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Thymidylate synthase expression in circulating tumor cells: A new tool to predict 5-fluorouracil resistance in metastatic colorectal cancer patients

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Thymidylate synthase (TYMS) is an important enzyme for 5-fluorouracil (5-FU) metabolism in metastatic colorectal cancer (mCRC) patients. The search for this enzyme in circulating tumor cells (CTCs) can be a powerful tool to follow-up cancer patients. mCRC patients were enrolled before the beginning of 5-FU-based chemotherapy. The blood was filtered on Isolation by Size of Epithelial Tumor Cells (ISET), and the analysis of TYMS expression in CTCs was made by immunocytochemistry. Additionally, we verified TYMS staining in primary tumors and metastases from the same patients. There were included 54 mCRC patients and 47 of them received 5-FU-based chemotherapy. The median CTCs number was 2 per mL. We were not able to analyze immunocytochemistry in 13 samples (9 patients with absence of CTCs and 4 samples due to technical reasons). Therefore, TYMS expression on CTCs was analyzed in 34 samples and was found positive in 9 (26.5%). Six of these patients had tumor progression after treatment with 5-FU. We found an association between CTC TYMS staining and disease progression (DP), although without statistical significance ($P = 0.07$). TYMS staining in primary tumors and metastases tissues did not have any correlation with disease progression ($P = 0.67$ and $P = 0.42$ respectively). Patients who had CTC count above the median (2 CTCs/mL) showed more TYMS expression ($P = 0.02$) corroborating with worse prognosis. Our results searching for TYMS staining in CTCs, primary tumors and metastases suggest that the analysis of TYMS can be useful tool as a 5-FU resistance predictor biomarker if analyzed in CTCs from mCRC patients.

Colorectal cancer (CRC) was the third most commonly diagnosed cancer in both men and women in the last 3 years in the United States.[1–3] For metastatic patients, the strategy for treatment is mostly 5-Fluorouracil-based chemotherapy, which shows high efficacy in a subset of patients. However, even those patients can experience disease progression due to 5-FU resistance.[4]

TYMS is a constitutive enzyme that catalyzes the reductive methylation of deoxyuridine monophosphate (dUMP) by CH_2H_4folate to produce deoxythymidine monophosphate (dTMP) and H_2folate leading to DNA replication and repair.[5] An upgrade of resistance to the treatment has been often correlated to increased levels of TYMS in cancer cells.[6] Others have demonstrated that intratumoral TYMS mRNA expression levels (in paraffin-embedded tissues) are independent predictive markers of survival for 5-FU and oxaliplatin combination therapy.[7] Although the presence of high levels of TYMS presents poor outcome, it becomes hard to evaluate since there are heterogeneity among the population, methods and techniques as well. Furthermore, the best way to detect the presence of TYMS and its real value remains unclear[8] as there is not a consensus about the role of TYMS expression in mCRC published in the last 20 years. Thence, analyzing prospectively this enzyme in circulating tumor cells (CTCs) can be helpful to predict the treatment response.

CTCs are believed to be responsible to detach from primary tumor, to get into blood circulation and lastly to be the

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What's new?

Currently, the common treatment strategy for metastatic colorectal cancer patients is 5-Fluorouracil-based chemotherapy, which shows high efficacy in a subset of patients. Even those patients, however, can experience disease progression due to 5-FU resistance. There are indications that the DNA replication and repair enzyme thymidylate synthase (TYMS) may be involved. Here, the authors set to measure circulating tumor cells levels and search for TYMS staining to correlate these findings with clinical outcome. The results suggest that circulating tumor cells represent a powerful tool to follow up 5-FU resistance in metastatic colorectal cancer patients in real time, by TYMS expression analysis.

factor that leads to dissemination and metastasis.[9,10] Detection and counting of CTCs levels in patients with advanced or metastatic colorectal cancer has been shown to be an independent prognostic biomarker, both in terms of progression-free survival (PFS) and overall survival (OS).[11,12] Then, research involving CTCs has been strengthened, and given the prognostic information that CTCs provide (*e.g.,* monitoring the disease status and therapy response, understanding the metastasis process and resistance mechanisms), several methods were developed in order to find them.[13] Recent studies have shown that is possible to determine chemoresistance profile of CTCs.[14,15] However, Thymidylate synthase (TYMS) expression on CTC was not related to date.

