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Review Article

Circulating tumor cells in breast cancer

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Abstract

Circulating tumor cell (CTC) measurement in peripheral blood of patients with breast cancer offers prognostic information. In this review, we will try to identify evidence that could be used for prognosis, predictive power to draw this tool to clinical utility. We reviewed 81 manuscripts, and categorized those in discovery datasets, prognostic factors in metastatic breast cancer, identification of clinical utility in early breast cancer and in novel approaches. With each patient responding differently to chemotherapy, more efficient markers would improve clinical outcome. Current CTC diagnostic techniques use epithelial markers predominantly; however, the most appropriate method is the measurement of circulating DNA. It has been hypothesized that micrometastasis occurs early in the development of tumors. That implies the presence of CTCs in nonmetastatic setting. The origin of stimulus for malignant transformation is yet unknown. The role of microenvironment as a stimulus is also being investigated. It has been shown that CTCs vary in numbers with chemotherapy. The markers, which are followed-up in the primary tumors, are also being studied on the CTCs. There is discordance of the human epidermal growth factor receptor-2 status between the primary tumor and CTCs. This review summarizes our current knowledge about the CTCs. With genetic profiling and molecular characterization of CTCs, it is possible to overcome the diagnostic difficulties. Evidence for clinical utility of CTC as prognostic and predictive marker is increasing. Appropriate patient stratification according to CTC determination among other tests, would make personalized cancer therapy more feasible.

Keywords: Cellsearch, circulating tumor cells, epithelial cell adhesion molecule, metastatic breast cancer, molecular profiling of circulating tumor cells

INTRODUCTION

Breast cancer is the most common malignant tumor in women. About 12.8% of women born today have a lifetime risk of being diagnosed with it. Based on the US National Cancer Institute Surveillance, Epidemiology, and End Results database, the age adjusted incidence rate of breast cancer was 123.8 cases/100,000 women/year.[1] The highest incidence (25.2%) was between 55 and 64 years. Despite advances in detection and treatment strategies, most cancer-related deaths are due to metastatic growth. The 5 years relative survival for a metastasized cancer is 24.3%. The period 1990-2007 saw a decline in death rates for breast cancer women both above and below 50 years (2.0% and 3.2%/year, respectively). Surgery, radiation, endocrine therapy, trastuzumab, cytotoxic therapy, bisphosphonates, and other biological agents have contributed to this.

Adjuvant therapy decreases the probability of recurrences and developing metastatic breast cancer (MBC), but not complete

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prevention of these events. The seeding of tumor cells to distant sites occurs in parallel, independent, niche related adaptation tactics.[2] Hence, treatment strategies targeting primary tumor profiling alone, may not be sufficient for a beneficial outcome. Metastases originate from a selected subpopulation of cells residing in a heterogeneous primary tumor. Most likely, this is a multifactorial step and depends on the interaction of tumor cells and the microenvironment.

Circulating tumor cells (CTCs) are isolated epithelial cells possessing the same or similar tumor characteristics as the primary cancer; these cells have been identified in the peripheral blood of solid tumor cancers, including breast cancer. Detection of these rare cells on a background of numerous hematopoietic cells poses a great challenge.

Cellsearch® (Veridex, Warren, NJ, USA), a commercially available test, identifies cytokeratin (CK) (8, 18, and 19) positive, CD45 negative and nucleated cells as CTCs.[3] Hence, analysis of targets identified on the primary tumor is now directed on the CTCs.

