Simultaneous phenotypic and genetic characterization of single circulating tumor cells from colon cancer patients

María Campos1, Rafael Luque2, Juan Jiménez2, Rafael Martínez4, Fernando Warleta1, Cristina Sánchez-Quesada1, Miguel Delgado-Rodríguez5,6, Alfonso Calvo7 and José J. Gaforio1

1Immunology Division, Department of Health Sciences, Faculty of Experimental Sciences, University of Jaén, Campus Las Lagunillas Jaén, 2Departments of Pathology, 3Surgery, 4Gastroenterology, University Hospital of Jaén, Jaén, 5Division of Preventive Medicine and Public Health, Department of Health Sciences, Faculty of Experimental Sciences, University of Jaén, Campus Las Lagunillas, Jaén, 6Biomedical Network Research Center for Epidemiology and Public Health (CIBERESP), Ministry of Science and Innovation and 7Division of Oncology, Center for Applied Medical Research (CIMA) and Department of Histology and Pathology, University of Navarra, Pamplona, Spain

Summary. Since circulating tumor cells (CTCs) have metastatic potential, their genetic and phenotypic characteristics could provide crucial information to establish the most effective therapy. We assessed the clinical utility of a methodology that allows the simultaneous analysis of CTC phenotype and genotype in colon cancer patients and, in addition, whether this methodology could provide complementary information to that obtained by the primary tumor biopsy.

Thirty-three non-metastatic (stages 0-III) colon cancer patients and 9 healthy donor samples were evaluated. All peripheral blood samples (10 ml) were analyzed by cytokeratin immunomagnetic enrichment. Eight samples were analyzed by immunocytochemistry and 25 samples were analyzed by FICTION technique for simultaneous cytokeratin expression and chromosome 17 and ERBB2 gene status. A further study was carried out in one patient who showed CTC heterogeneity in chromosomal abnormalities. We analyzed HER2 protein expression on CTCs and FISH and HER2 protein expression in primary tumor of this patient.

Our results show that 9.09% of patients had cytokeratin-positive CTCs (CK+/CTCs in peripheral blood). One of the patients showed heterogeneity in chromosomal 17 abnormalities and two different CK expression patterns on CTCs: one CK+/CTCs and one CK-/CTCs. Furthermore, 63.33% of these CTCs overexpressed HER2 protein while the primary tumor of this patient was diploid and did not express HER2 protein.

We describe a methodology that allows the simultaneous genetic and phenotypic analysis of CTCs in colon cancer patients, which may provide essential information to select patients who might benefit from specific therapy.

Key words: CK+/CTCs, Colon cancer, ERBB2, FICTION, HER2

Introduction

Colon cancer is one of the most common types of tumors in developed countries (Jemal et al., 2011). Despite the improvement of survival rates achieved with the use of new chemotherapy agents, approximately 20% of stage II patients will die from recurrent disease (Le Voye et al., 2003), which shows the need for more effective therapies.

Different genetic alterations that have been described in colorectal cancer may represent novel candidates for tailored therapy, such as i) alterations in...
protooncogene C-MYC (located on chromosome 8) that encodes a transcription factor that is one of the most potent and frequently deregulated oncoproteins in humans cancers (Facchini and Penn, 1998); ii) alterations in the ERBB2 gene (located at chromosome 17), a member of the EGFR family, it is a common target for breast cancer, but recent studies have found overexpression or amplifications in other solid tumors, such as lung, gastric and colorectal cancers (Nathanson et al., 2003; Takenaka et al., 2011; Tsapralis et al., 2012). This offers the possibility of testing ERBB2-targeted drugs, including trastuzumab and lapatinib, in these subpopulations of patients (Kuwada et al., 2004; Kelly et al., 2010; Javle and Hsueh, 2010; Morishita et al., 2010; Choi et al., 2011). Overexpression or amplification of ERBB2 has been reported in 3%-14% of primary tumors from colorectal cancer patients (Dursun et al., 2001; Nathanson et al., 2003; Al-Kuraya et al., 2007; Kavanagh et al., 2009), and some authors have suggested that the addition of trastuzumab to chemotherapy might increase the clinical response in these colon cancer patients (Kuwada et al., 2004; Al-Kuraya et al., 2007; Kavanagh et al., 2009; Kelly et al., 2010; Javle and Hsueh, 2010; Morishita et al., 2010; Choi et al., 2011).

The detection of circulating tumor cells (CTCs) appears to be an important prognostic marker for different tumor types, such as breast cancer, where it serves as a progression marker and indicator of overall survival (Gaforio et al., 2003; Cristofanilli et al., 2004, 2005; Müller et al., 2006). In addition, the detection of CTCs identifies therapy resistant breast cancer patients (Cristofanilli et al., 2005; Camara et al., 2007; Pierga et al., 2008). The prognostic significance of CTCs in colorectal cancer is not clear yet, although recent reports suggest an association with overall survival and disease progression (Koch et al., 2006; Cohen et al., 2008; Sastre et al., 2008). However, little attention has been paid to the cytogenetic features of such cells.