Therefore, this study had as objective to verify CTCs levels and search for TYMS staining in these cells and in paraffin-embedded tissues from patients with metastatic colorectal cancer (mCRC) and to correlate these findings with clinical outcome.

Material and Methods

Patients and samples

Stage IV CRC patients were recruited at Clinical Oncology Department of A.C. Camargo Cancer Center, São Paulo, Brazil from July 2012 to December 2013. Blood was collected before the beginning of chemotherapy (diagnosis of metastasis or protocol change). All patients signed the informed consent form previously approved by our ethical committee (reference number: 1367/10). Patients who had submitted for any surgical procedure within 3 weeks before CTC detection were excluded. The clinicopathological data was obtained from medical record and response to treatment was evaluated by imaging examinations according to RECIST criteria (version 1.1). According to RECIST, disease progression was characterized by the tumor increase in size of >20% from smallest sum of diameters or appearance of one or more new lesions.[16]

CTC isolation

Basically, there are three approaches to detect CTCs: antibody-based capture assays, physical characteristic-based assays, such as ISET® (Isolation by Size of Epithelial Tumor Cells, Rarecells Diagnostics, France) technique, and functional assays.[17] Concerning these approaches, some authors published results comparing two methods (ISET and immunoaffinity), and showed that ISET detects more CTCs than immunoaffinity restricted methods in solid tumors.[18–20] In addition, ISET underwent technical and clinical validation.[21–23] For all these reasons, we decided to use ISET technology in our Center.

Each patient was submitted to collection of 8 ml of blood. Blood was collected on EDTA tubes and maintained under homogenization for up 4 hr at room temperature to avoid blood coagulation. The sample was filtered on ISET according to manufacturer's procedure[21] (Rarecells Diagnostics, Paris, France). Briefly, 8 ml blood was diluted 1:10 with the ISET filtration buffer, transferred to the ISET block and filtered through a polycarbonate membrane with calibrated, 8-μm-diameter, cylindrical pores. The ISET® system (Rarecells Diagnostics, Paris, France) is based on the principle that the majority of white blood cells are the smallest cells of the body and that CTCs are larger than 8 μm.[24] Thus, cells that have >8 μm in diameter were maintained on ISET membrane by negative pressure. After the filtration, membranes were washed once with phosphate-buffered saline (PBS), decoupled of the block, and allowed to air-dry. Membranes were stored at −20 °C until time of analysis. Cells were considered as CTCs if they have presence of hypercromatic nucleus, irregular shape, high cytoplasm nucleus ratio (>0.8) and cell size ≥12 μm.[22,25]

Immunocytochemistry in CTCs

The spots from ISET membranes were cut and submitted to immunocytochemistry (ICC) assay on 24-wells plate. Cells were hydrated with TBS 1X for 10 min and permeabilized with Triton X-100 for 5 min. From this step all incubations were rinsed with TBS 1X. Endogenous peroxides were blocked with 3% hydrogen peroxide in the dark for 15 min. Then, the spots were incubated with antibodies previously diluted on TBS 10% fetal calf serum for 1 hr with parafilm. The following antibodies were used: anti-TYMS, anti-CD45, anti-CD34 and CK-20. Information about the antibodies and positive controls (Fig. 1e) used in this study are available in Supporting Information Table 1. Anti-TYMS was used in order to verify if CTCs were expressing this resistance protein to 5-FU (Fig. 1d). Leukocytes and endothelial cells were identified by anti-CD45 antibody and anti-CD34 antibody respectively. Anti-cytokeratin 20 (CK-20) antibody was used to search for colorectal marker expression on CTCs. For CK-20 expression, we used HCT 116 cell line spiked in health blood donor as positive control (Supporting Information Fig.

