based on immunoaffinity termed negative enrichment. CTCs population is obtained by depletion of CD45 positive cells. Therefore, we can detect all types of CTCs, but we do not obtain such a pure population as in the positive enrichment technique. Here we employ, for instance, the EasySep Human CD45 Depletion Kit or Negative Enrichment Immunofluorescence and an In Situ Hybridization System.[15,16]

Methods based on the biophysical properties of tumor cells are very different. Here we have techniques based on size and deformability of tumor cells, techniques using density gradient centrifugation (RosetteSep-CTC, Accucyte Enrichment and CyteSealer), microfiltration in two (ISET, FMSA) and three dimensions (Resetteable Cell Trap, Cluster Chip), inertial focusing (Vortex, ClearCell FX), electrophoresis (DEPArray) or acoustophoresis (Acoustophoresis Chip).[10]

Most of the methods mentioned require special technical devices which causes CTCs isolation to be quite expensive. The costs and problems with standardization are the main reasons that CTCs cannot be commonly detected in a routine diagnostic process for monitoring cancer patients. Therefore, we had the idea to verify the usefulness of commonly available laboratory techniques, such as flow cytometry (FC) and qRT-PCR, for CTCs identification.

Aim

The aim of the study was to standardize the conditions of CTCs identification with the use of FC and qRT-PCR. Sensitivity of FC and qRT-PCR were assessed in 2 models prepared from lung cancer cell lines suspended in healthy donors' whole blood.

Material and methods

Flow cytometry

Cells from the A549 lung cancer cell line (American Type Culture Collection, Manassas, VA, USA) were cultured under standard conditions: 37°C, 5% CO2 and 95% humidity in a culture medium that consisted of Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, Saint Louis, MO, USA) and Dulbecco's Modified Eagle's Medium_Nutrient Mixture F-12 Ham (Sigma-Aldrich, Saint Louis, MO, USA) with addition of Fetal Bovine Serum (Biochrom GmbH, Berlin, Germany) and Penicillin-Streptomycin-Neomycin Solution Stabilized (Sigma-Aldrich, Saint Louis, MI, USA). 5 ml peripheral blood samples were taken from 6 healthy volunteers into heparinized tubes. The first portion of peripheral blood from each donor was rejected to avoid sample contamination with epithelial cells. Subsequently, the blood sample was divided into 5 cytometric tubes (1.0 ml per tube) and then 5 different suspensions were prepared (0 cancer cells/ml, 10/ml, 50/ml, 100/ml and 200/ml) of cancer cells from A549 lung cancer cell line in healthy donor blood with the use of an electronic pipette (Eppendorf, Hamburg, Germany). Before preparation of the spikes, cancer cells had been counted with a Countess Automated Cell Counter (ThermoFisher Scientific, Waltham, MA, USA).

The next part of the experiment consisted of intrinsic molecule labeling with monoclonal antibodies (mAbs) and sample acquisition with the use of BD FACSCanto II Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Mouse anti-human pancytokeratin FITC mAb was used (Miltenyi Biotec, Bergisch Gladbach, Germany). We did not identify expression of EpCAM on the surface of our cancer cell line, which is why we used only pancytokeratin mAb. After 20 minutes of incubation with mAb in the dark, each sample was incubated for 10 min with 3 ml of BD FACS Lysing Solution (Becton Dickinson, Franklin Lakes, NJ, USA) diluted in deionized water (1:9) at room temperature in the dark. Subsequently, samples were centrifuged for 5 min with 500 × g acceleration and washed in Phosphate Buffered Saline w/o Ca^{2+}, Mg^{2+} (PAA Laboratories GmbH, Pasching, Austria). In the last step samples were acquired. It was important to minimize the Electronic Abort Rate to avoid losing epithelial cells, so samples were acquired with the lowest available velocity. In consequence, average time of acquisition for one sample was 3 hours.

Real Time qRT-PCR

In the Real Time qRT-PCR experiment, we used two lung cancer cell lines – the first one was A549 as in the flow cytometry experiment and the second one was H1975 (American Type Culture Collection, Manassas, VA, USA). They were cultured under standard conditions. The H1975 cell line was cultured in RPMI 1640 Medium (PAA Laboratories GmbH, Pasching, Austria) with Fetal Bovine Serum (Biochrom GmbH, Berlin, Germany) and Penicillin-Streptomycin-Neomycin Solution Stabilized (Sigma-Aldrich, Saint Louis, MI, USA). After digestion with the use of Accutase Cell Detachment Solution (Corning, NY, USA) cancer cells were labelled with mouse anti-human EpCAM APC mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) and mouse anti-human pancytokeratin FITC mAb (Miltenyi Biotec, Bergisch Gladbach, Germany). In the last step, spikes of cancer cells in healthy donor blood were prepared.