In this study we analyzed CTCs of 33 non-metastatic colon cancer patients with the goal of evaluating ERBB2 status in such CTCs. Our methodology allows us to analyze simultaneously the ERBB2 phenotype and genotype of single cytokeratin positive (CK+)/CTCs isolated from peripheral blood (PB) by immunomagnetic separation and FICTION (Campos et al., 2008). We found that 3 out of 33 patients had detectable CK+/CTCs (9.09%) and 1 of them had CK+/CTCs with strong HER2 expression. CK+/CTCs in this patient were triploid for chromosome 17 and did not show ERBB2 amplification, while the primary tumor showed disomy for this chromosome and ERBB2 gene, and were negative for HER2 protein expression. Therefore we were able to identify CTCs in PB with different genetic and phenotypic characteristics to the ones found in primary tumor biopsy. Since CTCs are linked to tumor recurrence and metastasis, CTCs’ genotype and phenotype analysis could also complete the real status of the disease with metastatic and primary tumor analysis.

Materials and methods

Cell lines and controls

The human colon cancer cell lines Caco-2, HT-29, and human breast cell lines MCF-7, MDA-MB-231 were obtained from the American Type Cultured Collection (ATCC; Rockville, MD). The human breast cancer cell line SK-Br-3 (with ERBB2 amplification and overexpression) was obtained from Eucellbank (Barcelona, Spain). MCF-7 and MDA-MB-231 cells were grown in MEM with Earle’s salt (PAA Laboratories, Pasching, Austria), supplemented with 10% (v/v) heat-inactivated fetal calf serum (PAA Laboratories, Pasching, Austria) and 1% of a stock solution of penicillin-streptomycin (Sigma, St. Louis, MO). Caco-2, HT-29 and SK-Br-3 cells were grown in RPMI 1640 (PAA Laboratories, Pasching, Austria), plus 10% (v/v) heat-inactivated fetal calf serum and 1% of a stock solution of penicillin-streptomycin. Cells in the exponential growth phase were used for the experiments. These cancer cell lines were used as positive control. Briefly, we spiked 1000 of each tumor cell line in 10 ml of peripheral blood (PB) from 6 healthy donors (one sample of blood per each tumor cell line except for SK-Br-3 control that was duplicated), under an approved ethical protocol and signed informed consent. Previously, we validated the absence of CTCs in PB from healthy donors as negative controls. Besides these healthy donor samples, we also evaluated three PB samples of patients with diverticulosis disease (benign inflammatory disease) as negative controls. Positive controls, negative controls and patient’s samples were carried out separately to avoid cross contamination and were processed following our previously published FICTION protocol (Campos et al., 2008).

Study population and clinicopathologic examination

Informed consent was obtained from all participants following an explanation of the nature of the study, as approved by the research ethics board of our hospital. All patients were considered sporadic cases on the basis that no clinical antecedents of familial adenomatous poliposis were reported, and those who met the clinical criteria for hereditary nonpolyposis colon cancer (Amsterdam criteria) were excluded. Between June 2004 and November 2005, blood samples (10 mL) were taken from 33 non-metastatic colon cancer patients (stages 0-III) by venipuncture, the day before surgery. All patients were followed up until death or until August 11, 2007. Follow-up of the patients was carried out every 6 months and included recording of the development of local and distant tumor relapse and the survival state of the patients.

The following variables were obtained from the
medical records of the 33 patients: Age, gender, tumor location, pathologic stage, histological differentiation and type, lymph node metastases, tumor invasion, and evidence of polyps (defined by the presence of polyps in the surgical sample). Pathologic stage was assessed using tumor-node-metastases (TNM) classification (Table 1).

**Blood samples, density gradient separation and CK+/CTCs enrichment by immunomagnetic separation**

Peripheral venous blood samples (10 mL) were collected from colon cancer patients before surgery. PB samples were collected in heparinized tubes (BD Vacutainer, Becton Dickinson; Heidelberg, Germany) and were processed within 4 hr after collection. Eight PB samples were processed according to the methodology previously described by our group (Gaforio et al., 2003) and 25 PB samples were processed following our FICTION protocol (Campos et al., 2008).

Briefly, a double-density ficoll gradient was prepared per PB sample (Histopaque 1119 and Histopaque 1077 (Sigma, St. Louis, MO)). After centrifugation, the mononuclear cell fraction and the granulocyte fraction were isolated. Then tumor cells were immunoseparated using the Carcinoma Cell Enrichment and Detection Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), with some modifications. After nucleated cells were isolated, permeabilized and fixed, the samples were incubated with Fc-Blocking Reagent and immunomagnetically labeled with MACS anti-cytokeratin microbeads (microbeads conjugated to a monoclonal anti-cytokeratin 7/8 antibody) and stained with a blocking solution (10% rabbit serum (Sigma, St. Louis, MO)).