METASTATIC PATHWAY

The presence of tumor cells shed from the primary tumor could potentially predict the development of metastasis. It is likely that only a unique subpopulation of tumor cells possess the property to metastasize, e.g. cells that have the clonogenic potential and stem cell properties, a vast majority of other cells with "tumor cell" phenotype might not survive in the circulation.[4] To initiate the cascade, the tumor cells (growth and proliferation) must penetrate the basement membrane (local invasion) and surrounding tissue to enter into circulation (intravasation). Downregulation of epithelial cadherin and upregulation of neural cadherin breaks cell to cell adhesion (mesenchymal phenotype). Degradation of extracellular matrix (ECM) is mediated by matrix metalloproteinases and urokinase plasminogen activator system. Epithelial mesenchymal transition (EMT) has been suggested to induce these changes (self sufficiency of growth signals) (survival in circulation).[5] EMT related markers were found to be upregulated on CTCs and disseminated tumor cells (DTCs). Studies show the existence of a continuum in the development of CTC phenotypes ranging from epithelial to mesenchymal differentiation. EMT in CTCs, was analyzed in a study by Yu *et al*.[6] They found that CTCs in lobular type cancers were predominantly epithelial while those from triple negative and human epidermal growth factor receptor-2 (HER2) positive cancers were predominantly mesenchymal. Furthermore, there was a decline in mesenchymal CTCs in patients who show response to treatment. Role of transforming growth factor (TGF) beta in EMT has also been proposed.[7] EMT-related markers showed worse prognosis more accurately, than epithelial markers.[8] By including EMT markers, it is believed that most aggressive CTC phenotype can be identified. These cells show a potential to extravasation and adaptation to the microenvironment.[9]

Cancer cells in primary tumors are surrounded by complex microenvironment comprising of endothelial cells, stromal fibroblasts, and a variety of bone marrow derived cells, which include macrophages, neutrophils, and mesenchymal stem cells.[10] This microenvironment plays a critical role in metastasis. The proteases required for degradation of ECM and the cell surface proteins, are supplied by cancer cells, tumor associated macrophages or both. The tumor associated macrophage increases invasiveness through epidermal growth factors.[9] On entering the systemic circulation, CTCs are exposed to a hostile environment. Platelets form aggregates with tumor cells and protect them from natural killer cell mediated lysis.[10,11] Thus, high platelet count is associated with decreased survival. Conversely treatment with anticoagulants has been shown to decrease metastasis.

Certain tumors metastasize to specific organs. This tropism is mediated by chemokines like stromal cell-derived factor-1 and CCL21, which are expressed at sites of metastasis.[10] The corresponding receptor CXCR4 is overexpressed in the tumor cells. The niche (metastatic site) has to be primed for incoming tumor cells. Some studies say hematopoietic progenitor cells form the niche. On encountering the local microenvironment, DTCs revert back to an epithelial phenotype to allow adhesion and proliferation. Thus the secondary metastasis usually resembles the primary tumor phenotype. This reversal of EMT goes by mesenchymal epithelial transition (limitless replicative potential, tissue invasion and metastasis).[12] Finally, the newly formed micro-metastases in distant tissues exhibit a potential to neovascularization (angiogenesis) to create a new environment for survival in the host tissues. In another study, presence of tumor clumps in the peritumoral vascular spaces was found to have prognostic significance in addition to tumor size and axillary nodal status.[13]

The relapse rate of estrogen receptor (ER) positive breast cancer, 7 or more years after tumor removal is about 1%/year for 20 years without further adjuvant therapy and can be decreased by anti-estrogen treatment by about 50-60%.[14] Recurrence of tumor after a long disease free period has been noted as cancer dormancy. Dormancy is thought to be a period of growth restriction in CTCs. A study by Meng *et al*. found that 36% of patients in dormancy had CTCs without clinical evidence of disease.[15] They also found that CTC concentration remained steady in the subsequent blood tests

among these patients. CTCs have a very short half-life, which implies there must be a replicating population of tumor cells at secondary sites that replenish CTCs and keep them at low levels for many years. There are many hypotheses to explain dormancy: A small number of micrometastases, aging cells, immune surveillance, lack of angiogenic switch, interaction with the microenvironment causing cell cycle arrest.[16]

A large portion of tumor cells in bone marrow microenvironment have characteristics associated with stem cells, like the phenotype (aldehyde dehydrogenase 1, claudin low etc.,), resistance to chemotherapy and ability to differentiate into more mature cells.[17] There is accumulating evidence that the origin of cancer stem cells (CSCs) is linked to the EMT program.[18] In a study by Mani *et al*. to determine if EMT induced cells acquired stem cell traits, they found that most of the resulting cells with mesenchymal characteristics, acquired $CD44^{high}/CD24^{low}$ expression pattern, which is the phenotype ascribed to breast cancer stem cells (BCSCs). These cells are regulated by both intrinsic and extrinsic signals from the microenvironment.[19] Cytokines produced by endothelial cells and the HER2 gene are important regulators of CSCs. Tumor associated fibroblasts and macrophages and mesenchymal stem cells secrete interleukin 6 (IL6), IL8 and CXCL7, which in turn activates signal transducer and activator of transcription 3/nuclear factor-kappa B leading to self-renewal of BCSCs.[20] They have been shown to possess high levels of adenosine triphosphate binding cassette transporters, which can actively efflux drugs.[21] Furthermore, they are resistant to apoptosis, which can explain dormancy. In a prospective study, it was identified that small numbers of $CD44^{+/}CD24^{-/low}$ cells were able to give rise to tumors when passaged into immunocompromised mice.[22] Targeting these CSCs and their molecular survival pathway may decrease the clonogenic potential, survival and reactivation; this in turn, might lead to improved patients' outcome.