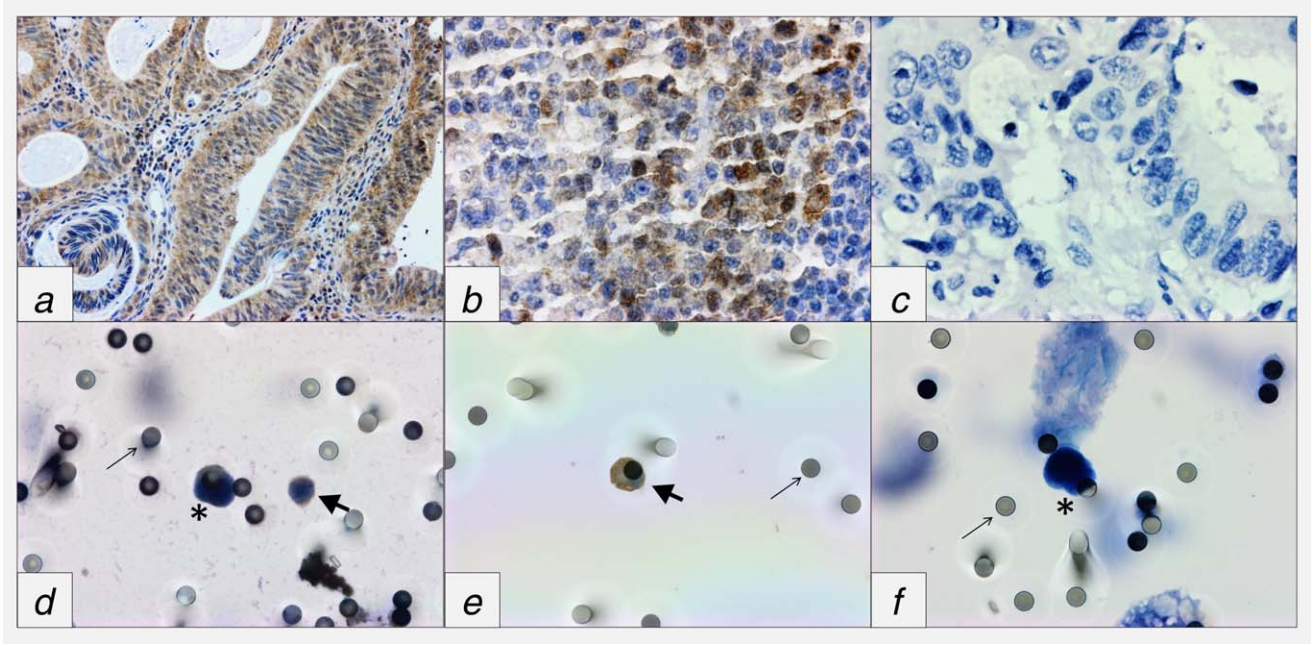

Figure 1. Immunostaining of Thymidylate synthase (TYMS). (*a*) Primary tumor tissue positive for TYMS. (*b*) Positive control, a normal palatine tonsil tissue. (*c*) Negative control, tumor tissue without antibody. (*d*) CTC TYMS positive. (*e*) Positive control, a white blood cell. (*f*) Negative control, a CTC without antibody. Thin arrows represent pores of ISET membrane, thick arrows show leukocytes and asterisks indicate CTCs. Images were taken at ×600 magnification using a light microscope (Research System Microscope BX61—Olympus, Tokyo, Japan) coupled to a digital camera (SC100—Olympus, Tokyo, Japan). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

1b). For negative control, a spot was evaluated without antibody (Fig. 1f and Supporting Information Fig. 1c).

After the antibody incubation, we used EnVision™+ Dual Link System-HRP (K4063, Dako) for 30 min and then, we incubated the spots for 10 min with DAB (SIGMAFAST# 3,3# - Diaminobenzidine tablets, Sigma–Aldrich). Following, cells were stained with haematoxylin and analyzed by light microscope (Research System Microscope BX61—Olympus). CTCs were quantified by 1 ml of blood and the count was performed as described by Krebs et al.[22]

Immunohistochemistry in specimens of primary tumors and metastases

The primary tumors' paraffin blocks were obtained by tissue bank archives (Department of Anatomic Pathology of A. C. Camargo Cancer Center). Slides obtained from these blocks were submitted to an immunohistochemistry (IHC) assay for anti-TYMS (Supporting Information Table 1). All reactions were accompanied by a positive control, in known positive tissue for TYMS, a normal palatine tonsil (Fig. 1b) and a negative control (removal of the primary antibody and withdrawal of the secondary complex) (Fig. 1c).

The histological section was deparaffinized in xylene, three baths of 5 min each, and rehydrated in alcohol 100%, 4 baths of 20 sec each, and then running water for 5 min. Antigen retrieval was done using either a citrate buffer (pH 6.0) and heated in a pressure cooker for 15 min.

The slides were placed in 3% hydrogen peroxide (10 V), three times for 5 min each, to block endogenous peroxides, and then washed in running water for 5 min. The sections were subjected to blocking nonspecific protein casein (Protein Block Serum-Free, DakoCytomation, Carpinteria) for 20 min at room temperature in a humid chamber.