**Detection of CK+/CTCs by immunocytochemistry**

Eight PB samples were processed by immunocytochemistry. After thawing, slides were stained with anti-cytokeratin 7/8 conjugated to FITC (isotype: mouse IgG2a. Miltenyi Biotec, Bergisch Gladbach, Germany) and further labeled with anti-FITC antibody conjugated to alkaline phosphatase (isotype: mouse IgG1, Miltenyi Biotec, Bergisch Gladbach, Germany). Then slides were air-dried overnight at room temperature and stored at -20°C without fixation.

**Detection of CK+/CTCs by FICTION (Fluorescence immunophenotyping and Interphase Cytogenetics as a Tool for Investigation of Neoplasm)**

Twenty five PB samples were processed by FICTION (Campos et al., 2008). Briefly, after thawing and fixation, these samples were incubated with a blocking solution (10% rabbit serum (Sigma, St. Louis, MO)) followed by primary antibody incubation with the bclonal mouse anti-AE1-AE3 antibody (BioGenex, San Francisco, CA).

**Table 1.** Correlation among CK+/CTCs and clinical/morphological variables.

<table>
<thead>
<tr>
<th>Clinical or pathological variables</th>
<th>Number of CK+/CTCs/10 ml</th>
<th>p value (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>2 (6.06%)</td>
<td>0.718</td>
</tr>
<tr>
<td>Female</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor location</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ascending colon</td>
<td>2 (6.06%)</td>
<td>0.477</td>
</tr>
<tr>
<td>Transverse colon</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Descending colon</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Sigmoid colon</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>Transverse-sigmoid colon</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td><strong>Grade of differentiation</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Well</td>
<td>2 (6.06%)</td>
<td>0.289</td>
</tr>
<tr>
<td>Moderate and moderate-poor</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td><strong>TNM</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0 (0.00%)</td>
<td>1.000</td>
</tr>
<tr>
<td>I</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>2 (6.06%)</td>
<td></td>
</tr>
<tr>
<td><strong>Histological type</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adenocarcinoma</td>
<td>3 (9.09%)</td>
<td>1.000</td>
</tr>
<tr>
<td>Mucinous adenocarcinoma</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Intramucosal adenocarcinoma</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor configuration</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Excrecent</td>
<td>0 (0.00%)</td>
<td>0.501</td>
</tr>
<tr>
<td>Excrecent-infiltrative</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Infiltrative</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>Polyploid</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td>Ulcerative</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>Ulcer vegetative</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>Vegetative</td>
<td>0 (0.00%)</td>
<td></td>
</tr>
<tr>
<td><strong>Tumor invasion</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphatic</td>
<td>2 (6.06%)</td>
<td>0.253</td>
</tr>
<tr>
<td>-</td>
<td>1 (3.03%)</td>
<td></td>
</tr>
<tr>
<td>Venous</td>
<td>0 (0.00%)</td>
<td>0.244</td>
</tr>
<tr>
<td>+</td>
<td>3 (9.09%)</td>
<td></td>
</tr>
<tr>
<td>Perineural (n=10)</td>
<td>1 (3.03%)</td>
<td>0.252</td>
</tr>
<tr>
<td>-</td>
<td>2 (6.06%)</td>
<td></td>
</tr>
</tbody>
</table>

2003). CK+/CTCs were counted separately by two expert researchers.
The development was conducted by applying 2 sequential layers of secondary antibodies: Alexa Fluor 350 rabbit anti-mouse IgG and goat anti-rabbit IgG (Invitrogen, Eugene, OR). After fluorescent immunophenotyping, slides were evaluated under a Leica TCS-DL confocal laser scanning system with argon and two helium neon lasers on a Leica DM IRB inverted microscope, with appropriate filter set. Blue fluorescent images were acquired with Leica DFC 300FX digital camera and processed with a Leica IM50 image manager. Confocal images were acquired and processed using Leica confocal LCS software (Leica Microsystems; Wetzlar, Germany).

After assessment of positive tumor cells on the slides, samples were fixed and dehydrated in series of ethanol (Panreac Química, Barcelona, Spain). Afterwards, samples were codenatured (85°C for 5 min) and hybridized (overnight at 37°C) with the LSI HER2/CEP17 multi-color probe, (ERBB2 (HER2/neu) probe labeled with SpectrumGreen and a chromosome enumeration probe CEP17 labeled with SpectrumAqua) (Abbott Molecular, Vysis; Des Plaines, IL). Posthybridization wash was performed at 72°C in 2xSodium chloride citrate buffer (SCC) (MP Biomedicals Europe, Illkirch, France)/0.3% Igepal (Sigma, St. Louis, MO) for 2 min, followed by another wash at room temperature. Microscopic evaluation was carried out with the Leica microscope described above by two expert researchers.

Analysis of hybridization

All CK+/CTCs were scored to determine the number of hybridization signals for each ERBB2 and CEP17 probe. The absolute copy numbers and the relative copy number ratio (ratio between the absolute number of ERBB2 signals in each CK+/CTC and the absolute number of their own chromosome 17 centromere signals) were determined. For FISH evaluation a [ERBB2]/CEP17 ratio value >1.5 was considered to be an increase copy number of the gene. Similarly, ratio value ≤0.7 was considered to be a decrease copy number of the gene, and ratios between 1.3 and 1.5 were considered as equivocal values, and were interpreted with caution.

