DETECÇÃO DE CÉLULAS TUMORAIS CIRCULANTES EM PACIENTES COM CÂNCER COLORRETAL E SUA CORRELAÇÃO COM EVOLUÇÃO CLÍNICA TUMORAL

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”Foi o tempo que dedicastes à tua rosa que a fez tão importante” (Antoine de Saint-Exupéry)

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RESUMO

Abdallah EA. **Detecção de células tumorais circulantes em pacientes com câncer colorretal e sua correlação com evolução clínica tumoral**. São Paulo; 2015. [Dissertação de Mestrado-Fundação Antônio Prudente].

O câncer colorretal (CCR) é atualmente a terceira neoplasia mais comum nos homens e a segunda nas mulheres, causando, em ambos os gêneros, aproximadamente 694 mil mortes no mundo. Aproximadamente 60% dos pacientes apresentarão metástase, sendo 30% destas, ao diagnóstico. Considerando que metástases à distância são responsáveis por 90% das mortes por câncer, é importante que novos biomarcadores sejam estudados. Tem sido demonstrado na literatura que a presença de Células Tumorais Circulantes (CTCs) está relacionada à pior prognóstico em diversas neoplasias, inclusive no CCR metastático (CCRm). Ainda, sabe-se que a falha no tratamento quimioterápico de CCR tem relação direta com resistência a drogas. Por este motivo, há necessidade em detectar os mecanismos relacionados a este evento. **Objetivo:** Avaliar os níveis de CTCs de pacientes com CCRm e sua correlação com a evolução clínica tumoral, além de avaliar nas CTCs e nos tecidos tumorais a expressão de proteínas associadas à resistência ao tratamento. **Métodos:** Foram coletados 8 mL de sangue periférico de pacientes com CCRm antes do início do tratamento quimioterápico e após 60 e 120 dias. O sangue foi filtrado no dispositivo ISET e as CTCs isoladas foram submetidas à técnica de imunocitoquímica (TYMS, MRP1, MRP4, ERCC1), buscando encontrar o perfil de resistência ao tratamento. **Resultados:** Foram analisadas CTCs no sangue de 54 pacientes com CCRm. Observamos que pacientes que tiveram CTCs acima da mediana na primeira coleta (baseline) (CTC1+) e abaixo da mediana na segunda coleta (follow-up) (CTC2-) tiveram melhor sobrevida livre de progressão (SLP) do que o inverso: CTC1- e se tornaram CTC2+ (14,7 vs. 6,9 meses; *P*= 0,06). Encontramos associação entre

expressão de TYMS nas CTCs e progressão da doença, apesar de falta de significância estatística (*P*= 0,07). A expressão da proteína MRP1 nas CTCs foi associada à pior SLP comparada aos pacientes com CTCs negativas (2,1 vs. 9,1 meses; *P*= 0,003). **Conclusão:** A avaliação da cinética das CTCs pode ser promissora na avaliação clínica de pacientes com CCRm. A análise de TYMS nas CTCs pode ser uma ferramenta útil como biomarcador de resistência a 5-Fluorouracil, do mesmo modo que a expressão de MRP1 nas CTCs foi preditora de resistência a irinotecano nos pacientes com CCRm.

SUMMARY

Abdallah EA. **[Detection of circulating tumor cells in colorectal cancer and its correlation with clinical outcome]**. São Paulo 2015. [Dissertação de Mestrado-Fundação Antônio Prudente].

Colorectal cancer (CRC) is the third most common type of cancer in man, and the second in woman, resulting, in both genders, approximately 694 thousand deaths worldwide. Approximately 60% of these patients will present metastasis; 30% of them at diagnosis. Taking in consideration that 90% of causes of cancer death is due to distant metastases, it is important to study new biomarkers. It has been shown in the literature that the presence of Circulating Tumor Cells (CTCs) is related to worst prognosis in many tumors, including CRC. The fail in chemotherapeutic treatment of CRC is directly related with drug resistance. For this reason, there is a need to detect the mechanisms related to this event. **Objective:** To evaluate CTCs levels from metastatic colorectal cancer (mCRC) patients and its correlation with clinical outcome. We aimed also to evaluate in CTCs and in tumor tissues the expression of proteins associated to treatment resistance. **Methodology:** It was collected 8 mL of peripheral blood from mCRC patients before the beginning of chemotherapeutic treatment and after 60 and 120 days. The blood was filtered in ISET device, and isolated CTCs were submitted to immunocytochemistry assay (TYMS, MRP1, MRP4, ERCC1), trying to find treatment resistance profile. **Results:** CTCs were analyzed from 54 mCRC patients. We observed that patients whose had CTCs above the median at baseline (CTC1+) and under the median at follow-up (CTC2-) had better progression free survival (PFS) than the inverse: CTC1- and became CTC2+ (14.7 vs. 6.9 months; P= 0.06). We found an association between TYMS expression in CTCs and disease progression, although without statistical significance (P= 0.07). The expression of MRP1 in CTCs was associated to worse PFS when compared to patients with CTCs MRP1 negative. **Conclusion:** The assessment of CTC kinetics can be promising in clinical evaluation in mCRC patients. TYMS analysis in CTCs can be a useful tool as 5-Fluorouracil resistance biomarker, likewise that the expression of MRP1 in CTCs was predictor of irinotecan resistance in mCRC patients.

LISTA DE ABREVIATURAS

5-FU	5-Fluorouracil
ABC	*ATP-Binding Cassete*
AJCC	*American Joint Commission on Cancer*
CCR	Câncer Colorretal
CCRm	Câncer Colorretal metastático
CPNPC	Câncer de Pulmão Não Pequenas Células
CTCs	Células Tumorais Circulantes
CTM	*Circulating Tumor Microemboli*
dCK	*Doxycytidine kinase*
DTCs	*Disseminating Tumor Cells*
dTTP	*2'-deoxythymidine 5'-triphosphate*
dTMP	*2'-deoxythymidine 5'-monophosphate*
dUMP	*2'-deoxyuridine 5'-monophosphate*
dUTP	*2'-deoxyuridine 5'-triphosphate*
EGFR	*Epidermal Growth Factor Receptor*
EMT	*Epithelial to Mesenchymal Transition*
ERCC1	*Excision Repair Cross Complementation 1*
FDA	*Food and Drug Administration*
FdUMP	*5-fluoro-2´-deoxyuridine -5´-monophosphate*
Hent1	*Human equilibrative nucleoside transporter-1*
HNPCC	*Hereditary Nonpolyposis Colorectal Cancer*
INCA	Instituto Nacional de Câncer
ISET	*Isolation by Size of Epithelial Tumor Cells*
MDR	*Multidrug Resistance*
MRP1	*Multidrug Resistance-associated Protein 1*
MRP4	*Multidrug Resistance-associated Protein 4*
PAF	Polipose Adenomatosa Familiar
P-gp	P-glicoproteína
SG	Sobrevida Global
SLP	Sobrevida Livre de Progressão
SLR	Sobrevida Livre de Recorrência
TYMS	*Thymidylate Synthase*
VEGF	*Vascular Endothelial Growth Factor*

ÍNDICE

1 INTRODUÇÃO

O Câncer Colorretal (CCR) é considerado a terceira neoplasia mais comum em homens e a segunda em mulheres. Esses valores representam 10% e 9,2%, respectivamente, entre todos os tipos de câncer. A incidência de CCR foi aproximadamente de 1,36 milhões de novos casos, causando 694 mil mortes no mundo em 2012. Cerca de 55% de todos os casos de CCR ocorrem em regiões mais desenvolvidas do mundo (FERLAY et al. 2012). No Brasil, de acordo com o Instituto Nacional de Câncer (INCA), a estimativa para 2014 foi de 15.070 casos novos de CCR em homens e 17.530 em mulheres (risco estimado de 15,44 e 17,24 a cada 100 mil homens e mulheres, respectivamente) (Ministério da Saúde 2014). Segundo o Registro de Câncer de Base Populacional da Universidade de São Paulo (RCBP-SP), foram notificados, em 2011, 1576 novos casos de CCR para homens e 1652 para mulheres no município de São Paulo (São Paulo. Registro de Câncer de São Paulo 2011).

Os principais fatores de risco à aquisição de CCR são: idade, hábitos dietéticos, estilo de vida e síndromes hereditárias. Pacientes com mais de 50 anos, com alta ingestão de carne e baixa ingestão de fibras, inatividade física, etilismo, tabagismo têm maior chance de desenvolver CCR. São condições hereditárias frequentemente associadas à CCR: a polipose adenomatosa familiar (PAF); o câncer colorretal hereditário sem polipose (HNPCC), também denominado síndrome de Lynch; síndrome do carcinoma

colorretal hereditário; polipose juvenil familiar; síndrome do adenoma plano e síndrome de Peutz-Jeghers (ANDRADE e PEREIRA 2007).

A detecção precoce deste tipo de neoplasia é essencial para determinação de melhor prognóstico para o paciente, visto que a presença de pólipos adenomatosos (não tumorais) pode ser detectada antes da transformação maligna ou transformação de adenoma para adenocarcinoma. O tempo estimado para esta transformação é superior a 10 anos. Depois de ser diagnosticado como tumor maligno, é necessário que haja o estadiamento do CCR, para determinação do prognóstico do paciente e escolha do tratamento (BINEFA et al. 2014).

O sistema de estadiamento para tumores malignos TNM da *American Joint Commission on Cancer*-AJCC (EDGE et al. 2010) é o mais utilizado para CCR e pode variar do estádio 0 a IV, que representam do melhor ao pior prognóstico, nesta ordem. A classificação varia de acordo com o tamanho do tumor e a profundidade em que penetrou a parede colorretal (T), envolvimento ou não de linfonodos (N) e presença ou não de metástase à distância (M).

O estádio IV da doença independe do tamanho e profundidade tumoral, bem como presença ou não de linfonodos acometidos. Se há a presença de metástase à distância, o tumor é considerado como pior prognóstico (EDGE et al. 2010).

Aproximadamente 60% dos pacientes com CCR desenvolverão metástase, sendo 30% no momento do diagnóstico (LEE et al. 2007). O CCR dissemina-se por contiguidade (invasão de tecidos vizinhos), implante

no peritônio ou órgãos intra-abdominais e pelas vias linfática e hematogênicas. Os principais órgãos acometidos por metástases são o fígado (75%), pulmões (15%), ossos (5%) e sistema nervoso central (5%). Em relação aos tipos histológicos, o CCR pode ser classificado em adenocarcinoma, carcinoma neuroendócrino, carcinoma adenoescamoso, carcinoma de células fusiformes, carcinoma de células escamosas (epidermóide) e carcinoma indiferenciado, sendo o adenocarcinoma o mais frequente, encontrado em até 90% dos casos (FLEMING et al. 2012).

O tratamento dos tumores de cólon e reto metastáticos é principalmente à base de quimioterapia e o objetivo deste tratamento dependerá do cenário clínico, e de fatores, como por exemplo, possibilidade de doença potencialmente ressecável ou não, e se não, o que é a realidade na maioria dos casos metastáticos, os objetivos mudam do cenário de cura, para tratamento paliativo, que visa prolongar a sobrevida global e manter a qualidade de vida pelo máximo de tempo possível (GLIMELIUS et al. 1994; DE GRAMONT et al. 2000).

A quimioterapia pode ser à base de 5-fluorouracil (5-FU), irinotecano, oxaliplatina e leucovorin (ácido folínico). Ainda, podem ser acrescentados anticorpos monoclonais, como bevacizumabe, cetuximabe ou panitumumabe, ou pequenas moléculas inibidoras de quinases angiogênicas, estromais e oncogênicas, como o regorafenibe. Estão disponíveis também, fluoropirimidinas orais, como capecitabina, S-1 e UFT (SCHRAG 2004).

O 5-FU foi desenvolvido em 1950 e desde então, tem sido utilizado como primeira linha de tratamento para câncer colorretal metastático. Recentemente, outras duas drogas foram incorporadas ao tratamento com o 5-FU: irinotecano (aprovado pelo *Food and Drug Administration* (FDA) em 1996) e ácido fólico (regime FOLFIRI) e oxaliplatina (aprovada pelo FDA em 2002) e ácido fólico (regime FOLFOX), que são relatados na literatura por aumentar a taxa de resposta e consequentemente, a sobrevida destes pacientes de 12 meses a vários anos (SALTZ et al. 2000; LETO e TRUSOLINO 2014). A aplicação de anticorpos monoclonais, como anti-*Epidermal Growth Factor Receptor* (EGFR) (cetuximabe/panitumumabe) e anti-*Vascular Endothelial Growth Factor* (VEGF) (bevacizumabe) (GROTHEY et al. 2008) dependerá de fatores moleculares do tumor. Por exemplo, o uso do anticorpo anti-EGFR como tratamento só é aprovado quando o gene *KRAS* não está mutado. A análise da mutação neste gene é utilizada na prática clínica como critério de elegibilidade para recebimento de anti-EGFR (KARAPETIS et al. 2008). As mutações mais frequentes no *KRAS* estão presentes no exon 2, códons 12 e 13. Estas mutações estão presentes em aproximadamente 40-45% dos tumores colorretais (DE ROOCK et al. 2010). Quando mutado, o *KRAS* é constitutivamente ativo, e independe da ligação do ligante (EGF) com seu receptor (EGFR), promovendo, assim, proliferação celular e como consequência, crescimento tumoral.

1.1 CÉLULAS TUMORAIS CIRCULANTES (CTCs)

Acredita-se que a disseminação do câncer necessita de Células Tumorais Circulantes (CTCs) e que essas células possam ser encontradas no sangue periférico de pacientes com câncer, especialmente aqueles que possuem tumores metastáticos (PARK et al. 2011). Uma das abordagens de 'biópsia líquida", é a detecção das CTCs, como uma análise substituta ou até mesmo complementar de biópsias convencionais, que muitas vezes são anatomicamente inacessíveis, podem oferecer riscos à saúde do indivíduo, além do risco de não fornecer todos os dados genômicos necessários, devido à alta complexidade e heterogeneidade intratumoral (revisado por KREBS et al. 2014).

Quantidades elevadas de CTCs têm sido relatadas na literatura como fator de prognóstico ruim (piores sobrevidas livre de progressão e global) em alguns tumores metastáticos: mama, colorretal e próstata (CRISTOFANILLI et al. 2004; DE BONO et al. 2008; COHEN et al. 2009; TOL et al. 2010). CTCs podem também fornecer dados preditivos, como os fornecidos por meio da análise da expressão de marcadores de resistência a tratamento (STOECKLEIN et al. 2008; GAZZANIGA et al. 2010; GRADILONE et al. 2011), e com isso ser um dos fatores determinantes de recorrência local e progressão tumoral.

Um estudo recente (HODGKINSON et al. 2014) mostrou a capacidade das CTCs provenientes de pacientes com câncer de pulmão de pequenas células em causar tumorigenicidade em camundongos

imunodeficientes, além de predizer "*in vivo*" o perfil de resposta ao tratamento pela característica destes tumores desenvolvidos, quando comparados ao perfil dos pacientes. Realizaram, ainda, caracterização genética e imunoistoquímica e encontraram ampla similaridade entre as CTCs, os tumores dos pacientes e os tumores desenvolvidos nos camundongos.

Apesar da análise de CTCs ter sido incluída em mais de 300 *clinical trials* abertos (http://clinicaltrials.gov), sua real utilidade clínica ainda está em debate, pois existem conhecimentos sobre CTC que ainda são limitados, como sua caracterização e a diversidade dos métodos de detecção (revisado por ALIX-PANABIÈRES e PANTEL 2014).

Muitas tecnologias para detecção de CTCs têm sido desenvolvidas e são divididas por suas abordagens: a) detecção por propriedades biológicas, como antígenos de superfície epiteliais (citoqueratinas+, EpCAM+) e depleção de leucócitos (CD45-); b) detecção por propriedades físicas das CTCs, como por filtração, densidade, campo elétrico (dieletroforese); c) ensaios combinados (baseados em propriedades biológicas e físicas) e; d) ensaios funcionais que dependem de um passo de pré-enriquecimento (revisado por KREBS et al. 2014; revisado por ALIX-PANABIÈRES e PANTEL 2014).