CIRCULATING TUMOR CELL DETECTION METHODS

In 1959, using cytological diagnosis, isolated cancer cells were identified in four cases by Nabar.[23] However, all the cytological methods were found to be low in specificity.

Owing to their rarity, blood needs to be enriched for detection, which can be carried out physically or biologically. Affinity methods based on the antigen expression, is the most commonly used strategy. Certain phenotypic characteristics have been used consistently to detect CTCs in peripheral blood. Positive selection of tumor cells with antibodies against epithelial cell adhesion molecule (EpCAM) is well developed. Subsequent immunocytochemistry detection is usually based on antibodies against CKs, broadly expressed by epithelial tumor cells and absent in hematopoietic cells.

The Cellsearch system is the first automated and Food and Drug Administration approved system for clinical use.[24] One major limitation of this method is considered to be the use of EpCAM expression as theoretically this is downregulated during EMT. Also all EpCAM expressing cells do not express CKs. Recent studies show that CTC markers may change over the course of therapy particularly with the use of biological therapy. Gain of mesenchymal markers such as vimentin and fibronectin was found to correlate with a worse prognosis than CKs.[25]

The major step stone in CTC detection using cell search system was the study published by Cristofanilli *et al*., which identified CTC as a significant predictor of progression free survival (PFS) and overall survival (OS) in MBC patients.[26] This was the first study to use a threshold level of five CTCs per 7.5 ml of blood to distinguish patients with an unfavorable prognosis from patients with favorable prognosis. Regardless of tumor histology, hormone receptor status, and HER2 status, CTC < 5/7.5 ml of blood predicted superior PFS and OS. The variation in its level following treatment has also been shown. However, no correlation was found between CTC detection and primary tumor response to neoadjuvant therapy.[27]

Another emerging technology within the past decade is the microfluidic platform. Also known as the CTC chip, it can handle very low blood volumes.[28] It consists of an array of microposts coated with EpCAM antibodies. The efficiency of cell capture is directly related to the duration of contact between the CTC and the micropost. Another new device from the same group is the Herring bone chip, which provides an enhanced platform.

Detection of viable CTCs/DTCs has been made possible by the EPISPOT assay.[29] Without direct contact with the target cells, CTCs are assessed based on the secreted or released proteins on a 48 h short-term culture. There is evidence for the release of full length CK 19 by viable tumor cells, which has been linked to the occurrence or progression of metastasis in breast cancer patients.[30]

There is a broad range of target genes used in messenger ribonucleic acid (mRNA) based assays to identify CTCs. Quantitative real time polymerase chain reaction (PCR) has improved detection of CTCs by allowing cut off values of marker transcript numbers where tumor cells deviate from normal cells.[31] Keratin 19 (KRT 19) is most commonly used target gene. The other available markers include mucin 1, mammoglobin, epidermal growth factor receptor (EGFR),

and KRT 20, which are also expressed at low levels in normal blood and bone marrow cells. The AdnaTest breast cancer select/detect uses reverse transcription-PCR to identify putative transcripts from EpCAM positive cells, isolated by immunomagnetic separation.[32] In a comparison study between AdnaTest and cell search, the concordance rate was found to be 73%.[33] However, there was variation in the positively reported cases by the two methods. In the DETECT study, it was shown that the CTC positivity assessed by AdnaTest had no association with clinical outcome parameters such as PFS or OS.[34]