The primary antibody was diluted in diluent containing 0.05 mol l^{-1} Tris-HCl buffer and 0.1% Tween 20 (antibody diluent with background reducing components, DakoCytomation, Carpinteria) and the slides were incubated at 4 °C, overnight (12–14 hr) in a humid chamber. After three washes with a PBS 1X buffer for 5 min each, the slides were incubated with a secondary antibody, containing a pool of anti-mouse, anti-rabbit or anti-goat antibodies using the *Kit Advance* ™ *HRP* (DakoCytomation, Carpinteria) for 1 hr in the darkroom, and washed with PBS three times for 5 min each. Staining was performed by using 3,3′ diaminobenzidine tetrachloride (DakoCytomation, Carpinteria). The specimens were counterstained with haematoxylin, dehydrated with alcohol and xylene and then mounted on slide.

The evaluation of the IHC study results was made for each antibody manually on Research System Microscope BX61—Olympus. We divided the results in absence of staining (0% stained cells) and presence of staining (≥1% stained cells).[26] All slides were reviewed by a pathologist of our Institution (VPA).

Statistical analysis

A descriptive analysis was done for each group of clinicopathological variables and of the received treatments. To

evaluate the differences among groups (those that expressed TYMS and those that did not express), the χ^2 test was used for variable categories and Fisher's exact test was used for small numbers. Survival curves were analyzed by Kaplan–Meier test and the difference between curves was calculated by Log Rank test. All statistical analysis was performed using the SPSS program for Windows, version 15. The p values were considered significant when ≤ 0.05.

Results

Patients

There were included 54 mCRC patients underwent palliative chemotherapy, all at stage IV of disease (100%). The median age was 59-years-old (30–81). The majority of patients were men (59.3%) and 81.5% were treated with FOLFOX or FOLFIRI. The most frequent localization of primary tumor was colon (66.67%) with metastases involving mostly the liver (64.82%) and of these, 24.07% were only in the liver and 40.75% were in liver plus other sites. The histological subtype adenocarcinoma was prevalent in all cases (100%) and the predominant histological grade was moderately differentiated (88.4%). Before CTC drawn, 10 patients (18.5%) were submitted to metastasectomy, and during CTC drawn, only 3 patients (5.55%) were submitted to an additional surgical procedure. CEA serum levels were collected at the approximate time of CTC collection and showed a median of 16.5 ng mL^{-1} (1.1–9,531) (data available in 49/54 patients). Patients' characteristics are shown in Table 1.

CTCs count

The median CTCs numbers detected by ISET in all patients was 2 CTCs mL^{-1} (0–31). Out of 54 patients, it was not possible to count two samples due to technical problems and 9 patients had absence of CTCs.

TYMS expression in CTCs, specimens of primary tumors, and metastases

Out of 47 patients that could be tested for TYMS expression on CTCs, because they were treated with 5-FU, we made ICC in CTCs only in 34 samples. We were not able to test the protein expression in CTCs from 13 patients because they did not have sufficient material (absence of CTCs on ISET membrane spots ($n = 9$) and material lost due improper technique ($n = 4$)). Among the 34 patients, two received 5-FU alone (5.88%), 20 received 5-FU in combination to irinotecan (58.82%) and 12 received 5-FU in combination to oxaliplatin (35.30%). Concerning TYMS expression on CTCs, 9 (26.5%) patients were found positive with cytoplasmatic expression. Six of these patients had tumor progression according to RECIST criteria,[16] in a median follow-up of 7.9 months (minimum of 36 days and maximum of 19.5 months) after the beginning of chemotherapeutic treatment (Supporting Information Fig. 2; Table 2). We performed also IHC in 29 primary tumor samples (26 positive *vs.* three negative) and 16 metastasis samples (15 positive *vs.* one negative).