Para todas as tecnologias, existem os prós e os contras, como por exemplo, CTCs que estejam passando pela transição epitélio-mesênquima do inglês *Epithelial to Mesenchymal Transition* (EMT) (ARMSTRONG et al. 2011; revisado por LIM et al. 2014), não serão capturadas pelos métodos

baseados em captura por antígenos epiteliais. Desta forma, pode ocorrer uma subestimação nos níveis de CTCs. Já a tecnologia *Isolation by Size of Epithelial Tumor Cells* (ISET), baseada em separação por tamanho celular, pode perder células com menos de 8 µm e ainda não é aprovado para prática clínica, apesar de já ter sido validado clinicamente (HOFMAN et al. 2011). Em contrapartida, o ISET é uma metodologia independente de antígenos, por isso permite caracterização das CTCs, além de capturar microêmbolos tumorais circulantes (CTMs, do inglês *Circulating Tumor Microemboli*), que parecem ter influência em pior prognóstico quando presentes, por apresentarem vantagens de sobrevivência na circulação sanguínea em relação às células que circulam sozinhas (FRIEDL e GILMOUR 2009; ILINA e FRIEDL 2009; KREBS et al. 2012; CHINEN et al. 2013).

Outra vantagem da tecnologia ISET é que por ser uma metodologia independente de antígenos, pode detectar CTCs de outras neoplasias não epiteliais, como melanoma (DE GIORGI et al. 2010; KHOJA et al. 2014) e sarcoma (CHINEN et al. 2014).

Figura 1 - Esquema do processo de filtração pela tecnologia ISET (*Isolation by Size of Epithelial Tumor Cells*). Adaptado de: http://www.rarecells.com/ctc-isolation.html.

1.2 CÉLULAS TUMORAIS CIRCULANTES (CTCs) E CÂNCER COLORRETAL METASTÁTICO

Estudos sugerem que o processo de metastatização ocorre antes mesmo de o tumor primário exibir características morfológicas de invasão (HÜSEMANN et al. 2008; KLEIN 2009), por isso a importância da detecção e o entendimento da função das CTCs se faz presente em diversas neoplasias.

Especificamente, no câncer colorretal, a presença de CTCs tem sido recentemente reportada, tanto em CCR localmente avançado (HIRAIWA et al. 2008; TOL et al. 2010), quanto metastático (KAIFI et al. 2013), utilizando diferentes tecnologias, e tem sido demonstrada como um potencial biomarcador para monitoramento da doença em tempo real, além de possibilitar estratificação do tratamento.

Para pacientes com doença não metastática a avaliação de CTCs também tem sido reportada. Em pacientes com estádios II e III, a presença de CTCs foi determinante para piores SG e SLP em relação àqueles pacientes com ausência de CTCs. Já nos pacientes com estádio I esta diferença não foi observada (IINUMA et al. 2011). A identificação de CTCs por RT-PCR após 24 horas da cirurgia com intenção curativa foi preditora de recorrência, e quando combinada com avaliação de positividade de linfonodos, foi relacionada a pior prognóstico, comparada a análise apenas de CTCs (ALLEN-MERSH et al. 2007).

RAHBARI et al. (2010) realizaram uma meta-análise, incluindo 36 estudos com CTCs de pacientes com CCR (isoladas do sangue periférico e sangue mesentérico ou veia porta) e com Células Tumorais Disseminadas (DTCs, do inglês *Disseminated Tumor Cells*), que são células tumorais encontradas na medula óssea. Foram incluídos 3094 pacientes com câncer colorretal em diversos estádios e demonstraram com a análise que tanto a presença de CTCs quanto de DTCs estava relacionada com pior sobrevida livre de recorrência (SLR) (HR= 3,24; IC 95%= 2,06 – 5,10) e SG (HR= 2,28; IC 95%= 1,55 – 3,38). Os autores conseguiram demonstrar também que apenas CTCs encontradas no sangue periférico eram capazes de determinar pior prognóstico (SLR: HR= 3,06; IC 95%= 1,74 – 5,38 e SG: HR= 2,70; IC 95%= 1,74 – 4,20). Outra meta-análise mais recente, publicada em 2013, GROOT-KOERKAMP et al., incluiu 16 estudos com 1491 pacientes com CCRm. Eles encontraram resultados similares ao citado acima: pacientes com CTCs tinham pior SLP (HR= 2,07; IC 95%= 1,74 – 3,51) e pior SG (HR= 2,47; IC 95%= 1,44 – 2,98) em relação aos pacientes sem CTCs.

1.3 RESISTÊNCIA ÀS DROGAS

A falha no tratamento quimioterápico tem relação direta com resistência às drogas. Muito tem se estudado para entender os mecanismos que as células tumorais apresentam para superar a ação dos fármacos. Existem dois conceitos de resistência: a resistência intrínseca e a adquirida.

A resistência intrínseca se refere a características pré-existentes no tumor que conferirão resistência, antes mesmo de sofrer qualquer modificação causada pela quimioterapia. Já a resistência adquirida se refere à modulação que o tumor ou uma subpopulação de células sofre após a exposição aos fármacos, por meio da seleção de alterações moleculares adicionais. Esta subpopulação de células resistentes que restam mesmo após tratamento é a que geralmente causará recorrência local e metástases à distância. A resistência tumoral pode ocorrer de diversas formas, como por mutações, alterações em vias de sinalização envolvidas em proliferação, na sobrevivência celular, apoptose, na desregulação de expressão gênica, entre outros (revisado por HOLOHAN et al. 2013).

A progressão do CCR depende de múltiplos passos de evolução molecular (TSAO et al. 1999), sendo considerada uma doença clonal, pelo fato de iniciar responsiva ao tratamento quimioterápico e se tornar, muitas vezes, resistente. Pelo fato de não haver diversificação das opções para tratamento, os agentes variam de FOLFIRI para FOLFOX, e vice-versa. Estes regimes foram estabelecidos como primeira linha de terapia para CCRm e não demonstram diferença entre si para tempo de progressão. O mesmo é verdade quando utilizados em segunda linha de tratamento, ou linhas posteriores com a troca dos agentes entre si (TOURNIGAND et al. 2004; COLUCCI et al. 2005).

O maior obstáculo atualmente está no desenvolvimento de novos biomarcadores a fim de melhorar a atividade dos agentes disponíveis, assim

como na determinação da melhor sequência de tratamento (revisado por YAFFEE et al. 2015).

1.4 TRANSPORTADORES E GENES ENVOLVIDOS EM RESISTÊNCIA ÀS DROGAS

Alguns biomarcadores promissores de resposta à quimioterapia em CCRm têm sido estudados. Dentre eles, Timidilato Sintase (TYMS), Topoisomerase I e ERCC1 (JENSEN et al. 2012). TYMS é uma enzima que participa de uma das vias de metabolismo do 5-FU. O mecanismo de ação do 5-FU pode variar dependendo da dose e da forma de administração. A infusão contínua resulta no anabolismo de 5-FU, formando principalmente o *5-fluoro-2´-deoxyuridine -5´-monophosphate* (FdUMP), que age como um potente inibidor da ligação de TYMS (SOMMER e SANTI 1974), o que leva ao desbalanço do *pool* de nucleotídeos, diminuindo a concentração de dTTP (*2'-deoxythymidine 5'-triphosphate*), aumentando a de dUTP (*2'-deoxyuridine 5'-triphosphate*) e, como consequência, levando à incorporação de uracil na cadeia de DNA (revisado por LONGLEY et al. 2003).

O tratamento com 5-FU em *bolus* favorece o anabolismo em FUTP e sua incorporação no RNA que interfere com o processamento normal do pré-RNAr. Dessa forma, resistência relacionada ao modo de administração do 5-FU pode envolver diversos fatores e isso é consistente com o fato de que linhagem de células de carcinoma de cólon resistentes ao tratamento com 5-FU em *bolus* permanecem sensíveis à exposição contínua. Um

mecanismo comum de resistência clínica ao *bolus* e à infusão está relacionado ao aumento dos níveis de TYMS e sua inibição. TYMS constitui uma enzima importante na síntese “de novo” de dTMPC (*2'-deoxythymidine 5'-monophosphate*), um precursor essencial de DNA, que catalisa a metilação de dUMP (*2'-deoxyuridine 5'-monophosphate*) a dTMP (SANTI et al. 1974). TYMS é o alvo das fluoropirimidinas. A quantidade intrínseca de TYMS parece ser um preditor de resposta dos pacientes ao 5-FU. Pacientes com CCRm com altos níveis de TYMS não respondem ao tratamento infusional com 5-FU, enquanto aqueles com baixos níveis apresentam melhores taxas de resposta (KIM et al. 2009; revisado por CALASCIBETTA et al. 2010). Contudo, apesar de muitos estudos relatarem SG ruim em tumores com alta expressão de TYMS, o real valor prognóstico desta enzima ainda não está estabelecido (ALLEGRA et al. 2002; CHEN et al. 2012).

A célula tumoral pode também usar como mecanismo de resistência o fenômeno chamado multirresistência a drogas (MDR, do inglês *Multidrug Resistance*), que pode ocorrer de diversas formas, simultâneas ou não, como, por exemplo, alterações no metabolismo celular, alterações no alvo molecular, aumento na capacidade de reparação do DNA, falha no mecanismo de apoptose e por meio da ativação do sistema de proteínas responsáveis por expelir compostos tóxicos, como fármacos e xenobióticos de dentro para fora da célula. Uma das características mais relevantes da MDR é a capacidade de expelir uma gama de quimioterápicos de estruturas e farmacologia analogicamente diferentes. As proteínas transportadoras se localizam principalmente na membrana plasmática e funcionam como

bombas de efluxo. Estas proteínas pertencem à família ABC (*ATP-binding cassete*) e utilizam energia derivada da hidrólise do ATP. Dentro desta família, já se tem descrito 50 membros subdivididos em 7 subfamílias (A-G) (http://nutrigene.4t.com/humanabc.htm). Especificamente, este estudo tem como foco duas proteínas da subfamília C, MRP1 e MRP4. A subfamília C tem 13 membros descritos, e 9 destes estão envolvidos primariamente em resistência a multidrogas (DEAN e ALLIKMETS 2001).

As três proteínas ABC mais estudadas e comprovadas por exercer função em MDR são: P-glicoproteína (P-gp/MDR1/*ABCB1*), MRP1 (*Multidrug Resistance-associated Protein 1*; *ABCC1*) e BCRP1 (MXR, ABCP*, ABCG2*). A proteína P-gp foi descoberta há mais de 30 anos (BORST e ELFERINK 2002; ECKFORD e SHAROM 2009).

A proteína MRP1 foi primeiramente clonada a partir de uma linhagem celular de câncer de pulmão (H69AR) resistente à doxorrubicina em 1992, não associada à superexpressão de P-gp (COLE et al. 1992). O gene da MRP1 (*ABCC1*) está na região 16p13.1 e codifica uma proteína com 1531 aminoácidos (COLE et al. 1992; SLOVAK et al. 1993). MRP1 está localizada na superfície basolateral da membrana epitelial de diversos tecidos, como pulmão, testículo, músculo esquelético, músculo cardíaco e placenta (revisado por SODANI et al. 2012).

Multidrug Resistance-associated Protein 4/*ABCC4* (MRP4) foi descoberta em 1996 em uma linhagem celular linfoide T. O gene *ABCC4* está na região 13q32.1 e codifica uma das menores proteínas MRPs, possuindo 1325 aminoácidos (revisado por SODANI et al. 2012). MRP4 foi

encontrada também na membrana basolateral, confirmada por exercer uma função protetora (LEGGAS et al. 2004) e excretora (CHEN et al. 2005).

Um trabalho realizado por GAZZANIGA et al. (2010) verificou nas CTCs proteínas relacionadas a MDR mais comumente usadas nos tumores sólidos. Foram avaliadas as seguintes proteínas: envolvida na resistência a antraciclinas, alcalóides da vinca, metotrexato, imatinibe, irinotecano, topotecano (MRP1), semelhante a MRP1, com exceção da cisplatina (MRP2), resistência a metotrexato, irinotecano, topotecano (MRP4), resistência a 5-FU, cisplatina e metotrexato (MRP5), resistência a taxanos e alcalóides da vinca (MRP7). Como gencitabina não é substrato para MRPs, dois genes associados ao seu transporte e metabolismo foram incluídos: *human equilibrative nucleoside transporter-1* (*Hent1*) e *deoxycytidine kinase* (*dCK*). Foram analisados 105 pacientes com diversos tumores sólidos antes do início da quimioterapia sistêmica, com intuito de predizer o perfil de sensibilidade das CTCs. O perfil de resistência às drogas nas CTCs correlacionou-se com SLP e sobrevida livre de doença em 24 meses ($p< 0,001$). De acordo com a resposta clínica, 21/32 pacientes em estádio IV apresentaram progressão de doença e dentre eles, o ensaio de quimiosensibilidade mostrou resistência em 20/21 e sensibilidade foi encontrada em 1/21. No grupo dos 11 pacientes com doença metastática com quadro estável, o ensaio de sensibilidade mostrou uma predição de resposta de 100%. Assim, o teste de resistência neste trabalho foi capaz de predizer a resposta ao tratamento em 98% dos pacientes, com 100% de especificidade. Nenhuma amostra de pacientes saudável foi positiva para

CTC. Os valores preditivos positivo e negativo encontrados foram de 96,5 e 100% respectivamente (GAZZANIGA et al. 2010).

Considerando o mecanismo de ação dos derivados da platina, estudos têm avaliado o papel de enzimas envolvidas no reparo de DNA como possíveis preditores de sensibilidade ou resistência a estas drogas, como *Excision Repair Cross-Complementation Group* 1 (ERCC1). ERCC1 é uma enzima de reparo a danos ao DNA e seu gene (*ERCC1*) está localizado na região 19q13.32. Especificamente, ERCC1 está envolvida na via de reparo de excisão de nucleotídeos, principalmente agindo nas lesões no DNA induzidas por exposições às luzes ultravioleta ou por compostos eletrofílicos, como a cisplatina (HOUTSMULLER et al. 1999), portanto desempenha um papel crucial na resistência às drogas.

Assim, o ERCC1 tem sido demonstrado na literatura como biomarcador potencial para selecionar os respondedores ao tratamento com quimioterapia a base de platina, principalmente em Câncer de Pulmão Não Pequenas Células (CPNPC), quando avaliado em tecidos tumorais (OLAUSSEN et al. 2006; YAN et al. 2013). Alguns estudos têm mostrado uma melhor resposta para pacientes com baixos níveis de ERCC1 medidos por RT-PCR, sugerindo que quantidades reduzidas desta enzima afetam a capacidade das células de reparar os danos causados pela droga ao DNA (LORD et al. 2002; CEPPI et al. 2006).

Focando em CTCs, ERCC1 já foi avaliado em CPNPC (DAS et al. 2012), em câncer de mama (SOMLO et al. 2011) e em câncer de ovário (KUHLMANN et al. 2014), procurando sua expressão em CTCs de pacientes

tratados com platina. Para mama (SOMLO et al. 2011) e CPNPC (DAS et al. 2012), os autores usaram uma combinação de depleção magnética de células brancas seguido de fixação, bloqueio e marcação das CTCs com anticorpos conjugados com fluorocromos. SOMLO et al. (2011) analisaram pacientes com câncer de mama com CTCs detectáveis e compararam com os tumores primários. Eles encontraram fraca correlação entre expressão de ERCC1 em CTCs e tumores primários (N=11), e também entre tumores primários e metástases (N=8). DAS et al. (2012) avaliaram a expressão de ERCC1 em CTCs de 17 pacientes com CPNPC. Eles verificaram que a falta de expressão de ERCC1 foi correlacionada com melhor SLP (P< 0,02, HR: 4.2).

KUHLMANN et al. (2014) usaram uma combinação de enriquecimento imunomagnético de CTCs procurando por epítopos epiteliais, seguido de RT-PCR *multiplex*, com adição de marcadores para CA125 e ERCC1. Eles também avaliaram a expressão de ERCC1 por imunoistoquímica em tumores primários. Os pacientes com CTCs positivas para ERCC1 tiveram pior SLP e SG, quando comparados com aqueles com CTCs negativas (P= 0,026 e P= 0,009, respectivamente). A expressão de ERCC1 nas CTCs foi também correlacionada com resistência à platina (P= 0,01).

Diante de tudo que foi exposto, CTCs, apesar de bastante estudadas, ainda constituem uma área com muitas indagações. Nosso grupo se propôs a responder a algumas destas perguntas, avaliando os níveis de CTCs em pacientes com câncer colorretal metastático, assim como proteínas envolvidas na resistência ao tratamento quimioterápico, possibilitando o melhor entendimento da biologia tumoral.

