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# A Novel Chromatographic Method for Ep-CAM mRNA Detection in Peripheral Blood and Bone Marrow of Patients with Metastatic Colorectal Cancer

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**Abstract.** *Background: The aim of our study was to detect Ep-CAM mRNA in peripheral blood (PB) and bone marrow (BM) samples of patients with metastatic colorectal cancer, using a novel chromatographic method (molecular strip) for the detection of PCR amplified product and to confirm the results by the standard method of agarose gel electrophoresis. Patients and Methods: Thirty patients participated in the present study providing 27 PB and 26 BM samples. PB samples were also obtained from 20 healthy volunteers. PCR products were detected using the molecular strip and the results were confirmed by 1.5% agarose gel electrophoresis. Results: In total 26 out of 27 blood samples (96%) and 19 out of 26 bone marrow samples (73%) were found positive for Ep-CAM expression using both methods, whereas none of the 20 healthy controls was found positive for the above marker. Conclusion: Ep-CAM mRNA is highly expressive in peripheral blood of patients with metastatic colorectal cancer. Its prognostic significance should be evaluated in patients with resectable carcinomas. The new strip detection method is highly specific for the PCR product under investigation, reliable, easy to handle, fast and safe for the user.*

Ep-CAM (also known as EGP-2, EGP40, 17-1A, GA733-2, ESA, 323/A3) is a 40 kDA epithelial transmembrane glycoprotein expressed on most human epithelial cells. This molecule is encoded by the GA733-2 gene located on chromosomal region 4q (1). Ep-CAM mediates $Ca^{2+}$-independent homotypic cell-cell adhesions, however its exact role in carcinogenesis and tumor progression is still under investigation (2). A few studies suggest that Ep-CAM overexpression might inhibit metastases by preventing the shedding of cancer cells due to increased intercellular adhesion of tumor cells in primary lesions (3). Other studies suggest that Ep-CAM overexpression might promote tumor cell invasion and metastasis by suppressing cadherin-mediated cell adhesion (4). Thus, Ep-CAM overexpression in tumor tissues could serve as a prognostic factor in the outcome of the disease (5).

Even though the prognostic significance of Ep-CAM expression has been assessed in breast (6, 7), head and neck (8), lung (9) and prostatic (10) tumor tissue specimens by immunohistochemical staining, few studies have suggested that Ep-CAM detection in bone marrow and peripheral blood of cancer patients, using PCR techniques, could also serve as a specific marker for disseminated tumor cells. In that case, early detection of circulating tumor cells could identify high-risk patients (11).

We detected Ep-CAM antigen in unfractionated whole blood and bone marrow samples of patients with metastatic colorectal cancer and heavy tumor load. The presence of the antigen was evaluated through RT-PCR and the subsequent detection used a novel chromatographic molecular method (Ep-CAM detection®, Acron Genomics, USA). The results were confirmed by the standard method of agarose gel electrophoresis. The new chromatographic method is based on the design of a polyadenylated probe which, under the appropriate conditions, will bind to the biotinylated single-strand DNA of Ep-CAM. Subsequently, the mix is applied to a strip and the presence of the product can be analyzed chromatographically due to a chromogenic reaction (Figure 1).

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## Patients and Methods

Twenty-three patients with metastatic colorectal cancer provided both peripheral blood and bone marrow samples. Additionally, three patients provided only bone marrow and four patients provided only peripheral blood samples. In total, thirty patients participated in the present study providing twenty-seven peripheral blood and twenty-six bone marrow samples. Samples were taken at the time of diagnosis or during the first cycles of chemotherapy, but definitively before any documentation of disease response. Peripheral whole blood samples were also obtained from twenty healthy volunteers. Each sample consisted of 5 ml of bone marrow or blood, collected in tubes containing ethylene diamine tetra acetate (EDTA), as anticoagulant. Informed consert was obtained from patients before sample collection.

*Cell cultures.* Human COLO 205 (ATCC, Colorado, USA), established colon carcinoma cells expressing the membrane cancer antigen Ep-CAM, were grown at 37˚C with 5% $CO_2$ in RPMI 1640 supplemented with 2mmol/L L-glutamine and 10% bovine serum, penicillin (100 U/ml) and streptomycin (Sigma Aldrich, Germany). The media and supplements were purchased from Euroclone Ltd., UK.