Table 1. Colorectal cancer patients' clinicopathological characteristics

Variable	No.	%
Total number of patients	54	
Age at entry study, years		
Median (range)	59 (30–81)	
Gender		
Male	32	59.30
Female	22	40.70
Location of primary tumor		
Colon	36	66.67
Rectum	17	31.48
Colon and rectum	1	1.85
Histological grade (data available in 43/54 patients)		
Well-differentiated	5	11.60
Moderately differentiated	38	88.40
Location of metastases		
Hepatic	13	24.07
Hepatic and extra-hepatic	22	40.75
Other except hepatic	19	35.18
Treatment		
FOLFIRI	27	50.00
FOLFOX	17	31.50
5-FU	3	5.55
Other	7	12.95
Metastasectomy pre CTC drawn		
Yes	10	18.50
No	44	81.50
Additional surgery during CTC drawn		
Yes	3	5.55
No	51	94.45
Cetuximab		
Yes	11	20.40
No	43	79.60
Histological type		
Adenocarcinoma	51	94.45
Tubular adenocarcinoma	2	3.70
Mucinous adenocarcinoma	1	1.85
Median CTC/ml number (range)		
Baseline (52/54)	2 (0–31)	
Median CEA serum level (ng/ml) (range)		
Baseline (49/54)	16.5 (1.1–9,531)	

Abbreviations: CTC, circulating tumor cells; CEA, carcinoembryonic antigen.

Because of heterogeneity found inside some tumor tissue samples (Figs. 2*a*, 2*b* and 2*c*) as well in metastases tissue (Figs. 2*d*, 2*e* and 2*f*), we dichotomized the IHC results as

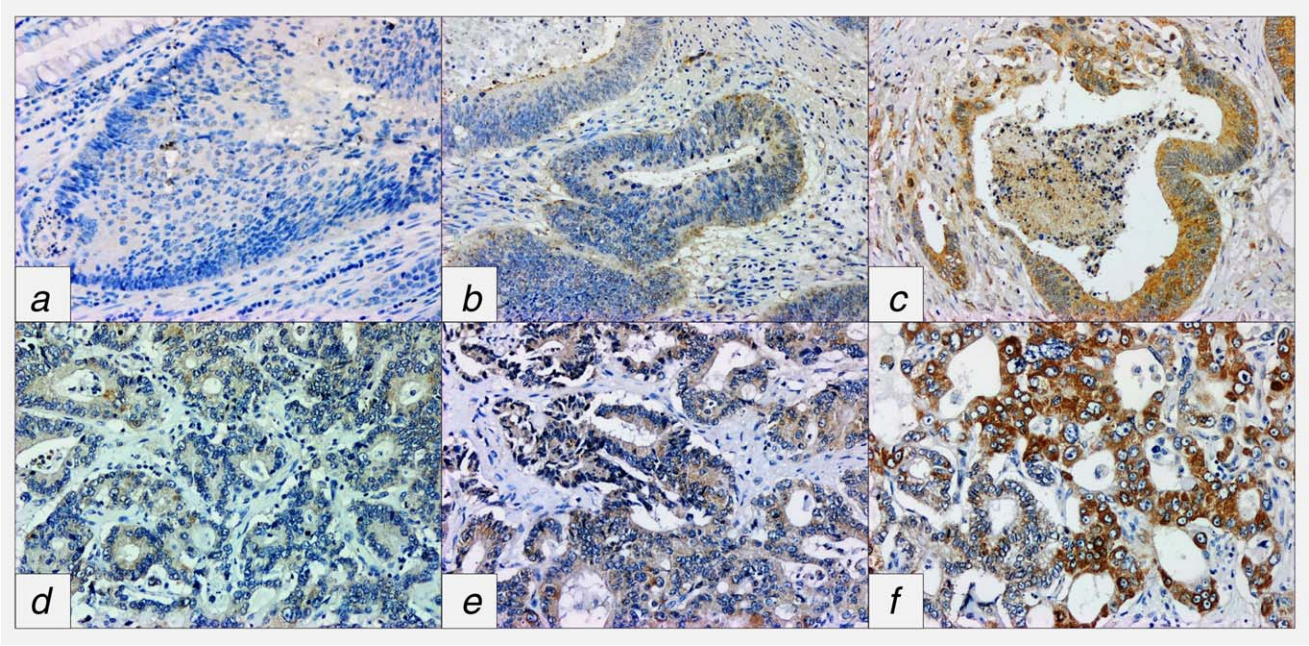

Figure 2. Immunostaining showing heterogeneity of TYMS expression in the same sample. Figures (*a*), (*b*) and (*c*) present primary tumor from the same patient with no expression, moderate and strong expression of TYMS respectively. Figures (*d*), (*e*) and (*f*) present metastasis tissue from another patient with different grades of staining (weak, moderate and strong expression of TYMS respectively). Images were taken at ×200 magnification using a light microscope (Research System Microscope BX61—Olympus, Tokyo, Japan) coupled to a digital camera (SC100—Olympus, Tokyo, Japan). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Table 2. Clinical features of colorectal cancer patients with TYMS protein expression on CTCs