2 OBJETIVOS

2.1 OBJETIVO GERAL

Filtrar CTCs do sangue periférico de pacientes com câncer colorretal metastático, contar estas CTCs e, assim, correlacionar seus níveis com evolução clínica tumoral.

2.2 OBJETIVOS ESPECÍFICOS

1 Determinar a correlação entre a expressão dos transportadores MRP-1, MRP-4 e de genes de resistência às drogas (ERCC1, TYMS) nas CTCs (por imunocitoquímica) com progressão tumoral;

2 Determinar a correlação entre a expressão dos transportadores MRP-1, MRP-4, e de genes de resistência às drogas (ERCC1, TYMS) nas CTCs, nos tumores e nas metástases com progressão tumoral.

3 ARTIGO 1 – SUBMETIDO

Early Detection of Poor Outcome in Metastatic Colorectal Cancer Patients: Tumor kinetics evaluated by ISET

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Short Title: CTCs evaluated by ISET in mCRC

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Keywords: circulating tumor cells, metastatic colorectal cancer, kinetics, ISET

Abstract

Background: Colorectal cancer (CRC) is the third most prevalent cancer worldwide. New prognostic markers are needed to identify patients with worse prognosis and circulating tumor cells (CTCs) have shown to be a promising tool. **Patients and methods:** Prospective study of 54 patients with metastatic CRC (mCRC), conducted by collecting blood in three times together with image exams for evaluation of therapeutic response. CTC isolation and counting were made by ISET (Rarecells, France). **Results:** The median age was 59 years old (31-81). Among patients, 60% (n= 32) were wild type KRAS (wt KRAS) and 54% (n= 29) were exposed to monoclonal antibodies (cetuximab or bevacizumab) along with systemic treatment. Comparing the baseline CTC level (CTC1) with the level at first follow-up (CTC 2), CTC1 positive patients who became negative were classified with a favorable evolution (n=14), with median progression-free survival (PFS) of 14.7 months. This was higher than patients with an unfavorable evolution (CTC1 – and CTC2 +) (n= 13), who were CTC1 negative and became CTC2 + (6.9 months; p= 0.06). Patients with wt KRAS with favorable kinetic had higher PFS (14.7 months) in comparison to those wt KRAS with unfavorable kinetic (9.4 months; p = 0.02). Moreover, patients whose imaging studies showed radiological progression had an increased quantification of CTCs at CTC2 compared to those without progression (p= 0.04). **Conclusion:** This study made possible the presentation of ISET for counting CTCs in mCRC patients. It also showed that CTC kinetics analysis can be promising in the clinical evaluation of these patients.

Introduction

Colorectal cancer is the third most common cancer in the world, with an estimated 1.36 million new cases per year, approximately 600,000 of whom will die from the disease[1]. Around 50 to 60% of patients with this cancer will present metastasis eventually; 30% at diagnosis [2-4]. The last decade was characterized by progress in the availability of new drugs with distinct mechanism of action and a positive impact on sequential treatment of metastatic colorectal cancer (mCRC), including important gains in median survival rates for these patients, which currently exceed 24 months [5-9]. Despite improvement in survival rates, the increased cost in treatment is not necessarily reflected in the health and well-being of patients, or in the ability to identify ideal treatments for specific patients.

As a disease associated with many prognostic factors including performance status, age, gender, weight loss, an increase in carcinoembryonic marker (CEA), number of metastatic sites, among others [10-11], various studies have attempted to identify biomarkers to improve prognostic standards[12]. The objective is to individualize therapeutic strategies and identify patients with worse prognoses or those on their way to rapid progression and who could benefit from a more aggressive therapy [13-15].

Circulating tumor cells (CTCs) studies have demonstrated promising results in an attempt to help the segment of patients with metastatic cancer [15]. Various studies have shown CTCs to be predictors of progression-free survival (PFS) and overall survival (OS) rates in patients with breast cancer

and metastatic prostate cancer [17-20]. In patients with mCRC, some publications describe a correlation of the presence of CTCs to prognosis [21-23]. The presence of at least 3 CTCs/7.5 ml of blood in these patients at the time of diagnosis and in the follow-up would be an important independent prognostic factor for worse PFS and OS. These values, when used in conjunction with imaging exams, could provide additional diagnostic information[24-25].

There are basically three kinds of methods to separate CTCs: immunological (based in capture antibodies), such as assays CellSearch® System, based on physical properties of cells (e.g.: ISET, Rarecells, Paris, France) and the functional assays, as EPISPOT. ISET System is a physical-based assay that consists in separating cells by size. It has an advantage of isolating CTCs in a marker independent manner. So, if these cells have epithelial markers (EpCAM and citokeratin (CK)), downregulated they will not be loosen by ISET [25]. In comparison with CellSearch® System (Veridex, LLC) it seems that ISET can capture larger amount of tumor cells than CellSearch system, without risk of overestimate counts, as this last one system can capture other non-malignant cells expressing EpCAM and CK. To date, all studies with CTC kinetics were made using CellSearch system [26-29].

The purpose of our study, therefore, was to evaluate clinical evolution and therapeutic response with conventional image exams, based on CTCs counts made by an isolation by size method (ISET, Rarecells, France) in patients with mCRC in sequential phases of treatment.

Materials and Methods

Patients

Participation in the study was limited to patients who signed an informed consent form (ICF). All participants had 8mL of blood collected at every scheduled visit. Patients included in the study fulfilled the following criteria: 18 years of age or older; ECOG 0-2 without organ dysfunction; metastatic disease confirmed by pathological and/or radiological analysis; patients who had started chemotherapy for metastatic disease; the extent of the disease determined by a physical exam and by imaging; disease measureable by RECIST criteria version 1.1 (Response Evaluation Criteria in Solid Tumors) [30].

Assay Methods

We used the ISET technology (Isolation by Size of Epithelial Tumor Cells, Rarecells; France) for analysis and characterization of CTCs. The peripheral blood samples of patients with metastatic colorectal cancer were collected in EDTA tubes (8 mL) and maintained under homogenization at room temperature for up to 4 hours. Before being filtered on ISET, the samples were diluted in a 1:10 buffer (ISET Buffer™). After 10 minutes of incubation, the samples were filtered, washed with PBS, removed from the ISET Block™ and, after drying in the dark at room temperature, stored at -20ºC, until analysis.

To count the CTCs, ISET membrane spots (n=4) were cut and submitted to immunocytochemistry with anti-CD45 antibody (1:100; clone

2B11 + PD7/26, Dako™), a surface leukocyte marker in order to differentiate them from the CTCs. The reaction was done as described previously by Chinen et al.[31]. After assembly, the slides were examined under a white light microscope. The CTCs were characterized based on the following criteria: negative cells for CD45, size of nucleus equal to or larger than 12μm, hyperchromatic and irregular nucleus, visible presence of cytoplasm and a high nucleus-cytoplasm ratio (> 50%) [32].

Study Design

This was a prospective study done by collecting blood (plasma) from patients with metastatic colorectal cancer (mCRC) treated at the AC Camargo Cancer Center in São Paulo, Brazil. The patients' blood was collected in three sessions, baseline and follow-ups, every two months in an attempt to reconcile cell collections with pictures of the images for evaluation of the therapeutic response.

Patients were evaluated using computed tomography and/or magnetic resonance of the thorax, abdomen, and pelvis before the first collection of CTCs, as well as approximately every 2 to 4 months during treatment, or as indicated. The images were interpreted using RECIST criteria version 1.1 [30] (Response Evaluation Criteria in Solid Tumors), and classified as complete response, partial response, stable disease, or disease in progression.

Statistical analysis methods

The distributions of absolute and relative frequencies were presented for the clinical-pathological variables and also the qualitative treatment and the standard deviation mean for age. The Kaplan-Meier estimator and the Log-Rank test were used in the analysis of progression-free survival. Variance Analysis of Repeated Measurements was used to access CTC evolution during the evaluations. The level of significance adopted was less than 5% and the analyses were performed in the program SPSS for Windows version 15.

Results

Between the months July 2012 and December 2013, we recruited 54 patients diagnosed with mCRC. The median age was 59 years old (31-81 years), mostly male (n = 31; 59%). At the time of the inclusion of the study, all patients had metastatic disease, including 65% (n = 35) who had at least one metastatic site and 7% (n = 04) with more than two metastatic sites. Only 30% (n=16) of the patients was treated with first line chemotherapy, at the moment of the study entry. Most had received previous treatment prior to study. KRAS wild patients constituted 60% (n = 32) of the study population and during follow-up 54% (n = 29) of the patients were exposed to monoclonal antibodies along with systemic treatment (Table 1).

Table 1: Characteristics of the studied population

Characteristics	N (%)
Median age (minimum - maximum)	59 years (31-81 years)
Gender	
Male	31 (59)
Female	23 (41)
KRAS	
Wild	32 (60)
Mutated	21 (40)
Use of antibodies	
No	25 (46)
Bevacizumab	19 (36)
Cetuximab	10 (18)
Liver metastasis	
Yes	25 (46)
No	29 (54)
Number of sites metastasized	
1	35 (65)
2	15 (28)
>2	4 (7)
Lines of treatment, pre CTC1	
0	16 (30)
1	20 (36)
>= 2	18 (34)

The quantitative analysis of CTCs by the ISET method was done at three distinct times during follow-ups for each patient: baseline (CTC 1), first follow-up performed between the 6th and the 8th week of treatment (CTC2), and the second follow-up, done between the 12th and 16th weeks of treatment (CTC3). Because there is not yet a cut-off value for counting CTCs for the ISET method, we standardized it with the median cut-off value of each time. So, the cut-off for CTC1 was 2.0 CTCs/mL (0-31 CTCs/mL), CTC2 was 3.0 CTCs/mL (0-47 CTCs/mL), and CTC3 was 3.00 CTCs/mL (0-77 CTCs mL). Values below established cut-offs were considered as CTC negative (CTC −) and equal to or higher as CTC positive (CTC +). Illustrative pictures of CTCs and leucocytes can be seen in Figure 1.

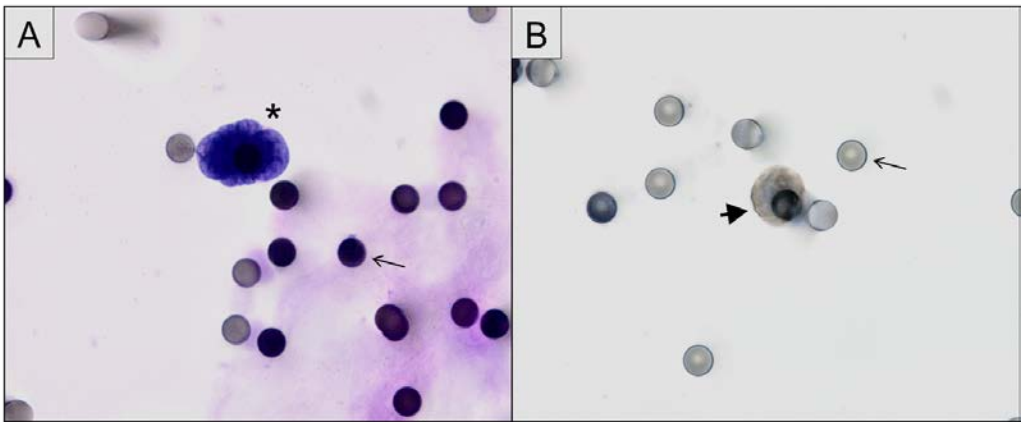

Figure 1: (A) CTC visualized by haematoxilin-eosin. (B) Immunostaining of leucocyte with CD45 and counterstaining with DAB. Asterisk indicates CTC, thin arrows represent pores of ISET membrane and thick arrow shows leukocyte. Both images were taken at x600 magnification using a light microscope (Research System Microscope BX61 – Olympus, Tokyo, Japan) coupled to a digital camera (SC100 – Olympus, Tokyo, Japan).

Out of 54 patients enrolled in the study, 33 (62%) had the three blood sample collected for analysis (CTC1, CTC2 and CTC3). Baseline sample (CTC1) was not obtained for one patient (the sample was collected but the blood coagulated). First follow-up (CTC2) was not obtained for 12 patients and second follow-up (CTC3) sample was not collected for 21 patients. The reason for not obtaining the samples (CTC2 and CTC3) were most due to lost of follow up, death or not re-consenting for a new blood draw.

CTC values monitored at three different times allowed the analysis of CTC kinetics. Comparing the baseline CTC level (CTC1) with the level at CTC 2, CTC1 positive patients who became negative were classified with a favorable evolution (CTC 1 + and CTC 2 −) (n=14), and had a progression-free survival (PFS) median of 14.7 months. This was higher than patients with an unfavorable evolution (CTC1 – and CTC2 +) (n= 13), who were

CTC1 negative and became CTC2 +, with a median PFS of 6.9 months (p = 0.06) (Figure 2). In patients without modification in the kinetics of the CTCs, (CTC1 + and CTC2 + or CTC1 – and CTC2 −) there was not a difference between the PFS curves (10.3 and 10.4 months, respectively; p= 0.60) (Figure 3). However, for those patients with no change in CTC´s kinetics (based on established cut-off), we observed that the pure analysis of CTC counts could help to define the disease. Patients that had increased CTCs numbers during follow-up showed worst prognosis in relation to those who had a decrease in CTCs counts (Table 2). The correlation between the second collection and the third collection did not demonstrate significant statistical differences (p = 0.79). Figure 4 shows the radiological evolution (progression of the disease) with a CTC count increase in a patient (patient 1) of the study to demonstrate the data presented.

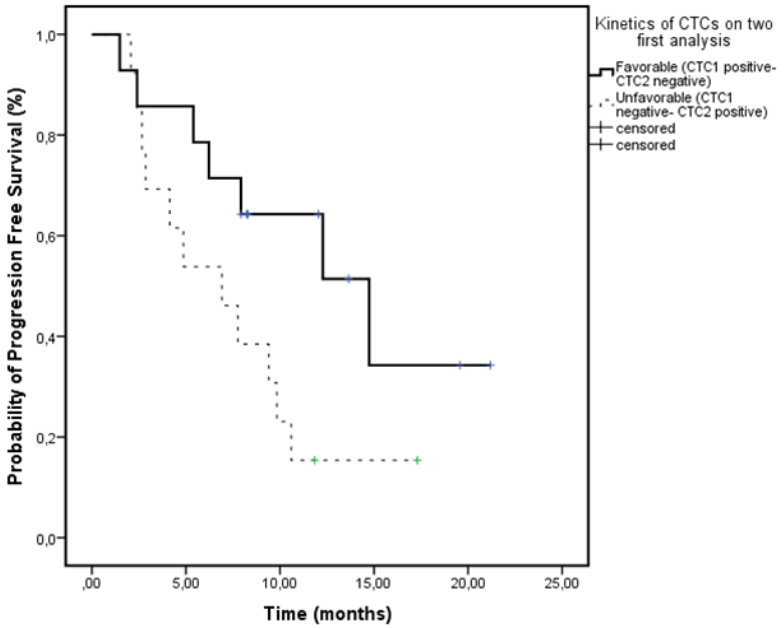

Figure 2: Progression free survival evaluation in patients with CTC kinetics on the first two analyses (CTC1 and CTC2). Continuous line: positive patients (CTC1+ and CTC2-). Dotted line: patients with unfavorable kinetics (CTC1 - and CTC2 +) (p = 0.06).