Statistical analysis

The patient characteristics were related to the presence of CK+/CTCs in PB by using Fisher’s exact test. When a variable was ordered a trend analysis was applied. Actuarial curves for progression-free survival (PFS) and overall survival (OS) were calculated by the Kaplan-Meier method. PFS and OS were calculated from the date of the primary detection of CK+ cells in PB to the date of disease progression or death. Disease progression was defined as metastatic recurrence of non-metastatic patients.

Study of patient with identification number (IN) 28

Due to the genetic characteristics of the CK+/CTCs found in IN 28 patient, we conducted a further study of this patient by assessing several tumor markers, genetic characteristic of the primary tumor and the expression of HER2 protein in CK+/CTCs of this patient.

Immunohistochemistry of primary tumor

Immunostaining of primary tumor sample was assessed by Department of Pathology (University Hospital of Jaén) on routinely processed paraffin sections using the citrate buffer (pH 6) (Master Diagnostica, Granada, Spain) as an antigen-retrieval method. Sections were examined with monoclonal antibodies (mAbs) recognizing p53 (Novocastra, Newcastle upon Tyne, UK); cyclin D1 (Dako, Glostrup, Denmark); HER2 (Dako, Glostrup, Denmark); Ki-67 (Concepta Biosystems, Barcelona, Spain); CD31 (Novocastra, Newcastle upon Tyne, UK); and CD34 (Novocastra, Newcastle upon Tyne, UK). Immunohistochemical staining was performed with a sensitive polymer-based system (Dako EnVision system, Glostrup, Denmark) with diaminobenzidine (DAB) solution as a chromogen (Dako, Glostrup, Denmark). All incubations were carried out at room temperature. The sample was counterstained with Mayer’s haematoxylin (Merck, Darmstadt, Germany) and mounted using standard procedures. Positive and negative (substituting the primary antibody by distilled water) controls were included in the kit. All recommended prognostic factors by the American College of Pathologists were recorded.

The assessment of immunohistochemistry was conducted using an Olympus BH-2 microscope (Olympus; Hertfordshire, United Kingdom) attached to a Nikon Coolpix 5400 camera for acquisition of digital images. All markers were quantitated using random fields that were recorded as digital images under high power view (x400) and counting a mean of 1000 cells per marker with the aid of the ImageJ software for image analysis (ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://rsb.info.nih.gov/ij/, 1997-2007).

FISH of primary tumor

The ERBB2 evaluation by FISH was performed on two sections (3 µm) from paraffin-embedded tissue sample. Briefly, after paraffin was removed the sections were rehydrated in ethanol series and treated with hydrochloric acid (Panreac Química, Barcelona, Spain), followed by a treatment with 8% Sodium thiocyanate (Panreac Química, Barcelona, Spain) and digested with 0.025% pepsin (Sigma, St. Louis, MO) in hydrochloric acid. Samples were then rinsed with 2xSSC, dehydrated in ethanol series and air dried at room temperature.

Next, dual-color probe Poseidon Repeat Free™
ERBB2, HER2/Neu (17q12) & SE 17 Control probe (red color for ERBB2, and green for SE17) (Kreatech Diagnostics, Amsterdam, The Netherlands) were added to slides and codenaturation (75°C for 10 min), hybridization (overnight at 37°C) and posthybridization wash (72°C in 1xSSC for 2 min, followed by 1xSSC at room temperature) were performed following the Kreatech Diagnostic’s recommended protocol. Slides were mounted with DAPI I counterstain (Abbott Molecular, Vysis; Des Plaines, IL).

Evaluation of the hybridization was done with a Zeiss Axioplan 2 epifluorescence microscope (Carl Zeiss; Jena, Germany), with the appropriate filters set. ISIS software (MetaSystems; Altslussheim, Germany) was used to capture the images. 100 tumor nuclei were examined and alterations of the number of signals were evaluated following standard procedures.

Immunostaining of CK+/CTCs with anti-HER2 mAb (HercepTest, Dako)

After FICTION evaluation, IN 28 sample was dismounted, rehydrated and the anti-HER2 antibody was applied as described in the section Immunohistochemistry of primary tumor. Positive controls, in which FICTION had been evaluated, were included in the immunostaining of CK+/CTCs with anti-HER2, in order to have a control of the immunocytochemistry. These controls followed the same process as described for the IN28 sample, except for one of the SK-Br-3 controls that was used as a negative control (substituting the primary antibody with distilled water to assure that crossed reactions or false positive results did not occur).

Analysis of HER2 expression

Tumor cell lines and CTCs from IN 28 sample were scored according to manufacturer’s guidelines. Score 0 was defined as no staining of tumor cells, score 1+, as faint membrane staining of these cells; score 2+, as weak to moderate membrane staining; and a score of 3+, as strong staining of the entire membrane with perinuclear reinforcement. Samples were considered negative when a score 0 or 1+ staining were found in >10% of tumor cells, while they were considered positive when score 2+ or 3+ was observed in >10% of these cells. Microscopic evaluation was carried out with the Leica microscope described above.