Patients	Gender	Primary tumor localization	CTC TYMS positive (1 spot)[1]	CTC count at baseline (1 ml)	Disease progression	Time of progression (months)[2]
1	Male	Colon	2	3	No	–
2	Male	Colon	1	19	Yes	9.28
3	Male	Rectum	9	5	Yes	9.11
4	Male	Rectum	10	11	Yes	5.23
5	Male	Colon	3	2	Yes	12.27
6	Male	Colon	8	1	No	–
7	Female	Rectum	1	6	Yes	8.42
8	Male	Colon	39	14	No	–
9	Male	Rectum	1	12	Yes	1.48

Abbreviations: CTC, circulating tumor cell; TYMS, thymidylate synthase.
[1]Cells were counted as positive in one spot of the membrane is corresponding to ~0.8 ml blood, considering that we collected 8 ml of blood and that the membrane has 10 spots.
[2]Time of disease progression from the date of the first blood collection.

absence of staining (0% stained cells) and presence of staining (≥1% stained cells). Among patients with CTC TYMS positive and DP, three had also tumor tissue positive (Fig. 1a). Patients who had CTC count above the median (2 CTCs mL^{-1}) showed more CTC TYMS expression ($p = 0.02$; Table 3). This find can be better visualized in Table 2. Interestingly, there are patients with high CTC counting and with few cells stained for TYMS (patients 2 and 9) and the inverse is also true, patients with low CTC counting and with intense TYMS staining (patients 6 and 8). By χ^2 analysis, CTC TYMS positivity was persistent, but not significant in patients who had DP ($p = 0.07$), which means that the event (DP) occurred in 66.7% of patients CTC TYMS positive against 32% of patients CTC TYMS negative (Table 3). The same was not observed in primary tumors tested (53.8% *vs.* 66.7% respectively, $p = 0.67$) and neither on material from metastasis tested (60% and 100% respectively, $p = 0.42$). The average time of Progression-Free Survival (PFS) for all patients was

Table 3. Description and percentages of TYMS expression in the three sites analyzed, its association with disease progression and with CTC count

	Disease progression			CTC count		
TYMS analysis (n)	No (%)	Yes (%)	*p* values	<2/ml	≥2/ml	*p* values
TYMS staining in CTCs (27)						
Negative (25)	17 (68)	8 (32)	0.07	15 (60)	10 (40)	0.02
Positive (9)	3 (33.3)	6 (66.7)		1 (11.1)	8 (88.9)	
TYMS staining in primary tumors (28)						
Negative (3)	1 (33.3)	2 (66.7)	0.67	2 (66.7)	1 (33.3)	>0.99
Positive (26)	12 (46.2)	14 (53.8)		12 (50)[1]	12 (50)[1]	
TYMS staining in metastases tissues (16)						
Negative (1)	0 (0)	1 (100)	0.42	0 (0)	1 (100)	0.46
Positive (15)	6 (40)	9 (60)		8 (57.1)[1]	6 (42.9)[1]	

Abbreviations: CTC, circulating tumor cell; TYMS, thymidylate synthase.
[1]Two patients tested to TYMS in primary tumor have no CTC count, and one of them have tested TYMS in metastasis tissue also.

Table 4. Comparison of TYMS expression in CTCs, primary tumors and metastases from colorectal cancer patients

Gender	CTC TYMS expression	Tumor Tissue TYMS expression	Metastasis Tissue TYMS expression
Male	Yes	Yes	Yes
Male	Yes	Yes	Yes
Female	Yes	No	Yes
Female	No	Yes	No
Female	No	Yes	Yes
Male	No	Yes	Yes
Male	No	Yes	Yes
Male	No	Yes	Yes
Female	No	Yes	Yes

Abbreviations: CTC, circulating tumor cell; TYMS, thymidylate synthase.