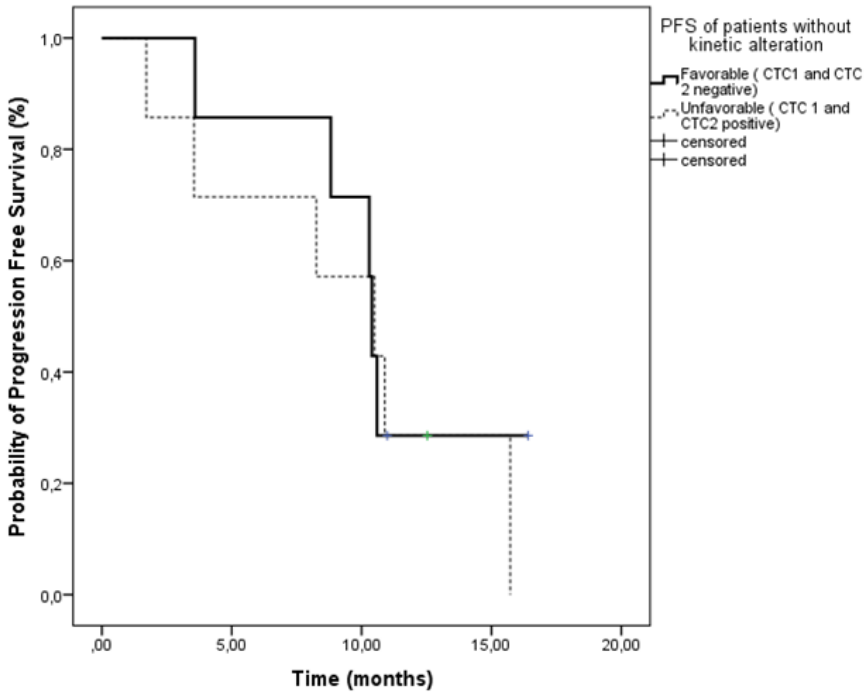

Figure 3: Progression-free survival (PFS) evaluation in months, in patients without kinetic alteration of CTCs (CTC1 + -> + CTC2) and (CTC1 - -> CTC2 -). Continuous line: patients with positive CTC maintenance. Dotted line: patients with negative CTC maintenance (p = 0.60).

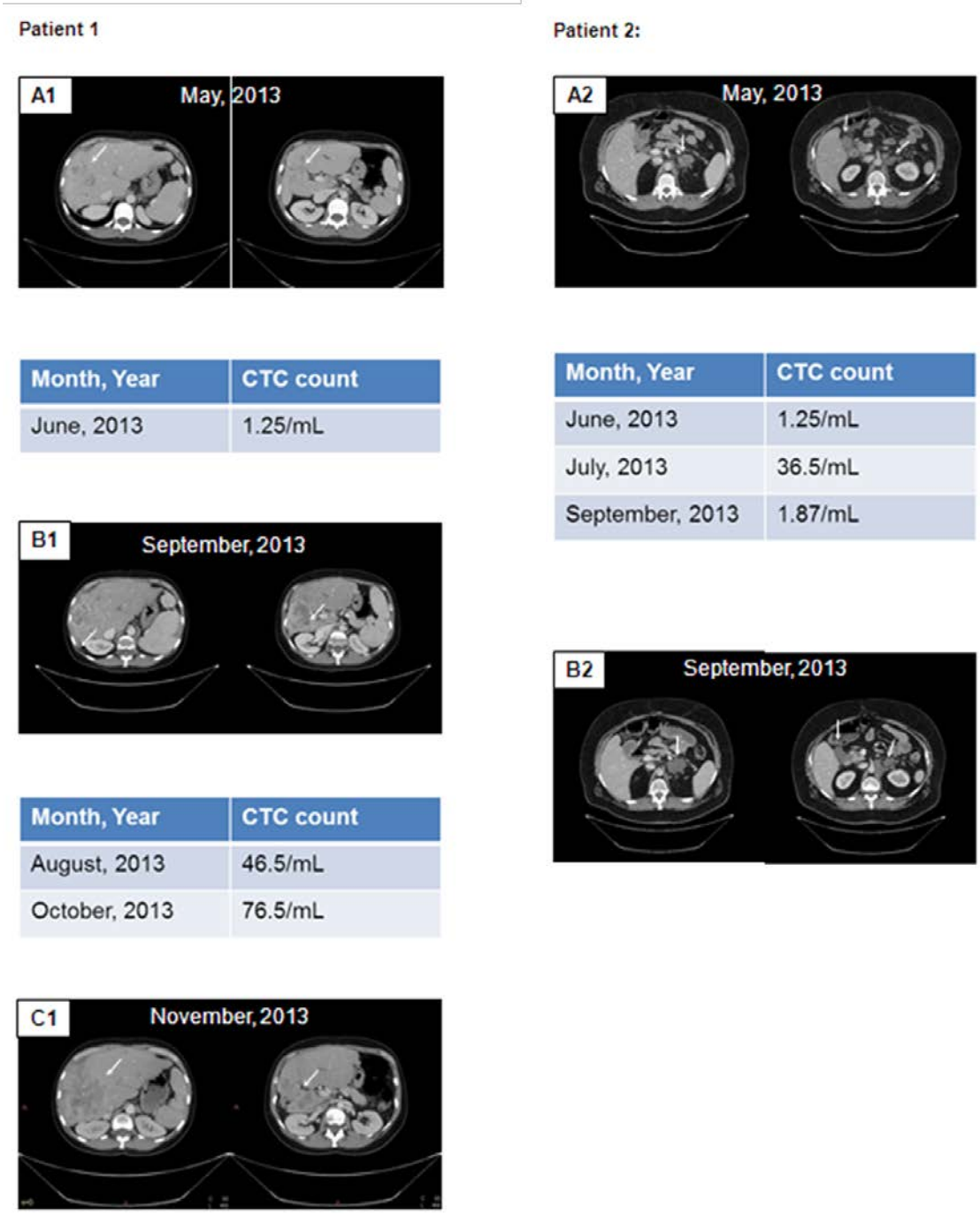

Figure 4: Radiological evolution and CTC counting evolution or development. Patient 1. Illustration A1: Image of hepatic lesion evidence (arrows) performed before the collection of CTC1 (May / 2013); Illustration B1: Image (September / 2013) of hepatic lesion evidence (arrows) in progression when compared with A1 image after the collection of CTC2; Illustration C1: Image of hepatic lesion evidence (arrows) in progression when compared with image B1 performed after collection of CTC 3 (November / 2013). Patient 2. Illustration A2: Image of lymph node involvement evidence (arrows) performed before the collection of CTC1 (May / 2013); Illustration B2: Image (September / 2013) of evidence of progression of lymph node disease (arrows) compared with illustration A2.

Table 2: Individual values of CTC´s counts of patients whose CTC kinetics based on established cut-off did not show alteration

	CTC 1 (CTCs/ml)	CTC 1 (+ or -)	CTC 2 (CTCs/ml)	CTC 2 (+ or -)	Kinetics Evolution (CTC1 and CTC 2)	PFS (months)
Patient A	46.0	+	6.0	+	NA	11,4
Patient B	11.0	+	31.0	+	NA	5,4
Patient C	4.0	+	24.0	+	NA	2,7
Patient D	0	-	0	-	NA	NP
Patient F	2.0	-	1.0	-	NA	NP
Patient G	12.0	+	11.0	+	NA	NP
Patient H	29.0	+	3.0	+	NA	4,3
Patient I	0	-	0	-	NA	6,9
Patient J	0	-	0	-	NA	10,4
Patient K	4.0	+	11.0	+	NA	6,6
Patient L	8.0	+	3.0	+	NA	3,5
Patient M	1.0	-	1.0	-	NA	4,4
Patient N	0	-	1.0	-	NA	10,5
Patient O	3.0	+	8.0	+	NA	11,1

Abbreviations: NA (No Alteration); NP (No Progression)

Based on the CTC kinetic profile, we correlated the favorable patients and the unfavorable with a KRAS mutation. Patients with mutated KRAS (KRAS mt) who presented a favorable CTC´s kinetic evolution had a PFS of 5.3 months, higher than the KRAS mt patients who had unfavorable CTC kinetic evolution (2.4 months). In addition, the KRAS wild (KRAS wt) patients with a favorable trend showed median PFS of 14.7 months, higher than the KRAS wt patients with a unfavorable kinetic of 9.4 months (p = 0.02) (Figure 5).

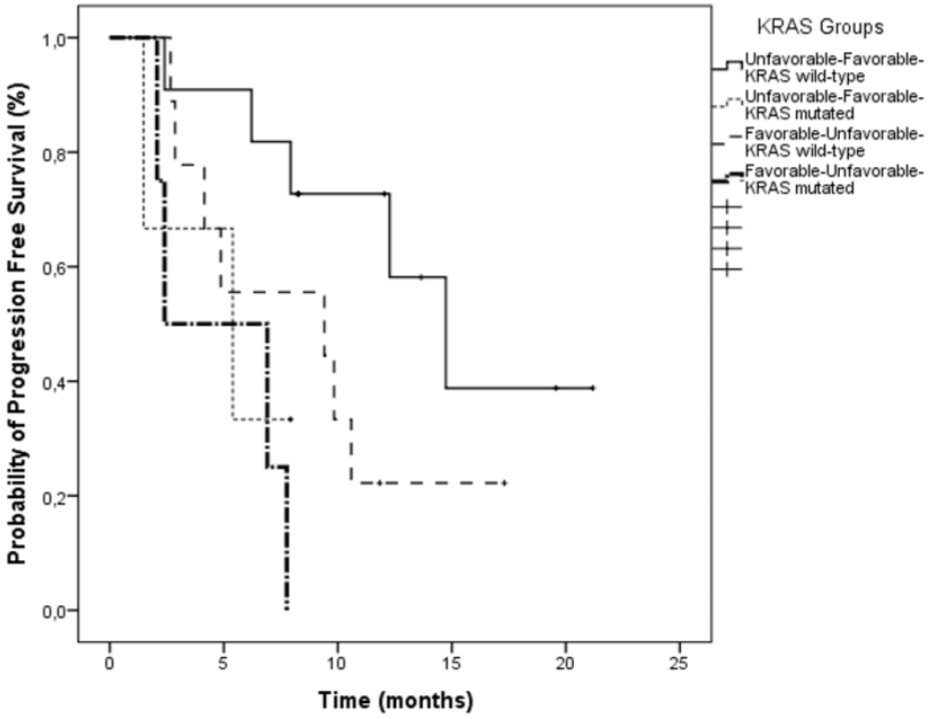

Figure 5: Progression-free survival based on CTCs kinetics and the status of the KRAS mutation. Continuous line: patients with KRAS wild favorable kinetics (CTC1 and CTC + 2). Dotted line: patients with mutated KRAS favorable kinetics (CTC1 and CTC + 2). Thin dashed line: patients with KRAS wild unfavorable kinetics (CTC1 - and CTC2 +). Thick dashed line: patients with mutated KRAS with unfavorable kinetics (CTC1 - and CTC2 +) (p = 0.02).

In relation to treatment with anti-angiogenic therapies and CTC kinetic profile, patients that used anti-VEGF who presented a favorable CTC kinetic (n=6) had a PFS of 18.2 months, higher than those with an unfavorable kinetic (n=5; PFS= 5.6 months; p = 0.007) (Figure 6).

In addition to the quantification of CTCs, patients were also monitored through the evaluation of images for each collection of cells up to 4 months after the collection of CTC3. We observed that the patients whose image exams showed radiological progression consistent with RECIST criteria (28)

(20/32) demonstrated higher average CTC quantification at the second time (CTC2) compared with those that did not show progression in the image exams (12/32), and this difference was statistically significant (p = 0.04) (Figure 7). To illustrate this correlation, we show in Figure 4 the radiological development with the cell count of patient 2, whose CTC elevation at the second time correlated with evidence of disease progression in images done after the CTC3 collection.

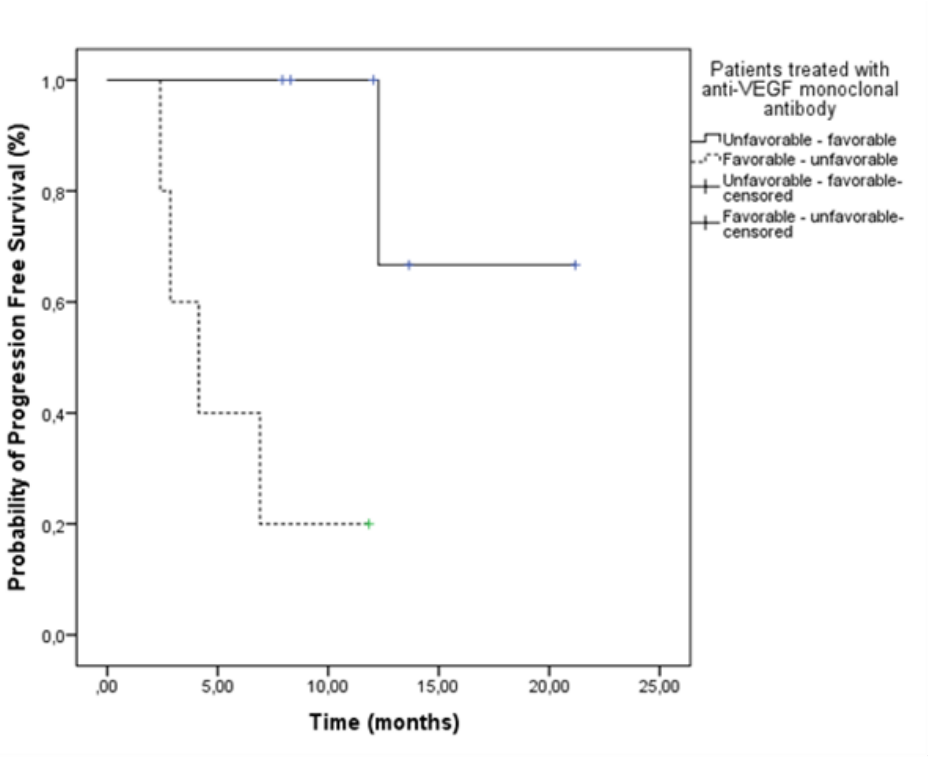

Figure 6: Progression-free survival based on CTCs kinetics and the treatment with anti-VEGF monoclonal antibody. Continuous line: patients treated with anti-VEGF with favorable kinetics (CTC1+ and CTC 2-). Dotted line: patients treated with anti-VEGF with unfavorable kinetics (CTC1- and CTC2 +) (p = 0.007).

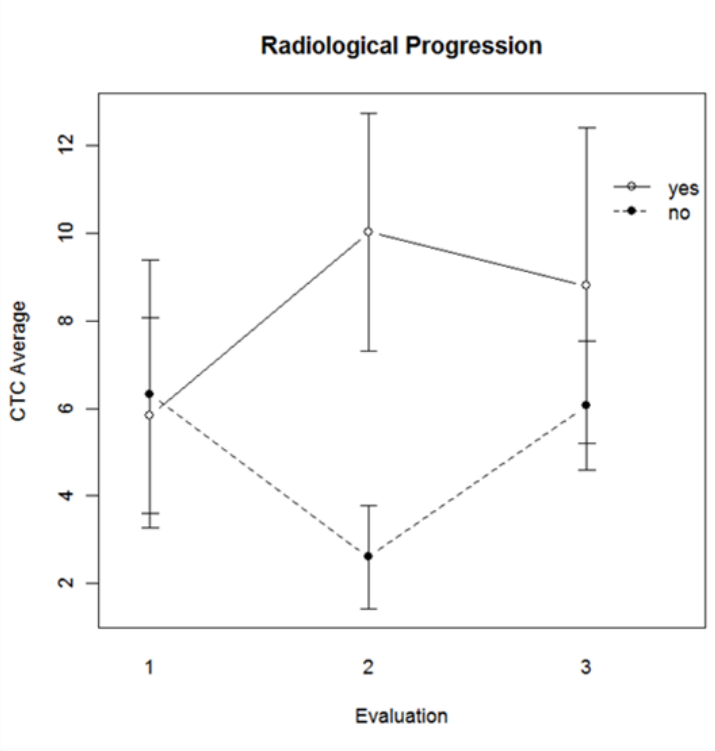

Figure 7: Correlation between CTC quantification and radiological progression. Continuous line: patients with radiological progression and increased CTC quantification in the second collection (CTC2). Dotted line: patients without radiographic progression that did not have higher CTC in the second collection (CTC2) (p = 0.04).

Discussion

Treatment of patients with mCRC in the last ten years has become substantially more complex, extending survival rates of these patients through a therapeutic arsenal composed of the following: fluoropyrimidine, oxaliplatin, irinotecan and targeted therapies (bevacizumab, panitunumab, cetuximab, and regorafenib aflibercept) [25,33]. From this point, oncologists need useful tools to evaluate the intensity and the best sequence of systemic treatment in order to provide patients with access to all potentially effective drugs during the evolution of the patient's disease. The definition of treatment beginning with one, two or three medications, associated or not with

biological agents (anti-VEGF, anti-EGFR and the tyrosine kinase inhibitor) becomes more difficult due to clinical and pathological complexities of mCRC and the absence of non-radiological markers that are useful for defining therapeutic strategies. In this way, evaluating the dynamics of CTCs could guide us in choosing a systemic therapy and predicting the clinical response from favorable and unfavorable patients [34-35].