In a study to identify microRNAs capable of discriminating CTC positive/negative MBC cases, it was found that miR200 family is an excellent indicator of the CTC status and also the prognosis.[35] Circulating DNA fragments carrying tumor specific sequence alterations are found in cell free fraction of blood.[36] These are released from the apoptotic cells. Analysis of loss of heterozygosity in circulating DNA may provide additional information and combination of such studies could improve the assessment of cancer stage and prognosis. Dawson *et al*. found a superior sensitivity of circulating tumor DNA (97%) when compared to CTCs (67%).[37,38]

METASTATIC BREAST CANCER

In metastatic disease, treatment is usually palliative; so treatment has to be judiciously planned to delay progression and induce clinical improvements, which in turn usually results in the alleviation of symptoms. In a study by Müller *et al*., CTCs were detected in 39.7% of patients with metastatic disease at first blood examination.[39] They analyzed blood samples from 47 randomly selected patients before and during systemic therapy. Only nine patients out of 47 had CTCs. But, none of these CTCs turned positive for proliferation associated Ki 67 antigen. This reinforces the heterogeneity of CTC population.

The prognostic power of these CTCs was established in many studies. Hayes *et al*. published follow-up data on the 177 patients from Cristofanilli study.[40] In this prospective longitudinal study, CTCs were assessed at specified intervals after the start of treatment. Patients with elevated CTCs (>5 CTC per 7.5 ml of blood) at any time point during the treatment had a rapid progression of disease (PFS: 1.3-3.6 months) and an early death (OS: 6.3-10.9 months). Patients who had >5 CTCs at baseline, but eventually dropped to <5 CTCs after therapy had the same risk of death as patients who never had elevated CTCs (median OS: 19.8 months). CTCs were found to be low in patients under remission. But, patients were receiving different treatments.

In breast cancer, the commonly used serum markers are carcinoembryonic antigen (CEA), CA27.29 and CA15-3. These are cell surface glycoproteins.[41] Study reported retrieval of 75% of patients with metastatic relapse by assessing CEA and CA 15-3. Addition of CTC increases detection rate to 90%. There was no clear superiority of CTC over other serum markers. Cristofanilli *et al*. showed that CTCs are not just a reflection of tumor burden, but cells with unique properties.[42] Their detection was independent of disease phenotype or line of treatment. In a multicenter study correlation was found between radiographic response and CTC variation after starting treatment.[43] But, imaging is not done as early as 3-4 weeks after initiation. Hence CTC assessment was found more feasible than imaging for monitoring treatment efficacy.

The clinical impact of CTCs in various immunohistochemical subtypes of breast cancer, was shown in a study by Giordano.[44] They found that baseline CTC enumeration had good prognostic value in all breast cancer subtypes except HER2+ cancer. In a study by Giuliano *et al*. the effect of different first line systemic treatment on the prognostic impact of CTCs were studied.[45] They had four different treatment groups-endocrine therapy, chemotherapy alone, chemotherapy plus bevacizumab (angiogenesis inhibitor) and chemotherapy plus HER2 targeting drugs. In both the groups with endocrine treatment and chemotherapy alone, high CTC was associated with a worse prognosis. On the other hand, patients in other two groups receiving either HER2 targeted treatment or biological agent did not maintain the negative prognostic value of high CTCs at the baseline, suggesting a therapeutic benefit from these agents.

High dose chemotherapy with hematological support is used to treat high-grade tumors. Immunocytologic and culture based detection methods revealed 10-16% tumor cell contamination in the peripheral blood stem cell (PBSC) hematopoietic support.[46-48] In a subsequent study to compare the incidence and quantity of contamination between PBSC and bone marrow, it was found that PBSC tumor involvement (22.5%) is less likely than bone marrow tumor cell involvement (70%).[49] Progenitor cells are mobilized from bone marrow by administering colony-stimulating factors. Are tumor cells also mobilized along with stem cells? In a subsequent study by Ross to compare the contamination between mobilized and nonmobilized grafts, there was no significant difference.[50] Glück *et al*. in their study found that the contamination dropped significantly after one cycle of induction chemotherapy but further cycles may not be beneficial.[51] The clinical significance of reinfused tumor cells is discussed controversially.[52-54] Most purging methods commonly employed deplete the graft of lymphocytes, which might increase the risk of recurrence.[55]