4 ml peripheral blood samples were taken from 6 healthy volunteers and placed into tubes with EDTA. The first portion of blood from each donor, as in the flow cytomeric model, was used for another procedure, because we wanted to avoid sample contamination with epithelial cells from the injection. Subsequently, the blood sample was divided into 5 eppendorf tubes (400 µl per tube) and then 5 different suspensions (0 cancer cells/0.4 ml, 10/0.4 ml, 50/0.4 ml, 100/0.4 ml and 200/0.4 ml) of cancer cells from A549 and H1975 lung cancer cell lines in healthy donor blood with the use of BD FACSAria III Cell Sorter were prepared. The population that we focused on was gated according to the scheme presented in Fig. 1.

In this model, cancer cells were sorted to 0.4 ml of blood because this is the volume dedicated for the MagCore HF16 Plus Automated Nucleic Acid Extractor (RBC Bioscience Corporation, New Taipei City, Taiwan) that was used for RNA isolation. The procedure of isolation was prepared according to the manufacturer's protocol with the use of kit number 601. RNA purification was prepared in an automated extractor to standardize the method. Subsequent steps were reverse transcription with the use of High Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham,

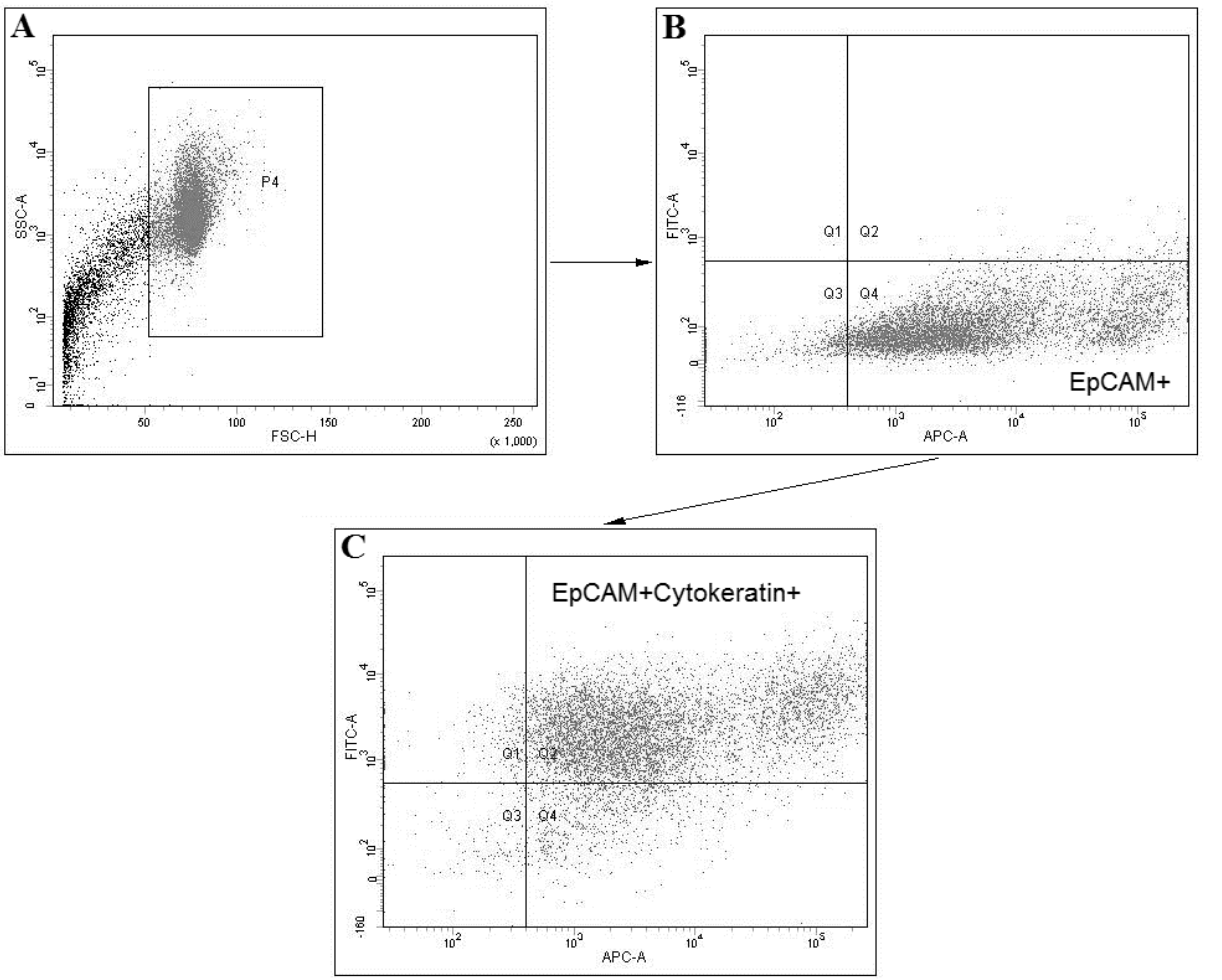

Fig. 1. Before sorting it is necessary to identify proper cell population. The scheme of gating lung cancer cell line cells in FC which were subsequently sorted to several spikes. A. P4 – H1975 lung cancer cell line population; B. Q4 – EpCAM positive H1975 lung cancer cell line population (extracellular staining); C. Q2 – EpCAM and pancytokeratin positive H1975 lung cancer cell line population (intracellular staining)

MA, USA) and the assessment of mRNA expression for KRT19 gene with the use of TaqMan Gene Expression Assay (ThermoFisher Scientific, Waltham, MA, USA). As a housekeeping gene control, we used TaqMan GAPDH Control Reagents (human) (ThermoFisher Scientific, Waltham, MA, USA). We pooled cDNA from '0' samples and we used ΔCt from samples '0' as a calibrator for normalization of qRT-PCR results. qRT-PCR was conducted with the use of Light Cycler 480 II Instrument (Roche, Basel, Switzerland).

Statistical analysis

Obtained flow cytometric and Real Time qRT-PCR data were collected in Microsoft Excel (Microsoft, Redmond, WA, USA) and analyzed by Statistica 13.0 PL software (StatSoft Polska, Cracov, Poland). The ANOVA Friedman test was used to verify the differences between the number of cells detected in several spikes. The significance of the differences between the predicted and detected cell number was assessed with the use of the Wilcoxon test. The percentage of samples with a lower number of detected CK+ cells than predicted in several spikes was calculated in frequency tables. We calculated correlation ratios (R2) and we described sensitivity of FC and Real Time qRT-PCR.