Our study makes it possible to infer the importance of the evaluation of CTC kinetics as a prognostic marking parameter to differentiate favorable patients with a PFS median of 14.7 months from unfavorable patients with a PFS median of 6.9 months ($p = 0.06$). We attribute the non-significant statistic to the small sample size, although this result correlates with a recent published study that demonstrates a correlation of CTC kinetics in metastatic breast cancer with prognosis and evaluation of response to the treatment adopted, supporting the value of our results [36]. This study with 356 patients demonstrated that patients with favorable CTC kinetics had a higher propensity to respond to the proposed treatment, supporting the evaluation that the dynamic counting of CTCs could better reflect the prognosis and the aggression of the disease than the isolated counting of cells [36]. Cohen and colleagues in their publications on mCRC also demonstrated the importance of CTC kinetics with evidence of increased survival for patients with unfavorable modification to favorable [24,37]. We might consider the possibility that the evaluation of CTC kinetics for patients with mCRC could become an important tool and could be pursued in further studies in order to improve clinical applications.

Although we used a method for isolating and counting CTCs that is still not FDA approved, such as CellSearch System, this method has passed through technical [32,38] and clinical [39] validations and has the advantage of allowing the visualization of quantified cells. ISET is an independent method to isolate CTCs, which allows the cytopathological identification of these cells. It is based on the principle that tumor cells from organs are much larger than leucocytes, with a nuclei of 24 µm or larger, so, they are retained on the ISET filter, whose pores have 8 µm, bigger than the majority of leucocytes [40]. As ISET method is size based, it do not depend on antigen markers and is not expected to have selection bias, false positive (epithelial non-tumor cells detected as tumor cells in healthy patients) and false negative (circulating tumor cells with mesenchymal markers not detected) results, as seen by CellSearch [40].

Even without a defined cut-off for ISET, established in our study to the median CTC number of each phase, it was possible to classify patients as favorable and unfavorable and compare our results with studies that use standardized techniques such as CellSearch ™ System (Johnson & Johnson) [22-23,41-44]. However, we believe that the most important in terms of clinical practice is to observe each patient individually, independently of values of cut-off, and for this propose the ISET method would be more appropriate, as it would enable the quantification of CTCs with better accuracy. Moreover, as CTCs can be directly seen by ISET and counts can be made by ml of blood, it is probably that a cut-off is not necessary, as it

seems that which is mandatory is to follow-up patients based in elevation or decrease of their own CTC counts.

In addition, upon evaluating the KRAS status of patients included in our study, we found that those mutated with unfavorable kinetics had a worse PFS median (2.4 months) compared with those with mutated but favorable kinetics (PFS median 5.3 month p = 0.02). Although our sample was composed of only 30% of the patients in first-line treatment, it is important to correlate this data with the study that evaluated the first-line treatment of 1589 patients with mCRC. In this study, the first count higher than 3 CTCs/7.5 ml, associated with the KRAS mutation presented worse prognosis when these patients were compared with patients with CTC <3/7.5 ml [45]. This correlation allows us to support the importance of the CTC kinetics not addressed in the study and the possibility of evaluating patients previously treated with other lines of therapy. Another important point that should be investigated in further studies is the evaluation of the KRAS wt subgroup with unfavorable kinetics who presented a median PFS of 9.4 months, less than KRAS wt patients with favorable kinetics who had a PFS of 14.7 months (p=0.02). These findings demonstrate that in a subgroup which anti-EGFR blocking could allow better evolution, we are able to identify, based on CTC kinetics, patients who showed a worse response to the EGFR blocking. So, we are able to question if this therapeutic choice in the context of its toxicities and costs could provide substantial benefits for such patients. This data allows us to say that within the subgroups (mutated and non-mutated) it is possible to distinguish the evolution of patients based on CTC kinetics and,

with this, define therapeutic strategies designed for patients with worse prognosis.

Another important point that should be investigated in further studies is the evaluation of treatment with anti-angiogenic therapies that was possible through the use of ISET technique. Although with a limited number of patients, we are the first group to demonstrate that by ISET is possible to follow up patients under treatment with anti-angiogenic therapies. Other authors have demonstrated that by CellSearch isolation, there is a mask in CTC counts when patients receive those treatments[46-48]. Probably it happens because CTCs from patients that are treated with anti-angiogenic therapies lose their epithelial markers, the targets of CellSearch.

Finally, our study has demonstrated that counting CTCs could help us infer that the patients who present disease progression in images during follow-ups present, on average, higher CTC counts in the second phase ($p = 0.04$), making it possible to carefully monitor these patients and discuss the modification of early therapeutic plans. In this way, the analysis of kinetic of CTCs could help in the choice of treatment [21,25,49] and we could modify supposedly ineffective therapeutic schemes, avoiding possible unnecessary side effects, instituting new therapeutic regimes early to better control patient disease. Still, for patients with unfavorable kinetics or those that present higher CTCs during evolution, the more intensive clinical follow-up would be crucial, since this information combined with new clinical systems, the elevation of biological markers and/or clinical deterioration could help in the

modification of treatment, even without evidence of radiological progress at that time.

We conclude that, despite the limited sample, the study could demonstrated the feasibility of a new technique for counting CTCs in mCRC (ISET, Rarecells, France) and reinforced the notion that the evaluation of CTC kinetics could be promising in the clinical follow-up of these patients. In this way, our study proposed a new form of CTC evaluation, unlike isolated analysis in previous studies [23-24,35,50-51]. In addition, in the KRAS wt population, we may have a new tool capable of identifying a population that is genuinely responsive to anti-EGFR therapy. Our results paves the way for the development of further studies that validate this data, which is essential to monitoring the treatment of patients with mCRC.

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4 ARTIGO 2-PUBLICADO-INTERNATIONAL JOURNAL OF CANCER 2015;137:1397-405.

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

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Short title: TYMS in CTCs predict therapy resistance

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Keywords: Thymidylate Synthase; Chemotherapy Resistance; Metastatic Colorectal Cancer; Circulating Tumor Cells; Isolation by Size of Epithelial Tumor Cells.

Article category: Research article – Early detection and diagnosis

Novelty and impact of the work

Circulating Tumor Cells represent a powerful tool to follow-up 5-Fluorouracil resistance in metastatic colorectal cancer patients in a real time manner, by Thymidylate Synthase expression analysis.

Abstract

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) correlating 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.

Introduction

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 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 -20ºC 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. 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]

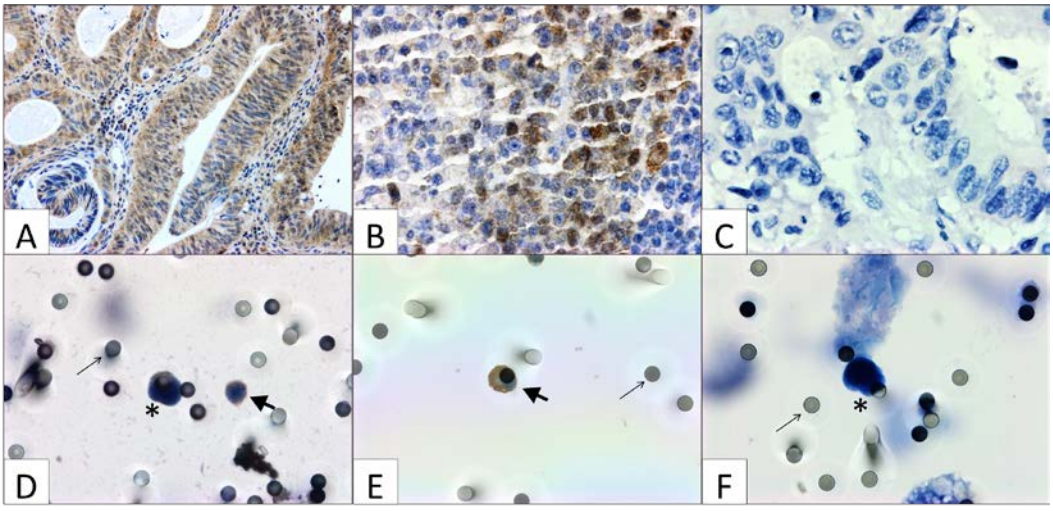

Figure 1. Immunostaining of Thymidylate synthase (TYMS). (A) Primary tumor tissue positive for TYMS. (B) Positive control, a normal palatine tonsil tissue. (C) Negative control, tumor tissue without antibody. (D) CTC TYMS positive. (E) Positive control, a white blood cell. (F) Negative control, a CTC without antibody. Thin arrows represent pores of ISET membrane, thick arrows show leukocytes and asterisks indicate CTCs. Images were taken at x600 magnification using a light microscope (Research System Microscope BX61 – Olympus, Tokyo, Japan) coupled to a digital camera (SC100 – Olympus, Tokyo, Japan).

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 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 4ºC, 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 TM HRP (DakoCytomation, Carpinteria) for 1 hr in the darkroom, and washed with PBS three times for 5 min each. Staining was performed by using 3,30 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 v2 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 who 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.

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 metastasis		
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 (0 - 31.0)	
Median CEA serum level (ng/ml) (range)		
Baseline (49/54)	16.5 (1.1 - 9531)	

Abbreviations: CTC **-** circulating tumor cells; CEA **-** carcinoembryonic antigen.

CTCs count

The median CTCs numbers detected by ISET in all patients was 2 CTCs mL21 (0–31). Out of 54 patients, it was not possible to count two samples due to technical problems (blood coagulation) 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, two samples which suffered coagulation and two lost in immunocytochemistry step)). 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).

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

Patients	Gender	Primary tumor localization	CTC TYMS positive (1 spot)*	CTC count at baseline (per ml)	Disease progression	Time of progression (months)**
1	Male	Colon	2	3.0	No	-
2	Male	Colon	1	19.0	Yes	9.28
3	Male	Rectum	9	5.0	Yes	9.11
4	Male	Rectum	10	11.0	Yes	5.23
5	Male	Colon	3	2.0	Yes	12.27
6	Male	Colon	8	1.0	No	-
7	Female	Rectum	1	6.0	Yes	8.42
8	Male	Colon	39	14.0	No	-
9	Male	Rectum	1	12.0	Yes	1.48

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

We performed also IHC in 29 primary tumor samples (26 positive vs. three negative) and 16 metastasis samples (15 positive vs. one negative). Because of heterogeneity found inside some tumor tissue samples (Figs. 2a 2b and 2c) as well in metastases tissue (Figs. 2d 2e and 2f), we dichotomized the IHC results as 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.

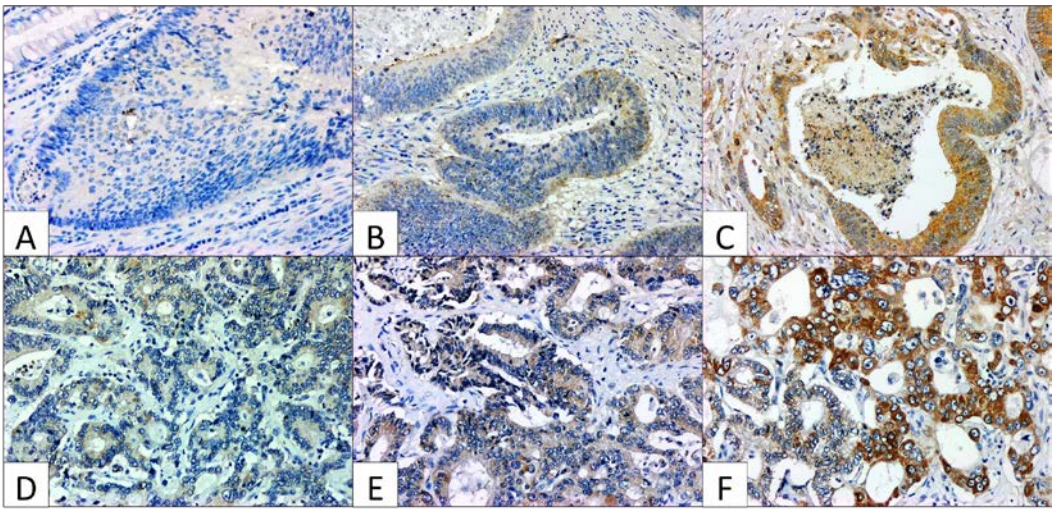

Figure 2. Immunostaining showing heterogeneity of TYMS expression in the same sample. Figures A, B and C present primary tumor from the same patient with no expression, moderate and strong expression of TYMS respectively. Figures D, E and F present metastasis tissue from another patient with different grades of staining (weak, moderate and strong expression of TYMS respectively). Images were taken at x200 magnification using a light microscope (Research System Microscope BX61 – Olympus, Tokyo, Japan) coupled to a digital camera (SC100 – Olympus, Tokyo, Japan).

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

TYMS analysis	Disease Progression		*P* value	CTC count		*P* value
	No (%)	Yes (%)		< 2.0/ml	≥ 2.0/ml	
TYMS staining in CTCs (34)						
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 (29)						
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)*	12 (50)*	
TYMS staining in metastasis tissue (16)						
Negative (1)	0 (0)	1 (100)	0.42	0 (0)	1 (100)	0.46
Positive (15)	6 (40)	9 (60)		8 (57.1)*	6 (42.9)*	

Abbreviations: CTC **-** circulating tumor cell; TYMS **-** Thymidylate synthase. *Two patients tested to TYMS in primary tumor have no CTC count, and one of them have tested TYMS in metastasis tissue also.

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 $\chi 2$ 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 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).

Table 4. Comparison of TYMS expression in CTCs, primary tumors and metastases from CRC 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.

Discussion

There are many studies in literature demonstrating prognostic and predictive values of TYMS presence in primary CRC, however, this role 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 angioinvasion. 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 angioinvasion. 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 reported previously,[36] there are differences between areas inside the tumor and metastasis staining. Because of this intra-tumor 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 CRC40 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 finding 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 cytokeratin (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, nontumor 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 nonmalignant cells expressing EpCAM and CK.[43] De Giorgi et al.[48] showed that by ISET System CTCs were not found 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, pyrosequencing, 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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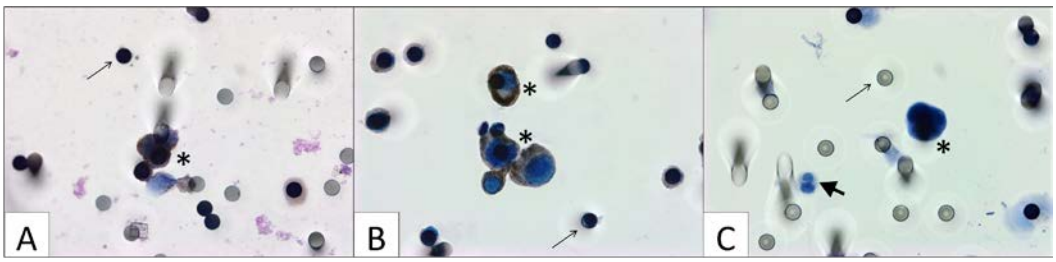
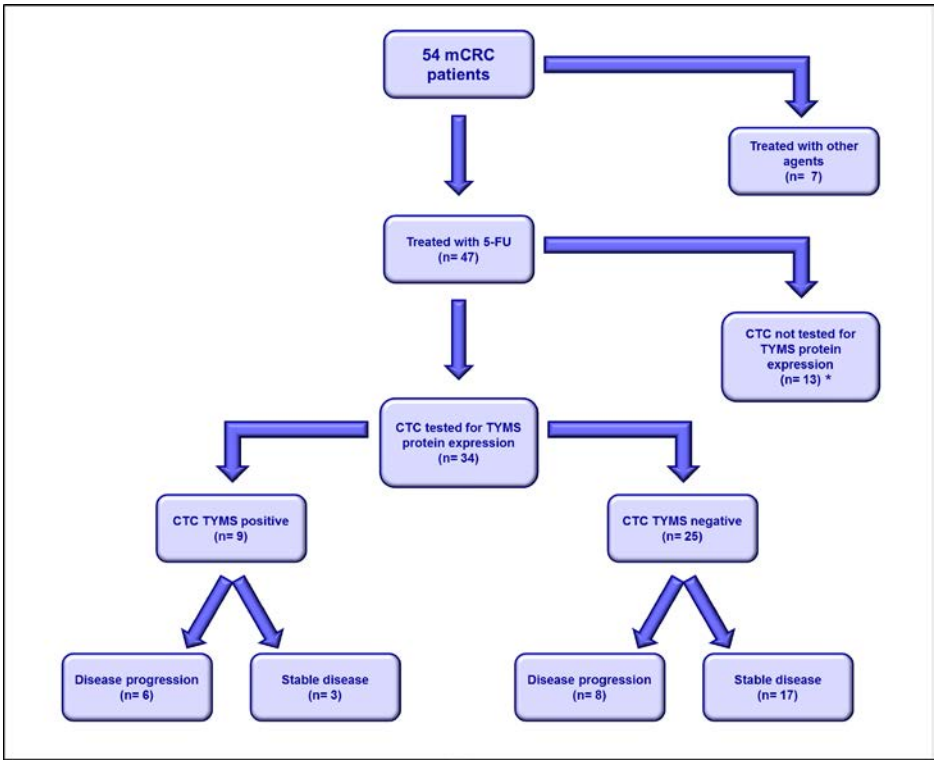
Table S1. Identification of antibodies applied in CTCs and paraffin-embedded tissues with its respective dilutions, clones, suppliers and positive controls.