Results

Detection of CK+/CTCs in colon cancer patients

Blood samples from 33 consecutive patients with colon cancer were taken to measure CK+/CTCs levels, and no statistical association was found between presence of CK+/CTCs and any of the variables studied (Table 1). The negative controls, PB samples from three patients with diverticulosis disease and 6 healthy volunteers were negative for CK+ cells detection.

CK+/CTCs were detected in 3 out of 33 PB samples (9.09%). The mean number of CK+/CTCs isolated in these 3 patients was 9.67 (range 1-26 CK+ cells), relationship between the presence of CK+/CTCs and OS or/and PFS was not found (median follow-up = 13 months; range = 0.5-29.9).

Regarding the three CK+/CTCs patients, one of them was analyzed by immunocytochemistry and showed one CK+/CTC with a strong cytoplasmic staining pattern, whereas the surrounding hematopoietic cells showed no CK expression (data not shown). The genetic characteristic of this CK+/CTC could not been studied due to technical problems. The other two samples were analyzed by FICTION and results are show in the section FICTION analysis on the immunoselected CK+/CTCs.

Characterization of tumor cells lines by FICTION and HER2 expression

We first established the characterization of the FICTION technique, using tumor cell lines spiked in blood samples from healthy volunteers.

Carcinoma cell lines in positive controls were unequivocally distinguished among the white blood cells by their CK blue-labelling (Fig. 1). Hybridization signals for ERBB2 and CEP17 were also clearly observed in both tumor and white blood cells (Fig. 1). All leukocytes showed two signals for the ERBB2 gene and centromere 17, thus serving as internal controls for the

### Table 2. Chromosomal 17 aneusomy according to HER2 protein expression scoring on CK+/CTCs from IN 28 patient.

<table>
<thead>
<tr>
<th>Chromosomal aneusomy</th>
<th>Number of CK+/CTCs</th>
<th>Percentage of CK+/CTCs</th>
<th>HER2 protein expression score</th>
<th>Total CK+/CTCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trisomy</td>
<td>23</td>
<td>88.46%</td>
<td>Number of CK+/CTCs</td>
<td>0   8   5   6  11  19</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percentage of CK+/CTCs</td>
<td>0.00% 42.11% 26.32% 31.58% 57.89%</td>
</tr>
<tr>
<td>Disomy</td>
<td>3</td>
<td>11.54%</td>
<td>Number of CK+/CTCs</td>
<td>0   0  2   0  2  2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percentage of CK+/CTCs</td>
<td>0.00% 0.00% 100.00% 0.00% 100.00%</td>
</tr>
<tr>
<td>Total CK+/CTCs</td>
<td>26</td>
<td></td>
<td>Number of CK+/CTCs</td>
<td>0   8  7   6  13  21</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Percentage of CK+/CTCs</td>
<td>0.00% 38.09% 33.33% 28.57% 61.90%</td>
</tr>
</tbody>
</table>
The five positive controls (SK-Br-3, Caco-2, HT-29, MCF-7 and MDA-MB-231) and SK-Br-3 negative control (in which we substituted the primary antibody for distilled water), were analyzed for the immunostaining with anti-HER2 protein. Different expression patterns were found on each positive control (Fig. 1). SK-Br-3, Caco-2 and HT-29 tumor cells showed a strong staining (3+ score). MCF-7 tumor cells displayed a moderate staining (annotated as 2+) and MDA-MB-231 cells were negative for HER2 protein expression.

**FICTION analysis on the immunoselected CK+/CTCs**

Criteria for CK+/CTCs identification consisted of nucleated cells with a malignant phenotype (Fig. 2 “Transmission column” shows a representative example of morphology of isolated CK+/CTCs), staining positive

---

**Fig. 1.** Fluorescent images (x100 objective) of PB from healthy volunteers spiked with different tumor cell lines [HT-29, Caco-2, SK-Br-3, MCF-7 and MDA-MB-231] processed by immunomagnetic enrichment and FICTION techniques. Column “Cytokeratin” displays the CK labelling of tumor cells which are clearly identified as blue fluorescent, evaluation by fluorescence microscopy. Columns “Chromosome 17” and “ErbB2” show the hybridization signals for CEP17 and ERBB2 (HER2/neu) respectively (overlay of different layers), evaluated by confocal microscopy. Column “Photomontage” displays the overlay of Cytokeratin (blue), Chromosome 17 (aqua or light blue) and ERBB2 (green). Column “Transmission” shows the morphology of tumor cells, analyzed with confocal microscopy. The expression of HER2 protein is shown in the last column “HER-2”. These cells are different from the ones photographed in the other columns. Scale bar: 12.32 µm
for cytokeratin (CK+ cells) in blue fluorescence. Results were expressed as number of CK+/CTCs per 10 mL PB. In addition, to ensure the efficiency of hybridization, a minimum of 50 morphologically intact and non-overlapping nuclei of normal cells (contaminated leukocytes) were scored per sample and used as internal control.