Tumor cells were found in the marrow in 30-60% of patients with metastatic disease, compared with only 1-2% in patients with primary disease. Several analyses have demonstrated that DTCs in Bone marrow at the time of initial diagnosis of breast cancer is a significant and independent prognostic factor of OS and breast cancer specific survival. Identification of micrometastasis at the initial presentation of breast cancer or after completion of adjuvant treatment could determine, which patient needs additional therapy.[56] In another study by Braun and Pantel, applying broad-spectrum anti-CK antibody A45-B/B3 for tumor cell detection, they found association of bone marrow micrometastasis with diagnosis of inflammatory breast cancer, distant metastasis and lymph node metastasis.[57] Most of these cells lack Ki67 positivity and seem to be resting G_0 phase. Hence they are likely to resist cytotoxic chemotherapy.

In a recent study to find the comparison between CTCs in peripheral blood and DTCs in bone marrow, it was found that the concordance rate increases from 69.4% in the primary situation to 75.6% during follow-up and 78.6% in metastatic disease.[58] Positivity for DTC was 23.1% at primary diagnosis, 31.7% during follow-up and 71% in metastatic situation. Correspondingly, CTC detection was 14.3%, 22% and 78.6% respectively. Bone marrow seems to be a stable homing site during cancer dormancy.

EARLY STAGE BREAST CANCER

In a nonmetastatic setting, the main treatment goals are complete eradication of the disease with reduction or prevention of recurrences. Neoadjuvant therapy, a concept where systemic treatment is given prior to definite surgery, is increasingly used in patients especially with larger tumors and clinically detectable lymph node involvement. No correlation between hematogeneous dissemination and lymphatic spread has been identified so far.[59] In one of the early studies to investigate micrometastasis in primary breast cancer patients, they found marrow involvement in 24% of node negative patients.[60] This percentage correlated with number of lymph node negative patients who relapsed within 5 years of presentation.

In a study by Lucci *et al*. to study the prognostic value of CTCs in early stage breast cancer, 73 patients had one or more CTCs, 29 patients had 2 or more CTCs and 16 patients had 3 or more CTCs per 7.5 ml of blood.[61] There was no correlation between primary tumor characteristics and detection of CTCs. Presence of CTC was associated with significantly shorter PFS. Hazard ratio increased with higher numbers of CTCs.

Krishnamurthy *et al*. conducted a study to evaluate the occurrence of DTCs in bone marrow and CTCs in peripheral blood and to find the correlation between their detection and the standard prognostic factors like tumor size, tumor histologic grade, ER status, progesterone receptor status, HER2 status and axillary lymph node status.[62] CTCs were present in 13 of 43 patients with T1 tumor and in 12 of 38 patients with T2 tumors. CTCs occurred in one of four patients with HER2 positive tumors and in 24 of 77 patients with HER2 negative tumors. CTCs occurred in 10 of 35 patients with lymph node positive disease compared with 15 of 46 patients with lymph node negative disease. There was no correlation between occurrence of CTCs and the standard prognostic factors. They suggested the possibility of independent modes of dissemination to different homing sites. The detection of CTCs and DTCs in lymph node negative early stage tumor questions the role of lymph node status in selecting patients for adjuvant chemotherapy.

In another study by Rack *et al*., 1767 patients with lymph node negative and node positive early breast cancer were evaluated for the presence of CTCs before and after treatment.[63] 10% of patients had >1 CTC before treatment, while 7% patients had >1 CTC after treatment. They found a significant correlation between presence of CTCs and lymph node positivity. Another study also reported correlation between CTC, bone marrow micro metastasis and primary tumor characteristics. A total of 38% (35/92) were CTC positive. Among these patients, 15 received neoadjuvant chemotherapy.[64] The authors found a positive correlation between HER2 status of primary tumor and detection of CTC.

The different molecular subtypes based on the receptors expressed by the primary tumor vary in their response to treatment and have independent clinical outcomes. Ignatiadis *et al*. in their study found that presence of CK 19 mRNA-positive CTCs predicted worse clinical outcome in the ER negative, triple negative and HER2 positive subgroups of patients.[65] The potential of CTCs to predict relapse and OS in early breast cancer patients may depend on timing of sampling, duration of follow-up and more importantly on the method of CTC detection. The detection of CTCs after adjuvant treatment is an independent adverse prognostic factor.