Antibody	Dilution	Clone	Supplier	Positive Control
Immunocytochemistry				
TYMS	1/230	3A1, monoclonal	Sigma-Aldrich	Leukocytes
CD45	1/100	Polyclonal	Sigma-Aldrich	Leukocytes
CD34	1/100	QBEnd-10, monoclonal	Dako	HUVEC cell line
CK20	1/100	Polyclonal	Sigma-Aldrich	HCT 116 cell line
Immunohistochemistry				
TYMS	1/100	3A1, monoclonal	Sigma-Aldrich	Palatine tonsil tissue
Abbreviations: TYMS - Thymidylate synthase.				



Supplementary Figure 1. Immunostaining with Cytokeratin 20 (CK20). (A) CTC CK20 positive. (B) Positive control, colon cancer cell line (HCT-116) stained with CK20. (C) Negative control (CTC without antibody). Thin arrows represent pores of ISET membrane, thick arrows show leukocytes and asterisks indicate CTCs. Images were taken at x600 magnification using a light microscope (Research System Microscope BX61 – Olympus, Tokyo, Japan) coupled to a digital camera (SC100 – Olympus, Tokyo, Japan).



Supplementary Figure 2. Flow chart of patients enrolled in this study. 54 mCRC patients were included and 47 received 5-FU-based chemotherapy. 34 of them had CTC tested for TYMS protein expression and 9 were found positive. 6/9 patients TYMS positive had disease progression in a median follow-up of 7.9 months. * Patients treated with 5-FU that were not possible to analyze TYMS protein expression: 9 had absence of CTCs and 4 samples due to technical reasons (n= 13). Abbreviations: mCRC – metastatic colorectal cancer; 5-FU – 5-Fluorouracil; CTC – circulating tumor cell; TYMS – Thymidylate synthase.

5 ARTIGO 3 - SUBMETIDO

MRP1 expression in CTCs confers resistance to irinotecan-based treatment in metastatic colorectal cancer

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Short title: MRP1 in CTCs confers resistance to treatment with irinotecan.

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Key Words: circulating tumor cells, metastatic colorectal cancer, ISET, multidrug resistance-associated protein 1, resistance

Article category: Research article – Tumor Markers and Signatures

Novelty and impact of the work: Circulating tumor cells can predict irinotecan resistance in metastatic colorectal cancer patients by their Multidrug Resistance-associated Protein 1 expression.

ABSTRACT

Circulating tumor cells are important markers of tumor progression and can reflect tumor behavior in metastatic colorectal cancer (mCRC). Identification of proteins that confer resistance to treatments is an important step to predict response and better select treatment for patients. Multidrug resistance-associated protein 1 (MRP1) and Multidrug resistance-associated protein 4 (MRP4) play a role in irinotecan-resistance, and Excision Repair Cross-Complementation group 1 (ERCC1) expression can confer platinum resistance. Here, we looked for immunocytochemical expression of MRP1, MRP4 and ERCC1 in CTCs isolated by ISET Technology from mCRC patients (n=19, 15 and 13 patients, respectively). Most of the patients included received FOLFIRI or FOLFOX chemotherapy (91.1%). We included 34 patients, and CTCs were identified in 30 (88.2%). The 4 out of 19 cases with MRP1 positive CTCs showed worse progression free survival (PFS) (2.1 months vs. 9.1 months; *P*= 0.003). None of the other proteins in CTCs had significant association with PFS. We analyzed also histological sections of primary tumors and metastases by immunohistochemistry, and found no association with clinicopathological characteristics. Our results show MRP1 as a potential biomarker of resistance to treatment with irinotecan and as consequence, worse prognosis, when found in CTCs from colorectal cancer patients.

INTRODUCTION

Colorectal cancer (CRC) is the third most common tumor in men and the second in women worldwide[1]. Although there are standard agents and strategies to this type of neoplasia, there are patients that, even responding to chemotherapy initially, become resistant. Circulating Tumor Cells (CTCs) are reported to cause distant micrometastasis and are a prominent investigation in the field. Previous studies have demonstrated that high CTCs level is correlated with poor progression free survival and overall survival (OS) in many neoplasms[2-4].

In a previous work[5], we verified Thymidylate Synthase (TYMS) expression in CTCs from patients treated with FOLFIRI/FOLFOX/5-Fluorouracil (5-FU). All of them received 5-FU-based chemotherapy, so we focused in TYMS expression. However, we observed that patients without TYMS expression in their CTCs also had disease progression after a period of treatment. Therefore, we decided to look in these CTCs the expression of other proteins that could be responsible for drug resistance through different mechanisms.

Multidrug Resistance (MDR) is a relevant phenomenon that can occur in cancer cells, as per overproducing drug-transporting proteins, increasing efflux of a broad class of hydrophobic cytotoxic drugs[6]. Multidrug resistance proteins (MRP) belong to the ATP binding cassette (ABC) transporter family[7] located to the cell membrane and act modulating absorption, distribution and excretion of many chemical compounds. MRPs expel cytotoxic molecules, like chemotherapeutic agents and protect the target cell from death. The multidrug resistance-associated protein 1 (MRP1; *ABCC1* gene) has been

described as a contributor to transport folate-based antimetabolites, anthracyclines, plant alkaloids, antiandrogens and camptothecins, such as irinotecan and topotecan[8-10]. MRP4 belongs to the same family of drug transporters; however, it confers resistance to irinotecan, topotecan and methotrexate[9]. Excision repair cross-complementation group 1 (ERCC1) is a protein involved in DNA damage and repair, that is described to confer resistance to platinum-based chemotherapy[11,12]. Thereby, these three proteins cooperate to tumor resistance to agents most usually combined with 5-Fluorouracil, in the treatment of metastatic colorectal cancer (mCRC) patients: oxaliplatin and irinotecan.

We hypothesize that by analyzing these proteins in CTCs, primary tumors and metastasis, we can identify good responders to chemotherapy in mCRC patients.

Therefore, our objective was to verify if the expression of multidrug resistance proteins MRP1, MRP4 and ERCC1 in CTCs, primary tumors and metastasis by immunocytochemistry/immunohistochemistry can predict response to FOLFIRI or FOLFOX chemotherapy in mCRC patients, combined or not with monoclonal antibodies.

MATERIALS AND METHODS

Prospective study made by blood collection (8 mL) from patients with metastatic CRC, to isolate CTCs and search for MRP1, MRP4 and ERCC1 proteins staining in CTCs and in paraffin embedded tissues (primary tumor and metastasis) from the same patients recruited.

Patients and samples

Blood from mCRC patients who previously signed the informed consented at Clinical Oncology Department of A.C. Camargo Cancer Center, São Paulo, Brazil were prospectively collected from July 2012 to December 2013. Blood was collected (8mL) in EDTA tubes before the beginning of irinotecan/oxaliplatin/5-FU-based chemotherapy (at diagnosis of metastases, disease progression or protocol change). Inclusion criteria were stage IV CRC patients who had initiating chemotherapy treatment with ECOG 0-2 without organ dysfunction, disease measureable by RECIST (Response Evaluation Criteria in Solid Tumors) criteria (version 1.1)[13]. Patients who had been submitted to any surgical procedure within 3 weeks before CTC detection were excluded.

CTC Isolation

We isolated CTC using the ISET method as previously reported[5]. In summary, 8 ml blood was diluted with the ISET buffer, then transferred to the ISET block and filtered. Membranes were maintained at -4°C until time of immunostaining analysis. CTCs fixed on spots from ISET membranes were stained by immunocytochemistry and counterstained with hematoxylin. CTCs were counted in 4 spots of the membrane and quantified in 1 ml of blood in accordance to Krebs et al[14].

CTC Immunostaining

To characterize CTCs as resistant or sensitive to treatment, we performed an immunocytochemistry assay using a protocol previously described[5]. The

following antibodies were chosen accordingly to the treatment: anti-MRP1 (ABCC-1, Polyclonal, Sigma 1:100, code: HPA002380), and anti-MRP4 (ABCC-4, Polyclonal, Sigma 1:100, code: HPA002476) were used in order to verify if CTCs from patients who underwent irinotecan/5-FU-based chemotherapy expressed these proteins on cell surface or cytoplasm. We used anti-ERCC1 (Polyclonal, Sigma-Aldrich 1:100, code: SAB4500795) in CTCs from patients who underwent oxaliplatin/5-FU-based chemotherapy. For all reactions and all antibodies (MRP1, MRP4 and ERCC1), we used A-549 cell line, accordingly to The Human Protein Atlas (http://www.proteinatlas.org/)[15] expressing these proteins, “spiked” in healthy donor and filtered on ISET as a positive control (Figures 2B, 2F, 2J). To confirm that cells analyzed were not leucocytes, we used anti-CD45 antibody (Polyclonal, Sigma 1:100, code: HPA000440). The detailed information about antibodies is shown in Supplementary Table 1.

The evaluation of the immunostaining results was made manually on Research System Microscope BX61 – Olympus coupled to SC100 high-resolution digital color camera – Olympus.

Immunohistochemistry

The primary tumors paraffin blocks were obtained from A.C. Camargo Cancer Center Tissue Bank Archive. Slides obtained from these blocks were submitted to IHC staining. All reactions were accompanied by a positive control, in known positive tissue for each antibody (Supplementary Table 1), and a negative control (removal of the primary antibody and withdrawal of the secondary complex).

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

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

The primary antibody was diluted in diluent containing 0.05 mol/L Tris-HCl buffer and 0.1% Tween 20 (Antibody Diluent with Background Reducing Components, DakoCytomation, Carpinteria, USA) and the slides were incubated at 4° C, overnight (12-14 hours) in a humid chamber. After three washes with a PBS 1X buffer for 5 minutes 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 TM HRP (DakoCytomation, Carpinteria, USA) for one hour in the darkroom, and washed with PBS three times for 5 minutes each. Staining was performed by using 3,3' diaminobenzidine tetrachloride (DakoCytomation, Carpinteria, USA). 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 coupled to C100 high resolution digital color camera – Olympus.

Statistical analysis

A descriptive analysis was done for each clinical-pathological variable and group of treatments. To evaluate differences between groups (those that expressed MRP1 and those that did not express), the $\chi 2$ test was used for variable categories and Fisher's exact test was used for small numbers. Survival curves were analyzed by Kaplan-Meier method and the difference between curves was calculated by the Log Rank test. The Cox proportional hazards regression model was used to run the multivariate analysis. All statistical analyses were performed using the SPSS program for Windows, version 15. The P values were considered significant when ≤ 0.05.

RESULTS

Patients

Thirty-four patients who underwent FOLFIRI- or FOLFOX-based chemotherapy were included, wherein 19 patients received FOLFIRI, 10 patients received FOLFOX regimen; two patients received only Irinotecan with or without monoclonal antibodies. Three patients received other agents (capecitabin plus irinotecan, capecitabin plus oxaliplatin and Regorafenib). The clinicopathological characteristics are available in Table 1. Twenty patients (58.8%) were male and primary tumor were predominantly found in the colon (n= 26; 76.5%). All tumors were classified as adenocarcinomas and 89.7% (29/34) of the patients with data available were classified as moderately differentiated. Twenty-two patients (64.7%) were treated with monoclonal antibodies wherein 8 (23.5%) used cetuximab and 14 (41.2%)

used bevacizumab. Twenty two out of 34 patients (64.7%) were submitted to two or more lines of therapy before the blood drawn used for the CTC assay. The mutational status of KRAS in the tumor was available in 31 medical records and 38.7% (n= 12) were found as KRAS mutated. Median follow-up time according to RECIST criteria was 8.4 months (95% CI 6 – 10.8 months).

Table 1. CRC patients' clinicopathological characteristics.

Variable	**Nº.**	**%**
Total number of patients	34	100
Age at entry study, years		
Median (range)	54.5 (30 - 81)	
Gender		
Male	20	58.8
Female	14	41.2
Location of primary tumor		
Colon	26	76.5
Rectum	8	23.5
Histological grade (data available in 29/34 patients)		
Well-differentiated	3	10.3
Moderately differentiated	26	89.7
Treatment		
FOLFIRI	19	55.9
FOLFOX	10	29.4
Irinotecan	2	5.9
Other*	3	8.8
Cetuximab		
Yes	8	23.5
No	26	76.5
Bevacizumab		
Yes	14	41.2
No	20	58.8
Line of chemotherapy		
First	12	35.3
Second or more	22	64.7
KRAS status (data available in 31/34 patients)		
Wild-type	19	61.3
Mutated	12	38.7
Median CTC/ml number (range)		
Baseline	2.3 (0 - 31.25)	
Median CEA serum level (ng/ml) (range)		
Baseline (30/34)	15.2 (1.1 - 9531)	

Abbreviations: CTC - circulating tumor cells; CEA - carcinoembryonic antigen.
*Other agents: XELIRI, XELOX and Regorafenib.

CTCs count and CTCs protein expression

CTCs counts were performed in all patients (n= 34) and in 4 (11.7%) no CTCs were found. The median number of CTCs per mL was 2.0 (0 – 31.0).

Immunocytochemistry was performed in patients who have previously detectable CTCs, and each antibody was tested accordingly with the treatment received by the patients. MRP1 was tested in 19 samples by immunocytochemistry and found positive in 4 (Figure 2A). Patients who had CTC MRP1 positive had shorter progression free survival (PFS) when compared with the patients with CTC MRP1 negative, 2.1 vs. 9.1 months (*P*= 0.003) as demonstrated in Figure 1A.

We analyzed the effect of MRP1+ CTCs on disease progression and time to progress for each patient included in the present study. As it can be seen in Table 3, we found 44% of concordance with these data (we did not analyze the case 3, because of loss of follow-up). We also observed that the median number of days for disease progression to patients with MRP1- CTC was 262 days, compared to 85 days in the MRP1+ CTC group. Of the 4 MRP1 positive patients, three (75%) had colon as primary tumor site and were KRAS mutated (75%). Two patients with both MRP1 positive and KRAS mutated progressed to death in an average time of 96 days.

Regarding MRP1- cases, 10 out of 15 (66.6%) had colon as primary tumor site. Four (26.6%) of them were KRAS mutated and only two (13.3%) progressed to death (average time of 267 days).

Concerning some bias of analyzing the same population of a work previously reported[5], we performed a Fisher's exact test in order to verify if the patients

resistant to irinotecan (who expressed MRP1 in CTCs) belonged to a subpopulation of the patients resistant to 5-FU (who expressed TYMS in CTCs). We found that the resistant population differed between these two groups (*P*= 0.54). None of patients CTC positive for MRP1 had also CTC positive for TYMS.

We attempted to detect MRP4 expression in CTCs for patients treated with irinotecan, and ERCC1 for patients treated with oxaliplatin and found 5 out of 15 (33.3%) and 6 out of 13 (46.1%) respectively (Figure 2E and 2I). None of these proteins had significant association with progression-free survival or any clinical-pathological characteristics. Despite the lack of statistical significance between the PFS curves for these two markers, we can see in Table 2 that the median time to progression was shorter in patients with positive CTCs than in patients with negative CTCs in both, MRP4 and ERCC1. In the case of CTCs MRP4 negative patients, the SLP increased two fold (7.5 vs. 3.4 months respectively; *P*= 0.64) comparing to the positive ones.