IN 21 Patient: A total of 2 intact cells were detected as CK+/CTCs in this patient. These CK+/CTCs were evaluated for the hybridization of ERBB2 gene signals. Both CK+/CTCs presented disomy for this chromosome and showed 2 copies for ERBB2 gene (data not shown).

IN 28 Patient: A total of 26 intact CK+/CTCs with a malignant phenotype were found in this patient. Among the 26 CK+/CTCs studied, 23 CK+/CTCs showed polysomy (trisomy) for chromosome 17 (88.46%) and 3 CK+/CTCs presented disomy for this chromosome (11.54%) (Table 2). The CEP17 average number of copies for these CK+/CTCs was 2.88 copies per cell. 20 CK+/CTCs (out of these 26 CK+/CTCs) were evaluated in terms of absolute copy number of ERBB2 signals. Four CK+/CTCs (4/20) presented 3 copies for this gene (20.00%) and 16 (16/20) showed 2 copies for ERBB2 gene (80.00%). In summary, amplification of ERBB2 gene was not detected. The ERBB2 gene status for these CK+/CTCs was 2.20 copies per cell. Figure 2, “ERBB2 and Chromosome 17 columns” shows a representative example of ERBB2 gene and chromosome 17 status of isolated CK+/CTCs.

**HER2 protein expression on CTCs**

Evaluation of HER2 protein expression was only possible in 21 of the 26 CK+/CTCs. HER2

---

**Fig. 2.** Fluorescent images (x100 objective) of CK+/CTCs isolated from PB from IN 28 patient. Column “Cytokeratin” displays the CK labelling of these cells, which are clearly identified by their blue fluorescence. Samples were analyzed by fluorescence microscopy. Columns “Chromosome 17” and “ErbB2” show the hybridization signals for CEP17 and ERBB2 (HER2/neu) respectively (overlay of different layers, confocal microscopy). Column “Photomontage” displays the overlay of Cytokeratin (blue), Chromosome 17 (aqua or light blue) and ERBB2 (green). Column “Transmission” shows the morphology of CK+ cells (confocal microscopy). The expression of HER2 protein is shown in the last column “HER-2”. All CK+/CTCs are the same as those photographed in the other columns. Scale bar: 12.32 µm
Fig. 4. Histopathological features of the tumor from IN 28 patient (stage II). A. Neoplastic glands with infiltrative pattern of growth affecting the muscular propria layer of the bowel. Haematoxylin-eosin. B. Detail from the neoplastic glands, atypical but well conformed, although fused glands are present. Note the presence of lymphovascular (small vessel) invasion (arrowhead). Haematoxylin-eosin. C-G. Immunohistochemical expression of the markers in the tumor, showing high microvessel density as seen by CD31 (C) and CD34 (D) immunostaining. The arrowhead corresponds to small vessel invasion. High proliferative index (Ki-67) (E), low p53 expression (F), high expression of cyclin D1 (G) and Herceptest (H) negative test define the molecular characterization of this tumor (insert into the lower right quadrant of figure: Positive control from intraductal breast carcinoma). All figures: Envision® method. A, x 10 objective; B, x 40 objective; C-K, x 20 objective.

Fig. 3. HER2 expression on CK-/CTCs (x100 objective) from IN 28 patient. The score of HER2 protein expression is shown in the lower right corner of the images. Scale bar: 12.32 µm.
overexpression was detected in 13 of 21 CK+/CTCs (61.90%); 6 CK+/CTCs showed a 3+ staining and 7 CK+/CTCs presented a 2+ staining. Eight CK+/CTCs (38.10%) displayed a faint membrane staining and were scored as negative-expression. Finally, 5 out of these 26 CK+/CTCs were not evaluable. Figure 2 “HER2 column” shows a representative example of HER2 expression on the isolated CK+/CTCs.

In addition, we found 9 tumor cells that were CK negative (CK-)/CTCs, but histopathological examination revealed that they were tumor cells. In these 9 CK-/CTCs, HER2 overexpression was detected in 6 of them, with a 3+ staining; and 3 tumor cells showed faint membrane staining (scored as negative for HER2 expression) (Fig. 3). In summary, 19 (13 CK+/CTCs and 6 CK-/CTCs) CTCs had HER2 overexpression (63.33%) and 11 (8 CK+/CTCs and 3 CK-/CTCs) CTCs were negative for HER2 (36.67%).

**Immunohistochemistry and FISH of the primary tumor tissue for IN 28 patient**

Focusing on IN 28 patient (stage II) we decided to make a deeper study of the primary tumor. Histological examination revealed an infiltrative adenocarcinoma (Fig. 4) without positive nodes or distant metastases (he was followed up until April 15, 2008). Mayer’s haematoxylin & eosin-stained slides showed a high grade of tumor aggressiveness (Fig. 4A; B). The tumor stained strongly for Ki-67 (up to 90% of stained cells) (Fig. 4E), cyclin D1 (about 50%) (Fig. 4G), with nearly nil expression of p53 (Fig. 4F) and no expression of HER2 (Fig. 4H). Immunohistochemical demonstration of CD31 and CD34 showed well-developed microvasculature as well as the presence of tumour cells within small vessels (Fig. 4C; D respectively).