Results

Flow cytometry

Using FC, we observed an increasing number of detected CK+ events from the first spike "0" to the last one - "200". The number of events acquired in each sample was 3×10^6. In the negative control, the median value was 0 false positive cells. In the subsequent four tubes from "10" to "200", the median was 5, 43.5, 58 and 78 number of events, respectively. The coefficient of correlation between spiked cell number and detected cell number was R2 = 0.8795 (Fig. 2). Flow cytometry was characterized by 66.67%, 76.33%, 60.33% and 51.83% sensitivity in groups 10 to 200, respectively. Mean sensitivity was 63.79%.

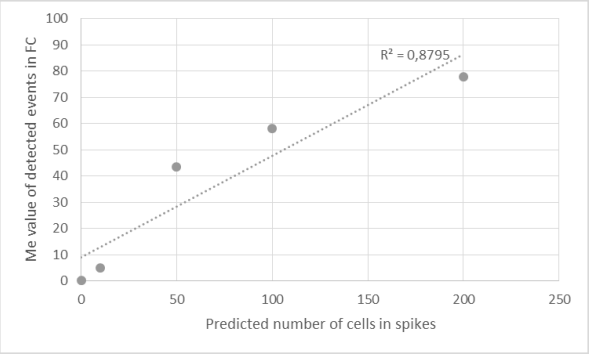

Fig. 2. The coefficient of correlation (R2) between spiked cell number and detected cell number in flow cytometry

According to the Wilcoxon test, there were not statistically significant differences between predicted and the real number of CK+ events in any group. However, dispersion in all spikes was very high (Table 1).

Table 1. A comparison between predicted and real numbers of CK+ events detected by flow cytometry

	Tube "10" [Me]	Tube "50" [Me]	Tube "100" [Me]	Tube "200" [Me]
Predicted number of cells	10	50	100	200
Detected number of cells	5	43.5	58	78

According to frequency tables, in 66.67% of spikes "10", fewer events were detected than predicted. In spikes "50" fewer events than predicted were detected in 50.00% of samples. Spikes "100" and "200" gave 62.50% and 75.00% fewer events than detected respectively.

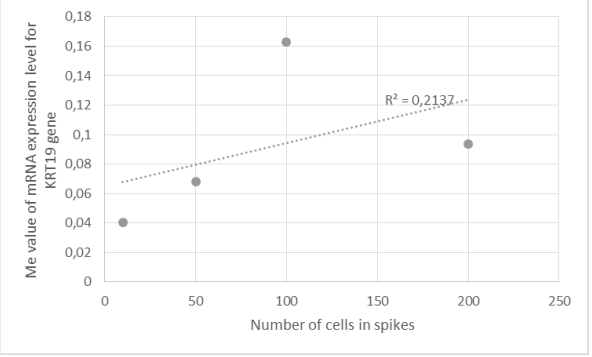

Fig. 3. Coefficient of correlation (R2) between spiked cell number and the level of mRNA expression for KRT19 gene in Real Time qRT-PCR

Real Time qRT-PCR

With the use of Real Time qRT-PCR, we were able to detect expression of mRNA for KRT19, but we did not observe a correlation between the increasing number of sorted cells in several spikes and the level of mRNA expression. There assumed increase of mRNA expression from the "10" to "200" spike in the model prepared with the use of the A549 cell line was not observed. The model with the H1975 cell line showed an increasing tendency in mRNA level, but the correlation ratio between the number of cells and mRNA level was very low (R2 = 0.2137) (Fig. 3).