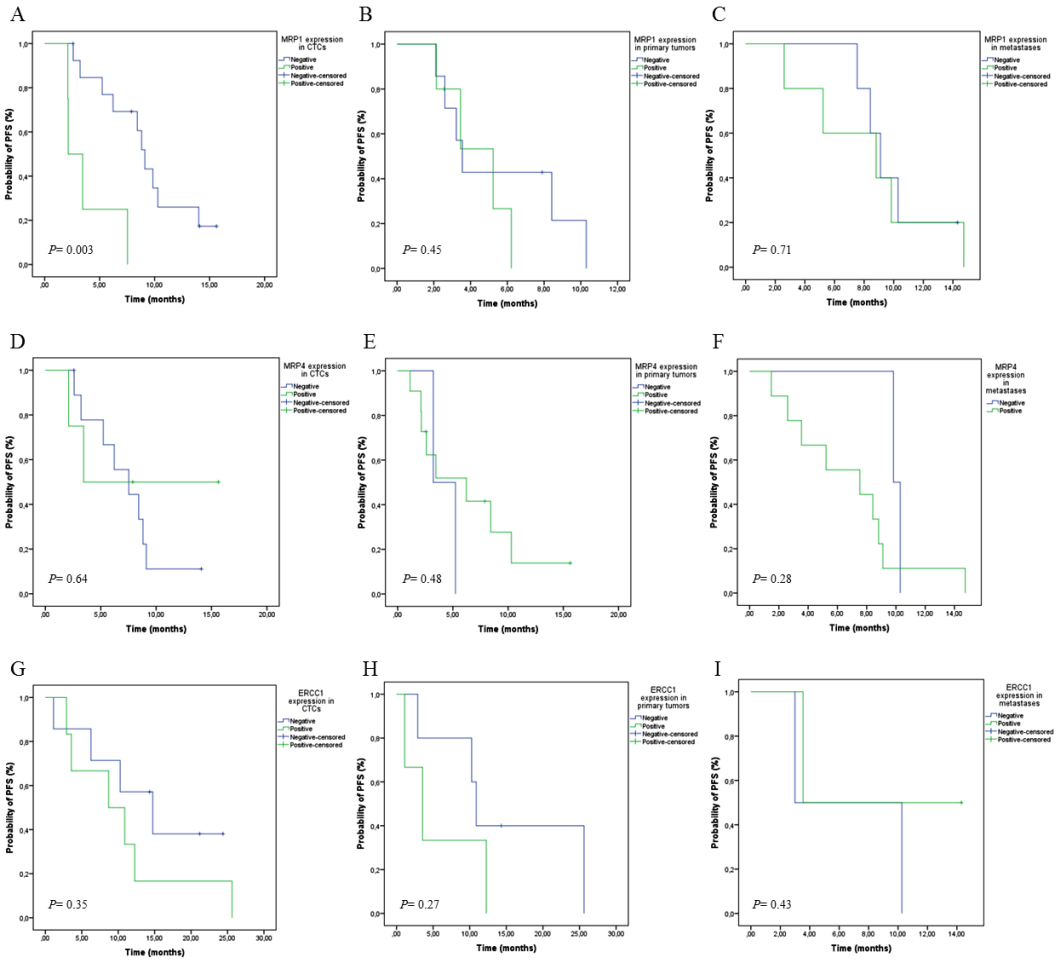

Figure 1. Progression free survival (PFS) of mCRC patients in relation to protein expression in CTCs. These cells were tested by immunocytochemistry for MRP1, MRP4 and ERCC1 antibodies, before the beginning of chemotherapeutic treatment. **A)** CTCs negative for MRP1: median PFS of 9.1 months vs. CTCs positive to MRP1: 2.1 months; P= 0.003. **B)** Primary tumors negative for MRP1: median PFS of 3.5 months vs. primary tumors positive for MRP1: 5.2 months; P= 0.45. **C)** Metastases negative for MRP1: median PFS of 9.1 months vs. metastases positive for MRP1: 8.8 months; P= 0.71. **D)** CTCs negative for MRP4: median PFS of 7.5 months vs. CTCs positive to MRP4: 3.4 months; P= 0.64. **E)** Primary tumors negative for MRP4: median PFS of 3.2 months vs. primary tumors positive for MRP1: 6.2 months; P= 0.48. **F)** Metastases negative for MRP4: median PFS of 9.8 months vs. metastases positive for MRP4: 7.5 months; P= 0.28. **G)** CTCs negative for ERCC1: median PFS of 14.7 months vs. CTCs positive to ERCC1: 8.6 months; P= 0.35. **H)** Primary tumors negative for ERCC1: median PFS of 10.8 months vs. primary tumors positive for ERCC1: 3.5 months; P= 0.27. **I)** Metastases negative for ERCC1: median PFS of 3 months vs. metastases positive for ERCC1: 3.5 months; P= 0.43.

Table 2. Median PFS (months) of negative and positive patients for the markers in CTCs, primary tumors and metastases.

CTC	MRP1-	MRP1+	*P*	MRP4-	MRP4+	*P*	ERCC1-	ERCC1+	*P*
Primary tumor	9.1	2.1	0.003	7.5	3.4	0.64	14.7	8.6	0.35
Metastasis	3.5	5.2	0.45	3.2	6.2	0.48	10.8	3.5	0.27
	9.1	8.8	0.71	9.8	7.5	0.28	3	3.5	0.43

Table 3. Patients' outcome according to CTC prevision by MRP1 staining (n= 19).

Patient ID	Tumor site	Treatment regiment	MRP1 assay	CTC prevision	Follow-up	CTC count/mL	Time to DP (days)
1	Rectum	FOLFIRI + Cetuximab	-	Sensitivity	SD	0.6	-
2	Rectum	FOLFIRI	-	Sensitivity	DP	11.2	159
3	Colon	Irinotecan + Cetuximab	-	Sensitivity	*	10	-
4	Rectum	FOLFIRI + Bevacizumab	-	Sensitivity	DP	5.9	256
5	Colon	FOLFIRI + Bevacizumab	-	Sensitivity	DP	1.6	426
6	Colon	FOLFIRI	-	Sensitivity	SD	13.7	-
7	Colon	FOLFIRI + Bevacizumab	-	Sensitivity	DP	1.2	98
8	Colon	FOLFIRI + Cetuximab	-	Sensitivity	DP	4.6	189
9	Colon	FOLFIRI + Cetuximab	-	Sensitivity	SD	4.3	-
10	Rectum	FOLFIRI + Bevacizumab	-	Sensitivity	DP	0	268
11	Colon	Irinotecan + Cetuximab	-	Sensitivity	DP	0.8	299
12	Colon	FOLFIRI	+	Resistance	PD	1.6	64
13	Colon	FOLFIRI + Bevacizumab	+	Resistance	PD	1.3	65
14	Colon	FOLFIRI + Cetuximab	-	Sensitivity	DP	0	313
15	Rectum	FOLFIRI + Bevacizumab	-	Sensitivity	SD	0	-
16	Colon	FOLFIRI + Bevacizumab	-	Sensitivity	DP	2.5	277
17	Colon	FOLFIRI + Cetuximab	+	Resistance	PD	11.8	105
18	Rectum	FOLFIRI + Bevacizumab	+	Resistance	PD	0.8	229
19	Colon	FOLFIRI + Bevacizumab	-	Sensitivity	DP	1.2	79

Immunohistochemistry

To verify MRP1 expression in tumors specimens, we analyzed 12 available tumor samples and 10 available metastasis samples by immunohistochemistry. We found cytoplasmic MRP1 staining in 5/12 (41.6%) of the primary tumors (Figure 2C), and in 5/10 (50%) of the metastases samples (Figure 2D), with no association to clinic-pathological characteristics or PFS. Considering the median time to disease progression, patients with MRP1 positive in primary tumors had better PFS than those MRP1 negative , although without statistical significance, probably because of the sample size (5.2 vs. 3.5 months respectively; *P*= 0.45). On the other hand, in metastasis, the expression of MRP1 did not change the PFS (8.8 vs. 9.1 months; *P*= 0.71).

Regarding MRP4 expression in tumors specimens, we evaluated 13 primary tumors samples 11 metastases samples. We found cytoplasmatic staining in 11/13 (84.6%) of primary tumors (Figure 2G) and in 9/11 (81.8%) of metastases tissues (Figure 2H); in both cases, there was little difference in PFS between those negative and positive staining, as can be seen in Table 2.

In the case of ERCC1, we have analyzed 8 primary tumors and 4 metastatic tissues. We have found 3/8 (37.5%) of primary tumors positive (Figure 2K), and 2/4 (50%) of metastases tissues positive (Figure 2L). Although having fewer samples analyzed for this marker, we could demonstrate without statistical significance, higher similarity concerning PFS between CTC and primary tumor, wherein patients negative for ERCC1 had longer PFS (CTC:

14.7 months; primary tumor: 10.8 months) compared with those patients positive for ERCC1 (CTC: 8.6 months; primary tumor: 3.5 months). In metastasis setting, the time did not change between the patients negative and positive for ERCC1 (3 vs. 3.5 months; *P*= 0.43).

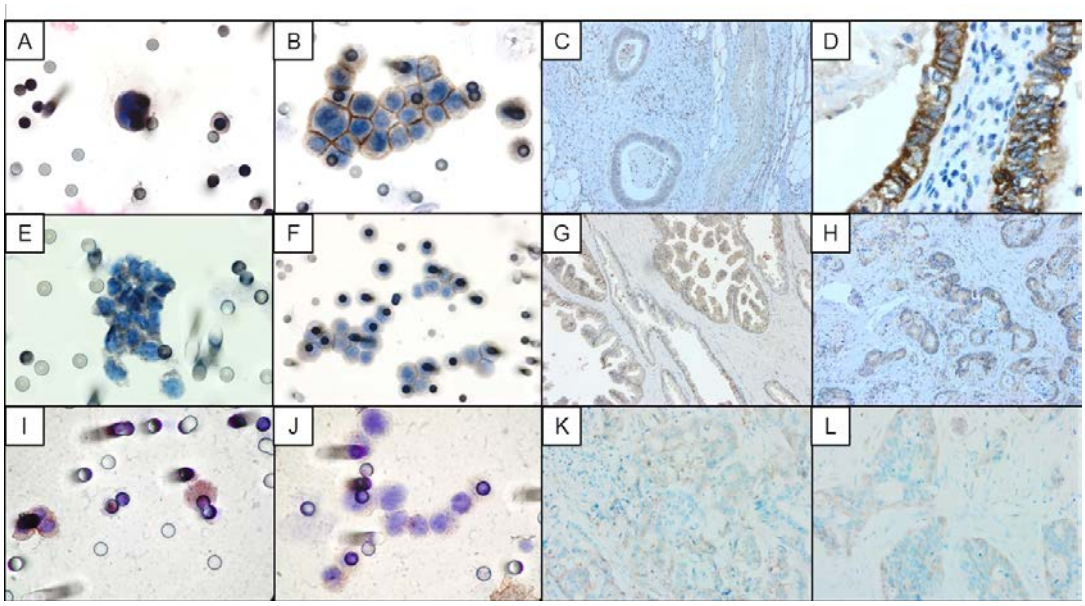

Figure 2. Immunostaining of CTCs, positive controls, primary tumors and metastases specimens. **A)** CTC from mCRC patient positive for MRP1 (x60). **B)** Positive control, A-549 cell line "spiked" in healthy blood and stained for MRP1 (x60). **C-D)** Primary tumor (x10) and metastasis (x60) positive for MRP1. **E)** Microemboli from a mCRC patient positive for MRP4 (x40). **F)** Positive control, A-549 cell line "spiked" in healthy blood and stained for MRP4 (x20). **G-H)** Primary tumor (x10) and metastasis (x10) positive for MRP4. **I)** CTCs from mCRC patient positive for ERCC1 (x60). **J)** Positive control, A-549 cell line "spiked" in healthy blood and stained for ERCC1 (x60). **K-L)** Primary tumor (x20) and metastasis (x20) weakly positive for ERCC1. All photomicrographies were taken using a light microscope (Research System Microscope BX61—Olympus, Tokyo, Japan) coupled to a digital camera (SC100—Olympus, Tokyo, Japan).

Comparison between the expression of MRP1, MRP4 and ERCC1 in CTCs, primary tumors and metastases

We could compare the expression of MRP1 in the three sites (CTC, primary tumor, and metastases) in four patients and found 50% (2/4) of concordance between them. Looking only at MRP1 expression in CTCs and primary tumors (n= 11), we observed 63.6% (7/11) of concordance. When we looked into CTCs and metastases (n= 8), the concordance decreased to 37.5% (3/8).

For MRP4, only three patients had the three sites evaluated for immunostaining and none of them had concordance. The same was observed in comparison between CTCs and metastases (n= 6), none of the patients expressed concordant MRP4 in these sites. On the other hand, looking into the expression of MRP4 in CTCs and primary tumors (n= 10), we found 60% (6/10) of concordance.

We made the comparison between the three sites for ERCC1 expression in three patients and found 66.6% (2/3) of concordance. Interestingly, these numbers remained the same when we compared CTCs with metastases (n= 3; 66.6%). Focusing in the comparison between CTCs and primary tumors, the number of patients analyzed increased (n= 8) and we found 50% (4/8) of concordance.

Multivariate analysis

We used the Cox proportional hazards regression model to run the multivariate analysis. We included those variables with the *P* value >0.25 in

the PFS analysis. There were included: the CEA levels at CTC collection (cut-off: 5 ng/mL; *P*= 0.21), the lines of chemotherapy before the CTC collection (*P*= 0.07), number of metastatic sites (1 vs. 2 or more; *P*= 0.01); MRP1 expression in CTCs (*P*= 0.003), and KRAS status in the primary tumor (*P*< 0.001). We verified that number of metastatic sites remained as independent prognostic factor to disease progression (HR: 5.9; *P*= 0.04) (Table 4).

Table 4. Multivariate analysis showing number of metastatic sites as an independent prognostic factor.

Variable	Category	HR	CI (95%)	*P* value
KRAS status	Wild-type	1	–	
	Mutated	2.9	0.7 – 11.3	0.11
MRP1 expression in CTCs	Negative	1	–	
	Positive	2.8	0.6 – 13.0	0.16
Number of metastatic sites	One	1	–	
	Two or more	5.9	1.0 – 34.0	0.04

DISCUSSION

So far, there are described 49 members of the ATP-binding cassette transporter family, which are grouped into seven subfamilies[16,17]. These membrane proteins have been reported as strong contributors to treatment failure, in different tumor types, such as breast cancer[18], primary central nervous system lymphoma[19], laryngeal squamous cell carcinoma[20] and also colorectal carcinoma[17,21]. ABC transporters have also been reported as important biomarkers in neuroblastomas[22] and glioblastomas[23], because of its capability to actively extruding a wide sort of drugs and/or its respective

substrates out of the cell. Specifically, in healthy tissues, MRP1 is extensively involved in the protection of cells from toxic xenobiotics and endogenous metabolites[24].

MRP1 protein analysis can be useful in clinical practice to select patients who will not benefit from irinotecan-based chemotherapy. Irinotecan is a topoisomerase I inhibitor, a semi-synthetic drug derived from camptothecin[25]. It is the conventional option combined with 5-FU and folic acid (the FOLFIRI regimen), for first- and second-line treatment of mCRC[26].

Our results on MRP-1 expression in CTCs brought out some interesting information. We found a significant better PFS in patients that do not express MRP1 (9.1 vs. 2.1 months; *P*= 0.003). It shows that in these compartment of the tumor (liquid biopsy), MRP-1 functions in favor of disease progression, removing the drug from the cells that are capable of migrating, cells may be responsible for tumor seeding in distant organs. Although a small percentage of CTCs is MRP1 positive (21%), this information is strongly predictive of worse PFS. Our results corroborate previous studies in the literature. Gazzaniga et al.[9] investigated this marker in CTCs using a combination of immunomagnetic separation and reverse transcriptase polymerase chain reaction (RT-PCR) and performed a molecular prediction in conformity to individually chemosensitivity profile.

They found that no MRP1 expression was associated with improved chemotherapy response. The same group[27] evaluated MRP1 and MRP2 messenger RNA expression in CTCs by RT-PCR from metastatic breast cancer patients treated with conventional anthracyclines or nonpegylated

liposomal doxorubicin, and observed that patients who had CTC with higher levels of these markers showed a significantly shorter PFS. The expression of genes related to drug sensitivity has been considered the next step towards the real personalized medicine. The higher levels of expression of MRP1 and MRP2 in colorectal tumors in comparison with normal tissues were already reported[17].