FISH analysis of two different sections demonstrated no ERBB2 amplification and disomic pattern for chromosome 17 (data not shown). The negative expression of HER2 is in line with the FISH result found in the primary tumor, but neither of them matched with the CK+/CTCs’ results of this patient.

**Discussion**

In this study, we analyzed genetic alterations of chromosome 17 and ERBB2 gene by FICTION in CK+/CTCs of 25 PB samples from a total of 33 non-metastatic colon cancer patients (stages 0-II). Some studies in breast cancer have demonstrated that HER2 expression can be acquired in CTCs from HER2 negative tumor during tumor progression (Meng et al., 2004; Fehm et al., 2007, 2010), and that detection of these CTCs with overexpression of HER2 could be associated with a worse prognosis (Wülfing et al., 2006). Therefore, we decided to study the genetic status of this gene in our series of patients. We have also chosen the analysis of ERBB2/HER2 protein because there is a monoclonal antibody against this protein that is nowadays being used in breast cancer treatment (Slamon et al., 2001; Madarnas et al., 2008). Furthermore, this treatment could be used in other types of tumors with ERBB2 amplifications or HER2 protein overexpression (Langer et al., 2004; Javle and Hsueh, 2010) and in patients with ERBB2-amplified or HER2+ CTCs (Meng et al., 2004).

It should be noted that few studies have been focused on the genetic characteristics of CTCs in cancer patients. Only two reports have applied cytogenetic techniques in search of chromosome aneuploidies in of CTCs from PB in metastatic (Fehm et al., 2002) and non-metastatic colon cancer patients (Wind et al., 2009). One of them indicates that CTCs were heterogeneous for chromosomal abnormalities and the gain of chromosomes was more frequent than the genetic loss (Fehm et al., 2002). These results are in agreement with findings in other tumors types, such as breast (Fehm et al., 2002) or prostate cancer (Fehm et al., 2002; Swennenhuis et al., 2009). Few studies have applied FISH technique to evaluate specific genes status in CTCs such as the concordance of ERBB2 amplification in CTCs and primary tumors in breast cancer patients (Meng et al., 2004); or the amplification of androgen receptor (AR) and MYC genes in CTCs from prostate cancer patients (Swennenhuis et al., 2009).

In this article, we have demonstrated the utility of a method that combines an immunomagnetic selection of CTCs from peripheral blood of colon cancer patients with FICTION technique. FICTION technique was developed by Weber-Matthiesen et al. (1992) to assign tumor cells a cytogenetically defined clone and to determine their specific cell lineage at one time. FICTION technique allows genetic and phenotypic CTC characterization without cell membrane destruction and subsequent cell relocalization, which typically is done in FISH, which the subsequent time consuming and loss of evaluable tumor cells (Swennenhuis et al., 2009). Furthermore, we demonstrate the feasibility to study second marker expression by immunocytochemistry on the same CTC.

The most interesting finding in this work was, apart from demonstrating the validity of the technique to analyze simultaneously CK+/CTCs’ phenotype and genotype, the discovery of a gain in chromosome 17 and HER2 overexpression in a patient with HER2 negative primary tumor. FISH analysis of the primary tumour demonstrated disomy for chromosome 17 and ERBB2 gene. A total of 26 CK+/CTCs were isolated from the PB of this colon cancer patient. These tumor cells were an heterogeneous cell population with basically two patterns. The predominant one (~88.00%) showed trisomy of chromosome 17 and the second pattern was characterized by disomy for this chromosome (~11.00%). Both populations of these CK+/CTCs presented 2 copies of ERBB2 gene (~80%) but neither of them had ERBB2 amplifications. In contrast, when we analyzed the expression of HER2 protein, 61.90% of the CK+/CTCs showed overexpression (63.33% if we
also counted CK-/CTCs). This patient was scored as HER2+ CTCs (more than 10% of CTCs were HER2+), and some studies showed that evaluation of only 10 CTCs could indicate the patient’s HER2 status (Meng et al., 2004, 2006; Cao et al., 2010).

Our finding of the existence of a heterogeneous CTC population in the same PB sample, and the tumor cells CK expression variability are in keeping with the results shown in metastatic colon (Fehm et al., 2002), prostate and breast cancer patients (Mikolajczyk et al., 2011). The fact that CTCs in this patient overexpressed HER2, whereas the primary tumor did not, can be explained by the possibility that these CTCs may come from an unusual subclone of tumor cells that are not readily detectable by primary tumor biopsy. Alternatively, CTCs could express HER2 de novo, since this may confer a survival advantage to metastatize. Obviously, we cannot conclude that colon cancer patients with HER2 overexpression in CTCs should be treated with trastuzumab (Herceptin) therapy. However, it is worth noticing that breast cancer patients with ERBB2-amplified CTCs were treated with trastuzumab and showed a partial or complete remission (Meng et al., 2004). Another few studies hypothesize that HER2 CTC status determination could be a tumoral marker for the use of HER2-targeted therapies (Fehm et al., 2007, 2010). Future studies would be needed to determine the convenience of targeting ERBB2/HER2 in colon cancer.