Discussion

Flow cytometry, a conventional method available as standard equipment in many medical facilities, may be an attractive way for detection of CTCs according to Takao et al.[17] Rapid readout of routine measurements, the capability of multicolor analysis, and the fact that size information is included in the data are the main advantages of this method. The same results were reported by Lu et al.[18] With the previous magnetic depletion of CD45+ cells, they detected CTCs with 87.5% sensitivity. Our FC results were characterized by 63.79% mean sensitivity. However, an important fact is that the mentioned research that focused on CTCs detection with FC were equipped with additional devices for enriching the population prior to using FACS. In our experiment, we wanted to use FC without any supplementary devices to avoid generation of additional costs. Nevertheless, many other techniques for CTCs detection also require pre-enrichment.[13,19] Therefore, it is possible that even if this step generates additional costs and cell loss, pre-enrichment is necessary to obtain an acceptable method sensitivity.

qRT-PCR is very often used in CTCs detection techniques only for molecular characterization of isolated populations.[20-22] Fu et al. assessed in CTCs expression of hTERT mRNA level, while Bao et al. prepared multimarker qRT-PCR and assessed mRNA expression for 8 genes.[20,22] In some papers, qRT-PCR was used for CTCs identification, but only after pre-enrichment with the use of MACS or another negative enrichment techniques.[21,23,24] According to our experiment, where we isolated mRNA directly from whole blood, the correlation between the number of spiked cancer cells and expression of mRNA for KRT19 was weak (R2 = 0.2137). One of the problems with using qRT-PCR for CTCs detection is the lack of a standardized set of markers characterizing this population. Koren et al. prepared a similar experiment for CTCs detection with the use of qRT-PCR and obtained strong positive linear relationship between the number of spiked cells and the level of mRNA expression (R2 = 0.998), but they assessed the expression of mRNA for KRT7.[25] On the other hand, there was a study which compared qRT-PCR with the CellSearch System and in this experiment Politaki et al. assessed the level of KRT19 mRNA expression and obtained concordant results in qRT-PCR and in the CellSearch platform in samples from patients with metastases breast cancer.[26]

Although, they took 20 ml of blood from patient and isolated mRNA from PBMC.

The main assumption of our study was that the methodology should be simple, but it was connected with many limitations. The most critical point in the study was preparation of CTCs models in whole blood of healthy donors. We have to consider the influence of pipetting errors and dilutions during preparation of the spikes. Cancer cells were obtained from cell culture which were scrapped or enzymatically digested, suspended in PBS and counted. Because of the large number of cells, suspensions needed to be diluted. All these steps are a source of error, which Koren et al. eliminated by preparation of the spikes with the use of micromanipulator system.[25] Also, during acquisition in FC, we lost part of events and similarly during centrifugation in procedure of intrinsic molecules staining. The situation most often occuring in FC was that the number of detected positive events was underestimated. A high number of false negative events in FC is associated with the risk of omitting CTCs. On the other hand, in several samples, we detected also false positive events in FC. This could be associated with low precision during preparation of the spikes.

Different CTCs detection assays are created that vary considerably in the protocols and markers used for CTCs isolation, the volume of blood analyzed as well as the definition of positivity. Therefore, comparisons between experiments are difficult. As a consequence, despite the fact that in multiple reports the presence of CTCs has been correlated with patient outcome, CTCs have not as yet been approved for prevalent clinical use.

Some groups of researchers are focused on detection of CTCs in lung cancer patients to replace invasive diagnostic procedures with 'liquid biopsy'.[27-29] Nonetheless, the gold standards today are still chest X-ray, bronchoscopy and solid biopsy.

Conclusion

To conclude, we have many methods to identify circulating tumor cells in use today. However, if they are to become useful for clinicians, they need to be standardized and simplified. For proper evaluation of FC and qRT-PCR usefulness in CTCs detection, highly precise equipment seems to be essential. Such commonly available and flexible methods seem to be a more attractive tool in diagnostic procedures than special devices created for CTCs detection. However, a set of the most characteristic markers for CTCs needs to be defined.

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