Here, we also analyzed MRP1 expression by immunohistochemistry in primary tumors specimens and the findings did not correlate with PFS such as observed in CTCs. Similarly, Moureau-Zabotto et al.[28], investigated by semiquantitative RT–PCR the expression of MRP1, MDR1 (Multidrug Resistance Protein 1) and GSTP1 (Glutathione S-Transferase Pi 1) in frozen samples of breast cancer tissue and did not find impact in neither PFS nor OS, excepting for GSTP1. Burger et al[29] evaluated mRNA levels of BCRP (breast cancer resistance protein), LRP (Lung Resistance-Related Protein), MRP1, MRP2 (Multidrug Resistance-associated Protein 2), and MDR1 in breast tumors. They found a correlation between the expression of BCRP and MRP1 and clinical outcome in the patients treated with anthracycline-based chemotherapy. Regarding colorectal cancer, Micsik et al.[30], measured functional activity of MDR1 and MRP1 by modified calcein-assay in primary colorectal tumors and compared to normal mucosa. They found no difference in MRP1 activity between these sites. Curiously, the activity of MDR1 was increased in normal mucosa when they compared with tumors. Altogether, these results show that maybe the primary tumor is not the best compartment to be analyzed when drug resistance has to be tested. Our

findings suggest that CTCs is the better cell to inform about drug resistance in mCRC.

Reinforcing the concept of different resistance mechanisms, we previously carried out a subpopulation of this study for the presence of TYMS staining in CTCs, to associate with tumor resistance to 5-FU agents[5]. Interestingly, we found that none of the patients irinotecan-resistant belonged to the population of 5-FU resistant (*P*= 0.54), indeed highlighting the different mechanisms of resistance.

Curiously, 75% of patients with CTCs positive for MRP1 had also KRAS mutated (the reason why they could not be treated with cetuximab) and patients CTC MRP1 negative were prevalently KRAS wild-type (69.3%). It reinforces the strong predictive value of this drug transporter protein. This finding could suggest an interaction between these pathways, and more studies are necessary to address if any connection really exists.

We compared the expression of MRP4 and ERCC1 proteins in CTCs, primary tumors and metastases specimens. We observed a two-fold increased PFS in patients who had CTCs negative for MRP4 compared with those positive (7.5 vs. 3.4 months, respectively; *P*= 0.64). The inverse result was seen in patients with primary tumors negative for this protein. For metastases, we found an increase of 2.3 months of PFS in the patients negative for MRP4 in relation to those positive (9.8 vs. 7.5 months, respectively; *P*= 0.28). MRP4 is able to transport drugs, and plays multiple physiological functions, as protecting the brain from cytotoxic effects from topotecan[31]. Gazzaniga et al.[9] verified by RT-PCR the MRP expression

profile in CTCs, including MRP1 as cited above, and also MRP4 in CTCs from patients treated with irinotecan. They showed no MRP4 expression in three patients who responded and weak MRP4 expression in three patients who did not respond to treatment with irinotecan, indicating that maybe this protein can be a good indicator of response to this chemotherapy.

Although also not statistically significant, ERCC1-negative CTCs and primary tumors had better PFS when compared with the positive ones (14.7 vs. 8.6 months; 10.8 vs. 3.5 months, respectively). In the case of metastasis, the time of PFS was almost the same. ERCC1 has been demonstrated in the literature as a potential biomarker to select responders to treatment with platinum-based chemotherapy, mainly in Non-Small Cell Lung Cancer (NSCLC), when evaluated in tumors specimens[32,33]. Focusing in CTCs, ERCC1 was already evaluated in NSCLC[11], in breast cancer[34], and in ovarian cancer[12], looking this expression in patients treated with platinum. The role of ERCC1 in CTCs is still controversial. Somlo et al.[34] found poor correlation between ERCC expression in CTCs and primary tumors (N=11), and also between primary tumor and metastasis (N=8). On the other hand, Das et al.[11] verified that the expression of ERCC1 in CTCs was correlated with worst PFS in NSCLC ($P< 0.02$, HR: 4.2). Kuhlmann et al.[12] analyzed the expression of ERCC1 in CTCs and in primary tumors of patients with ovarian cancer. Patients with CTCs positive for ERCC1 had poor PFS and OS, when compared with CTCs negative ($P= 0.026$ and $P= 0.009$, respectively). The expression of ERCC1 was correlated with platinum resistance ($P= 0.01$).

Altogether, our results have some limitations concerning the number of samples analyzed as well the availability of tumor tissues from primary and metastatic sites. Even considering these limitations, we could show a consistent data, mostly, in relation to CTC´s isolation and analysis. CTCs have a very heterogeneous and plastic phenotype. However, the methodology we used here to isolate and to characterize these cells (ISET, Rarecells, France) is considered feasible, as we isolate them in a marker independent manner and by this, do not lose cells due to downregulation of epithelial markers during epithelial-to-mesenchymal transition process that CTCs can suffer[35].

In the genomic research era, the personalized care challenge persists. It is necessary to understand tumor biology and chemotherapeutic resistance to overcome tumor heterogeneity. Here, knowing all limitations of our hypothesis generating study, we advocate that CTCs is a feasible and non-invasive manner to better select treatment for patients with mCRC. We believe that is urgent to make studies with large cohorts of patients to define the real role of CTCs and the expression of MRP-1 in these cells in the clinical practice.

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CONFLICT OF INTEREST

The authors have declared no conflicts of interest.

Supplementary table 1. Identification of antibodies used in CTCs and paraffin embedded tissues (tumors and metastases), the dilutions, clones, manufacturer and positive controls.

Antibody	Dilution	Clone	Code	Manufacturer	Positive control CTC	Positive control tissue
MRP-1 / ABCC1	1/100	Polyclonal	HPA002380	Sigma	A-549 cell line	Normal bowel
MRP-4 / ABCC4	1/100	Polyclonal	HPA002476	Sigma	A-549 cell line	Normal prostate
ERCC1	1/100	Polyclonal	SAB4500795	Sigma-Aldrich	A-549 cell line	Normal or tumor lung
CD-45	1/100	Polyclonal	M7165	Sigma-Aldrich	Leucocytes	-

6 CONCLUSÃO

- Com o presente trabalho, pudemos verificar que avaliar a cinética de CTCs pode ser uma maneira efetiva de monitorar a progressão da doença, quando os níveis isolados de CTCs por si só não fornecem informações prognósticas.
- Conseguimos, também, traçar um perfil de pacientes que serão resistentes a alguns agentes quimioterápicos. Encontramos a expressão de TYMS nas CTCs associada à resistência ao tratamento com 5-FU.
- Pacientes tratados com quimioterapia a base de irinotecano cujas CTCs expressaram MRP1 tiveram pior sobrevida livre de progressão quando comparados àqueles com CTCs negativas para esta proteína.
- Este estudo demonstrou que as CTCs podem ser um retrato da doença em seu estado atual, tanto pela análise de seus níveis, quanto pela caracterização do perfil de resistência às drogas. Mais estudos são necessários para aprofundamento neste assunto, que continua sob investigação em nosso laboratório.

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Anexo 1 – Carta de aprovação do Comitê de Ética em Pesquisa

A.C.Camargo Cancer Center

Comitê de Ética em Pesquisa - CEP

São Paulo, 09 de outubro de 2013.

A
Dra. Ludmilla Thomé Domingos Chinen.

Aluna: Emne Ali Abdallah (Mestrado).

Ref.: Projeto de Pesquisa nº. 1367/10B
"Detecção de células tumorais circulantes em pacientes com câncer colorretal e sua correlação com evolução clínica tumoral".

Os membros do Comitê de Ética em Pesquisa em Seres Humanos da Fundação Antonio Prudente – Hospital do Câncer - A.C. Camargo/SP, em sua última reunião de 08/10/2013, **tomaram conhecimento e aprovaram** os seguintes documentos:

- Solicitação de dispensa da submissão da documentação obrigatória e análise ética do projeto acima mencionado por se tratar de um projeto afiliado ao temático intitulado "*Detecção de células tumorais circulantes e correlação com evolução clínica tumoral*", registrado neste CEP sob nº 1367/10. O projeto afiliado em referência será a dissertação de Mestrado do aluno *Emne Ali Abdallah*.
- Projeto afiliado, datado de agosto de 2013;
- Termo de Consentimento Livre e Esclarecido para o projeto afiliado.

Atenciosamente,

Dr. Jefferson Luiz Gross
1º Vice-Coordenador do Comitê de Ética em Pesquisa

Anexo 2 - Termo de Consentimento Livre e Esclarecido

TERMO DE CONSENTIMENTO LIVRE E ESCLARECIDO

NOME DO ESTUDO: DETECÇÃO DE CÉLULAS TUMORAIS CIRCULANTES EM PACIENTES COM CÃNCER COLORRETAL E SUA CORRELAÇÃO COM EVOLUÇÃO CLÍNICA TUMORAL

Investigador Responsável: Dra. Ludmilla T. Domingos Chinen
Nome do (a) Voluntário (a): _____

Por favor, leia cuidadosamente este formulário, pois ele informa o que você necessita saber sobre os objetivos deste estudo. Se concordar em tomar parte neste estudo, deverá assinar e datar este formulário. A sua assinatura significa que recebeu as informações necessárias e que deseja participar deste estudo.

OBJETIVOS DO ESTUDO

Usar um equipamento laboratorial novo no mercado para detectar células tumorais circulantes (são células que se desprendem do tumor) no sangue periférico e correlacionar seus níveis com:
- marcadores tumorais sanguíneos, observados pelo exame de sangue que você faz a pedido do seu médico;
- resposta ao tratamento (verificaremos se há diminuição dos níveis dessas células durante e após o seu tratamento);
- tempo de vida após o tratamento.

PROCEDIMENTOS DO ESTUDO

Ao concordar em participar deste estudo, você será submetido a três coletas de sangue (aproximadamente 8 mL), em três momentos: antes do início do tratamento sistêmico, 60 e 120 dias após o início do tratamento. A identificação do seu material será feita por códigos para preservar sua confidencialidade.

RISCOS

O seu tratamento será exatamente o mesmo, caso você participe ou não deste estudo. Nenhum dano imediato ou tardio, que comprometa a sua saúde, poderá ser decorrente deste estudo. Os riscos a que você estará sujeito são os riscos inerentes a qualquer punção venosa como: dor local no ato da punção, sangramento no local, hematoma e raramente flebite (infecção na veia puncionada), mas isto será evitado pela limpeza adequada do local de punção e realização do procedimento por profissional capacitado. Apesar de raro, pode ser que ocorra equimose (quando o sangue sai para a pele, resultando em uma mancha azul ou púrpura, redonda não elevada ou irregular) após a coleta de sangue. Caso isso aconteça com você, não há nada a ser feito, a não ser esperar que desapareça (desaparece em até 7 dias). Os exames realizados em seu sangue não implicarão em nenhuma mudança em seu tratamento ou qualquer conduta médica que esteja sendo realizada ou venha a ser realizada no seu seguimento, uma vez que a pesquisa de CTCs no Brasil ainda é experimental, não havendo nenhum prejuízo para o sucesso das condutas médicas outras aos quais você esteja se submetendo.

BENEFÍCIOS

Não haverá benefícios de qualquer espécie para os voluntários, apenas a importância de contribuir para uma pesquisa científica cujos dados obtidos

poderão trazer benefícios para pessoas com câncer. A recusa em participar, não acarretará prejuízo na qualidade do tratamento. O pesquisador responsável se compromete a suspender a pesquisa imediatamente ao perceber algum risco ou dano à sua saúde, que não previsto neste termo de consentimento.

MÉTODOS ALTERNATIVOS EXISTENTES

Apesar de métodos alternativos serem aprovados e usados na prática clínica em outros países, no Brasil, esses métodos ainda não estão disponíveis.

ACOMPANHAMENTO, ASSISTÊNCIA E RESPONSÁVEIS

Os pesquisadores se comprometem a dar informação atualizada ao longo do estudo, caso este seja o seu desejo, porém, por se tratar de nova tecnologia, os resultados não poderão indicar cura ou piora do se quadro, pois ainda não temos valores de referência para estabelecer uma comparação. Contatos dos pesquisadores: Dra. Ludmilla T. Domingos Chinen (2189-5000, ramal 2776); Dr. Marcello F. Fanelli (2189-5000, ramal 2765).

CUSTOS

Não haverá qualquer custo ou forma de pagamento para os voluntários por sua participação neste estudo.

CARÁTER CONFIDENCIAL DOS REGISTROS

Seus registros médicos poderão ser consultados pela equipe de pesquisadores envolvidos neste estudo. Os dados obtidos pela análise do seu sangue são confidenciais, não sendo divulgada a identificação de nenhum participante ainda que informações do registro médico sejam utilizadas para publicação.

BASES DA PARTICIPAÇÃO

É importante que você saiba que a sua participação neste estudo é completamente voluntária e que você pode recusar-se a participar ou interromper sua participação a qualquer momento, sem penalidades ou perda de benefícios aos quais tem direito.

SALVAGUARDA DE CONFIDENCIALIDADE, SIGILO E PRIVACIDADE

A eventual inclusão dos resultados em publicação científica será feita de modo a manter seu anonimato. Você terá acesso aos seus dados de exames, atendimentos médicos e administração de terapia, quando solicitados.

ESCLARECIMENTOS SOBRE COMPENSAÇÕES OU DANOS RELACIONADOS À PESQUISA

Você não terá nenhum tipo de remuneração ao aceitar participar deste estudo. A pesquisa não envolve nenhuma forma de compensação financeira aos participantes. Não existe nenhum tipo de indenização para complicações causadas pelo tratamento.

ESCLARECIMENTOS SOBRE OUTROS DIREITOS DO PACIENTE SUJEITO À PESQUISA

A sua participação no estudo é voluntária. Você tem o direito de sair do estudo a qualquer momento e por qualquer motivo. Caso venha a abandonar o estudo ou decidir não participar do mesmo, o seu tratamento não será prejudicado. No entanto, se você decidir sair da pesquisa, deverá informar ao seu médico.

INFORMAÇÕES SOBRE NOMES, TELEFONES E ENDEREÇOS PARA CONTATOS

Esclarecimentos para questões sobre os direitos dos participantes na pesquisa e/ou danos relacionados à pesquisa, contatar os pesquisadores Dra. Ludmilla T. Domingos Chinen (2189 5000 ramal 2776) ou Dr. Marcello Ferreti Fanelli (2189 5000 ramal 2779). Se o pesquisador principal não fornecer as informações/esclarecimentos suficientes, por favor, entre em contato com o coordenador do Coordenador do Comitê de Ética em Seres Humanos da Fundação Antônio Prudente- Hospital do Câncer- A.C. Camargo/ SP, cujo horário de funcionamento é de segunda a quinta-feira, das 07:00 às 18:00h e de sexta-feira das 07:00 às 16:00h (Telefone 2189-5020; endereço: Rua Professor Antônio Prudente 211-Liberdade- São Paulo, SP). Você receberá cópia deste documento e o original será arquivado no prontuário do médico. Somente assine este documento se consentir integralmente com seus termos.

GARANTIA DE ESCLARECIMENTOS

Nós lhe estimulamos a fazer perguntas a qualquer momento do estudo. Se tiver perguntas relacionadas aos seus direitos como participante do estudo clínico, também pode contar com uma terceira pessoa imparcial, o Coordenador do Comitê de Ética do Hospital A.C.Camargo.

DECLARAÇÃO DE CONSENTIMENTO E ASSINATURA

Li as informações acima e entendi o propósito deste estudo assim como os benefícios e riscos potenciais da participação no mesmo. Tive a oportunidade de fazer perguntas e todas foram respondidas. Eu, por intermédio deste, dou livremente meu consentimento para participar neste estudo. Entendo que não serei submetido a nenhum exame adicional e não

receberei compensação monetária por minha participação neste estudo. Eu recebi uma cópia assinada deste formulário de consentimento.

_____ ____ / _____ / _____

Assinatura do (a) voluntário (a) dia mês ano

_____

Nome do (a) voluntário (a) - letra de forma

_____ ____ / ____ / _____

(Assinatura de Testemunha, se necessário) dia mês ano

Eu, abaixo assinado, expliquei completamente os detalhes relevantes deste estudo ao paciente indicado acima e/ou pessoa autorizada para consentir pelo paciente.

_____ ____ / ____ / ____

(Assinatura da pessoa que obteve o consentimento) dia mês ano