We have only detected 3 CK+/CTC in a total of 33 (9.09%) non-metastatic colon cancer patients. In addition, CK+/CTCs detection did not correlate with primary tumor characteristics or with PFS or OS. This percentage of CK+/CTC-positive patients is lower than those published in previous studies (Cohen et al., 2008; Sastre et al., 2008; Maestro et al., 2009). However, other studies have reported similar percentages of CTCs in PB to those shown in our study (Wind et al., 2009; Thorsteinsson et al., 2011). The main difference between these two sets of studies is the study population, which may explain the disparity found between these results.

In metastatic colorectal cancer patients (Cohen et al., 2008, 2009; Molnar et al., 2001), where the sample collection was taken postoperatively and before chemotherapy treatment was initiated, a number of CTCs higher than 2 or 3 correlated with shorter OS and/or PFS (Cohen et al., 2009; Maestro et al., 2009) and only the presence of this tumor cells identified chemotherapy resistant patients (Molnar et al., 2001).

In non-metastatic colon cancer patients, analysis of CTCs in peripheral blood before surgery or chemotherapy treatment rendered a similar percentage to the one found here. Wind et al. (2009) tested the detection of CTCs in PB and portal blood in different sets of samples in 31 non-metastatic colon cancer patients at different time points: before tumor mobilization, after tumor mobilization and post-surgery. This report showed a CTC detection rate of 4-7% in peripheral blood vs. 26-54% in portal blood, depending on when blood samples were obtained, and described no correlation between tumor cell detection and clinicopathologic characteristics or disease progression. Thorsteinsson et al. (2011) found that 5% of samples (1/20 non-metastatic colon cancer patients) before surgery were positive for CTCs whereas samples taken after surgery were negative. This study reported no correlation between CTC detection and the tumor characteristics, PFS or OS. Therefore, our results suggest that basal levels of CTC (or CK+/CTCs) in non-metastatic colon cancer patients may have no prognostic value, although further studies with larger series of patients should be carried out to address this issue.

Cohen et al. (2006) theorized that colorectal cancer biology could play an important role in CTCs’ recovery, since this cancer disseminated more frequently via portal blood than other epithelial tumors, which disseminated via peripheral blood, such as breast and prostate cancer. Thus, these authors suggested that metastatic colorectal cancer patients might be less likely to have CTCs in peripheral blood than metastatic breast or prostate cancer patients. Consequently, in non-metastatic patients it would be more noted. For example, Wind et al. (2009) detected a higher percentage of non-metastatic colon cancer patients with CTCs in portal than in peripheral blood (54%; 31%; 45% in portal blood vs. 7%; 4%; 4% in peripheral blood, these samples were taken before tumor mobilization, after tumor mobilization or post-surgery, respectively).

In the patient where we found gain of chromosome 17 in HER2 overexpression in CTCs, we may speculate that this would confer metastatic advantage. In breast cancer, it has been previously reported that HER2 overexpression can be acquired during tumor progression: CTCs may express HER2 although their primary tumor was considered as HER2 negative (Meng et al., 2004; Fehm et al., 2007, 2010). The detection of these CTCs with HER2 overexpression could be associated with a worse prognosis (Wülfing et al., 2006). Although the use of HER2-targeting drugs (such as trastuzumab) is not yet approved for colon cancer, clinical trials are underway to test its efficacy in the selected small population of HER2+ patients.

The CTCs are traditionally considered as one of the sources of the metastatic disease’s origin (Pantel and Brakenhoff, 2004; Paterlini-Brechot and Benali, 2007). Additionally, genetic changes can occur at any stage of tumor progression (Meng et al., 2004, 2006). Thus, we hypothesize that the CTCs analysis would be useful to know the real status of the disease and, in the future, could be considered as a routine analysis in cancer patients.

Conclusions

The conclusions from our study are as follows: (i) individualized CTC analysis using the technique described in the present article may provide additional information to conventional pathological diagnosis; (ii) chromosome 17 triploidy and HER2 overexpression can
appear in CTCs of early stage colon cancer patients.

Acknowledgements. This study was supported by the “Fundación de Investigación Médica Mutua Madrileña” (to J.J. Gaforio).

References


Fehm T., Becker S., Duer-Stoerzer S., Slotar K., Mueller V., Wallwiener D., Lane N., Solomayer E. and Uhr J. (2007). Determination of HER2 status using both serum HER2 levels and circulating tumor cells in patients with recurrent breast cancer whose primary tumor was HER2 negative or of unknown HER2 status. Breast Cancer Res. 9, R74.


systematic review. Cancer Treat. Rev. 34, 539-557.

Accepted May 6, 2013