10.21 months (95% CI 8.07–12.35). Patients who had CTC expressing TYMS had poor PFS in relation to those without TYMS expression (8.9 *vs.* 10.3 months respectively, $p = 0.42$). For patients with primary tumor TYMS positive and negative we found the same mean time of DP (9.4 *vs.* 9.3 months respectively, $p = 0.92$). On the other hand, we found an inverse correlation between negative and positive TYMS metastasis expression and PFS 3 *vs.* 10.35 months respectively, $p < 0.01$). These controversial results can be explained by the small subset of patients that had metastasis tissue negative (n= 1). We could compare TYMS expression from primary tumor, CTCs and metastasis in nine patients and observed that there were fairly similarity between primary tumor and metastasis (77.8%). Furthermore, we found more similarity between CTCs and metastasis (44.4%) than CTCs and primary tumor (22.2%). Agreement of TYMS expression among CTCs, primary tumor and metastasis were observed in two (22.2%) samples (Table 4).

All cells with malignant morphology were negative for endothelial and leukocytes markers (CD34 antibody and CD45 antibody respectively), and we also found 14/30 (47%) of CK-20 positivity (Supporting Information Fig. 1a).

Discussion

There are many studies in literature demonstrating prognostic and predictive values of TYMS presence in primary CRC, however, this rule as a marker of progression is not known.[27–31] A Systematic Review[8] analyzed hazard ratio of TYMS levels in 20 studies involving 887 advanced and 2610 localized CRC patients and concluded that low levels of TYMS seems to mean longer OS. van Triest *et al.*[32] assessed biochemically TYMS and Thymidine phosphorylase (TP) levels in 32 fresh frozen CRC samples and found that TP activity was two- to three-fold higher in the tumor tissue than in normal tissue but did not correlate with Dukes' stage, differentiation grade or angio-invasion. They also assessed IHC in paraffin-embedded tissues of the same patients and no correlation was found between TYMS staining and tumor histology, Dukes' stage or angio-invasion. A recent Chinese study[33] including 100 CRC patients demonstrated by quantitative RT-PCR significant association among higher TYMS levels and lymph node metastasis ($p < 0.001$), suggesting TYMS as an independent prognostic factor for recurrence and survival. Allegra *et al.*[34] investigated association between TYMS, Ki67 or p53 IHC with Disease-free survival (DFS) and OS and found no significant result. Similarly, a meta-analysis[35] analyzed 17 studies including 2,893 stage II and III CRC patients treated with surgery or adjuvant chemotherapy and concluded that TYMS expression does not predict DFS none OS.

Our results about the influence of TYMS expression on PFS in primary tumors and metastases were controversial. As related previously,[36] there are differences between areas inside the tumor and metastasis staining. Because of this intratumor heterogeneity observed in our samples (areas intensely, moderately and weakly stained in the same tissue) (Fig. 2),

we decided to analyze only positivity without measurement of staining as a manner to reduce bias. Even with this analysis we could not conclude about the role of TYMS in our tissues and metastases samples.

There is nothing on literature about the presence of TYMS on CTCs and its clinical impact in mCRC patients. In our sample, even with our low percentage of patients positive for TYMS in CTCs (26.5%), DP was strongly related to this Group (6/9) when compared with those negative for TYMS expression (8/25). Corroborating our findings, a previous case report[37] described that TYMS plays an important role in treatment resistance in Non-Small Cell Lung Cancer (NSCLC) by detecting this enzyme in CTCs. The authors found an association between high TYMS expression and poor clinical outcome in Pemetrexed-based treatment and theorize that this analysis act as a surrogate for tumor biopsies. Interestingly, we also found an association between number of CTCs (per mL) and expression of TYMS. CTCs expressing this marker were found more frequently in patients with higher number of these cells (more than 2 CTCs/mL; $p = 0.02$).

One of the points of resistance to treatment is the upregulation of drug-targets such as TYMS. There are two concepts on literature concerning drug resistance: intrinsic and acquired. The first one refers to factors that preexist on tumor constitution and will cause resistance to the target therapy; it is an "innate" tumor resistance. Acquired resistance concept proposes an induction of resistance during the treatment, which can be by activation of other escape pathway that will compensate the function as well by mutation of drug target among other adaptive responses.[38,39] The last kind of resistance affects a subset of cells. That is the reason why many tumors begin responsive and acquires resistance after a while. Our result with TYMS staining in CTCs reflects the "acquired" tumor resistance. In addition, the heterogeneity of expression of this marker in CTCs reflects the existence of subclones of resistant cells, as we found staining only in some CTCs of the same patient.