Amplification of the encoding gene c-erbB2 occurs in about 20-30% of early breast cancers.[66,67] HER2 positive CTCs are found to play a significant role in metastasis that one-time assessment may not be sufficient.[68] Several reports suggest that HER2 gene amplification may be acquired during progression of disease. In another study conducted

by Georgoulias in women with early stage breast cancer and HER 2 negative tumor, 51 (89%) of the 57 analyzed patients had HER2 expressing CTCs.[69] Trastuzumab is the first approved anti-HER2 agent used both in MBC as well as in early breast cancer, if HER2 positive. In a study by Pachmann *et al*., the benefit from 1 year of trastuzumab therapy in MBC lasted on average only for first 3 years.[70-72] CTC monitoring during trastuzumab treatment might point out patients who will do well. Now studies have shown that dual HER2 blockade by combining two or three agents with nonoverlapping mechanisms of action, improves cell death and tumor shrinkage in HER2 positive tumors.[73,74]

FUTURE DIRECTIONS

Nadal *et al*. analyzed five biomarkers in CTCs in non-MBC patients.[75] No correlation was found between EGFR, HER2 or topoisomerase 2A status of CTC and clinico-pathological characteristics. Anti-EGFR therapy can act over the entire HER-2 pathway downstream. The phosphatidylinositol 3 kinase pathway activated by transmembrane receptor tyrosine kinases' including epidermal growth factor family is encoded by PIK3CA gene.[76] In a recent study, laser capture microdissection analysis of some cases showed discrepancy in mutation of this gene within the tumor. Also, discrepancy was found between primary tumor and metastasis with a tendency towards gain of mutations in metastasis. CTC analysis in this direction may add to our knowledge about targeted therapy. Mammoglobin A is another specific molecular marker associated with poor disease free survival.[77]

Chimonidou *et al*. conducted a study to look for the status of tumor suppressor genes and metastasis suppressor gene on the CTCs.[78] They extracted the DNA from EpCAM positive cells. Using methylation specific PCR, the promoter sequences of cystin (CST6), breast cancer metastasis suppressor 1 (BRMS1) and SOX17 were analyzed. Methylation of these cancer related genes is an important biomarker for early detection and prognosis estimation. Cystin M (CST6) is an endogenous inhibitor of cathepsins L and B, which are downregulated in MBC. DNA hypermethylation in its locus impairs transcription. BRMS1 regulates multiple other genes leading to suppression of EGFR and downstream Akt signaling. SRY-Box 17 (sex determining region Y) regulates development and precursor cell function at least partly through repression of Wnt/β-catenin signaling pathway. Promoter region DNA hypermethylation, as in the earlier case, results in epigenetic silencing. CST6 promoter methylation in CTCs was observed in 10/56 patients with operable breast cancer and 10/27 patients with verified metastasis. Similarly BRMS1 and SOX17 promoter regions were highly methylated in CTCs.

A Powell *et al*. in their study divided CTCs into two major clusters based on their gene expression data.[79] Both the clusters shared some metastatic genes like the EMT associated genes. There was a high transcript level of PTEN gene in 83% of CTCs, which is inversely associated with TGF-beta expression. TGF-beta is an important regulator of breast development and its pathway is considered crucial in metastasis. Also, their data showed absence of cell adhesion protein CDH1 in CTCs.

Circulating tumor cell molecular profiling identifies diverse phenotypes, which require optimized treatment regimens to target all the subtypes of CTCs. Once the functional response of genes with higher level of transcription is identified in relation to clinical outcome, metastasis site and patient characteristics, new drugs can be developed. Recently, there has been a hypothesis about CTC hemodialysis in the postoperative course of breast cancer patients.[80] It needs to be validated in terms of curative effect and therapeutic periodicity. Circulating levels of extracellular domain of HER2 receptor can be monitored to detect recurrence and also to predict response.[81]

SUMMARY

Circulating tumor cell in MBC can be used as a tumor marker and to guide therapy decisions, more definite prospective studies are awaited. These will increase the predictive power of CTCs for therapy decisions. In early breast cancer, data are still immature for clinical use and need more validation studies. By the use of molecular markers on the CTCs themselves, it can become a powerful tool as predictive marker for therapy and also help decide upon which biological agents to use.

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