In our samples, to confirm tumor origin of CTCs, we used anti-CK-20 antibody, which is a marker of CRC[40] and we found only 47% of positivity (Supporting Information Fig. 1a). This shows the plasticity of these cells, as CTCs from well and moderately differentiated adenocarcinoma expressed more CK-20 in relation to those from undifferentiated adenocarcinoma. Lecharpentier *et al.*[41] investigated epithelial and mesenchymal markers in CTCs from 6 NSCLC patients and found co-expression of these markers in all samples. They also found three patients with CTCs expressing only vimentin and none expressing only keratin. Another found that points to the plasticity of these cells in our sample, is the difference found in expression of TYMS between primary tumors, CTCs and metastases (Table 4). We could compare TYMS expression from these three sites in 9 patients and observed that there were more similarity between primary tumors and metastases (77.8%) than between CTCs and metastases (44.4%) or CTCs and primary tumors (22.2%). The agreement among the three sites was found only in two samples (22.2%). Similar result was published by Mostert *et al.*[42] when comparing KRAS status in biological material from CRC patients. This group found an agreement of KRAS mutation between CTCs and primary tumors of 2.32% and between CTCs and metastases of 9.3%. Likewise, they believe that those results represent the tumor heterogeneity and reinforce the importance of mutation or protein expression analysis in CTCs, as these cells seem to be the real time reflection of the disease. In addition, although we did not reach a statistical significant result (Table 3), our findings with TYMS expression and DP showed that, probably, only CTCs are suitable to evaluate clinical outcome.

Finally, concerning isolation and characterization of CTCs, there are basically three kinds of methods to separate these cells: immunological (based in capture antibodies), assays based on physical properties of cells and the functional assays.[17] ISET System is a physical-based assay that consists in separating cells by size. One of the advantages of this method in comparison with immunomagnetic methods, among them, the CellSearch® System (Veridex, LLC), still the only method approved by FDA,[11,43] is that it isolates CTCs in a marker independent manner. Therefore, if circulating cells are passing through epithelial-to-mesenchymal transition and are downregulated of epithelial markers (EpCAM and citokeratin (CK)), these cells will be captured by ISET.[22] Even the functional methods require an initial enrichment step, which generally is made by using anti-EpCAM and anti-CK antibodies. Furthermore, by ISET is possible to capture Circulating Tumor Microemboli (CTM). It is already well established in literature that these CTMs are associated with higher tumor aggressiveness, leading to worse prognosis.[44,45] Another advantage of ISET is that it allows morphological observation of the cell, which in itself is already an identification factor. Hofman *et al.*[46] analyzed morphologically circulating cells with non-hematological characteristics (CNHCs) in different types of malignant tumors, non-malignant diseases, non-tumor diseases and healthy volunteers, and showed that cytomorphological analysis are relevant if it concerns isolated CTCs. They suggested that the methods to detect these cells should be combined to better reflect their presence, and if CTCs are in fact, malignant.

There are many studies comparing the ability of CellSearch® System (Veridex, LLC) and ISET® Technique in capturing CTCs.[19,20,22,43,47] In summary, it appears that ISET can captures larger amount of tumor cells than CellSearch system, without risk of overestimates counts, a risk that CellSearch system presents, because it can capture other non-malignant cells expressing EpCAM and CK.[43] De Giorgi *et al.*[48] showed that by ISET System CTCs were not find neither in the control group (healthy subjects) nor in a group of patients with melanoma *in situ*. They investigated CTCs also

in patients with invasive melanoma and metastatic melanoma, and found CTCs in 9 and 62.5%, respectively ($p < 0.001$). This demonstrates again, the high sensitivity and specificity of this method. In addition, by ISET is possible to perform immunocytochemical assays, allowing us not only the visualization of proteins that are involved in the entire metastasizing process,[49] but also molecular assays such as FISH, pirosequencing, CGH and Next Generation Sequencing. However, this technique is nonautomated and depends of a quick processing. After the blood collection in EDTA tubes, the blood must be maintained under homogenization and processed within 4 hrs at room temperature. The CellSearch system does not have the time or the homogenization as limiting factors, since it uses a collection tube (Veridex®) that keeps CTCs feasible up to 72 hours.[20,50]

By analysis of our results with TYMS expression in CTCs, it seems that these cells can show the current disease status and maybe can be used as a novel predictor biomarker of 5-FU resistance in mCRC patients. All clinical management of cancer is based on analysis of tissue sample, which is often collected years before metastasis development, or advance of disease. The detection of CTCs is a non-invasive method, based on a simple blood collection. We suggest further studies in order to verify predictive value of CTCs TYMS expression in a large cohort, including all stages of the disease.

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