*RNA extraction.* RNA extraction from 500 μl peripheral blood and 250 μl bone marrow were performed using the TRIR reagents according to the manufacturer's protocol (TRIR, Abgene, UK). Subsequently, each sample was incubated for 10 min following the addition of 750 μl of TRIR reagent on ice. RNA was then extracted by the addition of 250 μl of chloroform. The tube was vortexed for 5 min and then placed on ice for 10 min. The phases were separated by a 15-min centrifugation at 11,000g in a table-top microcentrifuge at 4˚C. The aqueous phase was transferred to a sterile tube, an equal volume of isopropanol was added and the mixture was incubated on ice for 10 min to precipitate RNA. After another 10-min centrifugation at 11,000g in a table-top microcentrifuge at 4˚C, the supernatant was discarded. One ml of 75% frozen ethanol was added to the tube and recentrifuged at 4˚C for 5 min. The contents of the tube were dried (5-10 min) at room temperature. The RNA should not dry completely before re-suspension in 40μl diethyl-pyrocarbonate-treated water. The quality and quantity of RNA was assessed by a spectrophotometer reading at 260/280.

*RNA extraction from cells.* Cells at density $5x10^9$ were suspended in 500 μl 1x PBS followed by RNA extraction using TRIR according to the manufacturer's protocol.

*Ep-CAM detection.* The production of cDNA and the strip application were performed according to the manufacturer's instructions and reagents included in the Ep-CAM detection kit (Medicon Hellas, S.A., Greece). In addition the RT-PCR products (10 μl) were analyzed on a 1.5% agarose gel (Abgene) in Tris-borate-EDTA buffer pH=8.3 and visualized by ethidium bromide staining (Sigma Aldrich, Germany).

*Production of c-DNA.* Reverse transcription was performed using the protocol according to the manufacturer's instructions (Acron Genomics, USA). First-strand c-DNA synthesis was done with reverse primer in a 20 μl reaction, which contained 1 μg of RNA after spectrophotometer analysis, 1mM each of d-ATP, d-CTP, d-GTP and d-TTP (Sigma Aldrich), 10U/μl Rnase inhibitor (Sigma Aldrich) and 6U reverse transcriptase (Abgene). The reaction was incubated for 10 min at 70˚C and 37˚C for 1h. The reverse transcriptase was denatured at 99˚C for 5 min and the solution cooled on ice. Five μl reaction was then used for amplification.

*Production of double-stranded DNA.* To ensure that the product of double-stranded amplification corresponded to mRNA, each amplification primer pair was designed to span a long intron beyond the limits of amplification possible with PCR. Using this strategy, only c-DNA corresponding to spliced mRNA, and not contaminating genomic DNA, can be successfully amplified. Amplification was performed with 5 μl of c-DNA in volume of 50μl containing 5μl 10x PCR buffer (100mM Tris-HCl, pH 8.3, 500mM KCl, Abgene), 4mM $MgCl_2$, 400μM dNTP's (Sigma Aldrich), 10pM forward primer, 10pM reverse primer (Medicon Hellas, S.A.) and 3U Taq polymerase (BioLabs, USA). For the detection of Ep-CAM mRNA following the initial denaturation step (94˚C for 2min.), 35 cycles were carried out (94˚C for 80sec, 53˚C for 20sec, 72˚C for 5sec) followed by a final elongation step at 72˚C for 5min. Polymerase chain reaction products (10μl) were separated by electrophoresis through an 1.5% agarose gel (Abgene) in Tris-borate-EDTA buffer pH=8.3 and visuali zed by ethidium bromide staining (Sigma Aldrich).

*Molecular strips analysis of PCR products.* Each PCR product sample was analyzed using a molecular strip provided with the kit according to the manufacturer (Acron Genomics). A control tube with distilled water instead of DNA template was included in every analysis of the amplification experiment and prepared after all samples had been pipetted. Ten μl PCR product, 1μl internal probe master and 1μl hybridization buffer were added to each analysis reaction tube. The reaction tubes were then incubated at 95˚C for 2 min and 37˚C for 5 min. While in the hybridization step, another reaction tube was taken for each PCR product sample and 200 μl of development buffer were added. Following the hybridization step, 5μl of the hybridization product sample was added to the specific area on the molecular strip up from the concrete marked area. The final result was obtained following incubation of the molecular strip in the development buffer for 7 min.

*Procedures to prevent contamination.* Disposable gloves were worn at all times and were changed frequently. Pipette tips with aerosol barriers and sterile microcentrifuge tubes were used for all manipulations and buffers autoclaved. Separate rooms within the laboratory, each with dedicated pipette, were designated for samples preparation, isolation of RNA, storage and detection of PCR products.

## Results

Among the 23 patients who provided both blood and bone marrow samples, 17 (74%) expressed Ep-CAM both in blood and bone marrow, whereas only 1 (4%) was negative in both. The remaining 5 patients (22%) were positive for Ep-CAM expression in their blood and negative in their bone marrow. Two of the 3 patients, who provided only

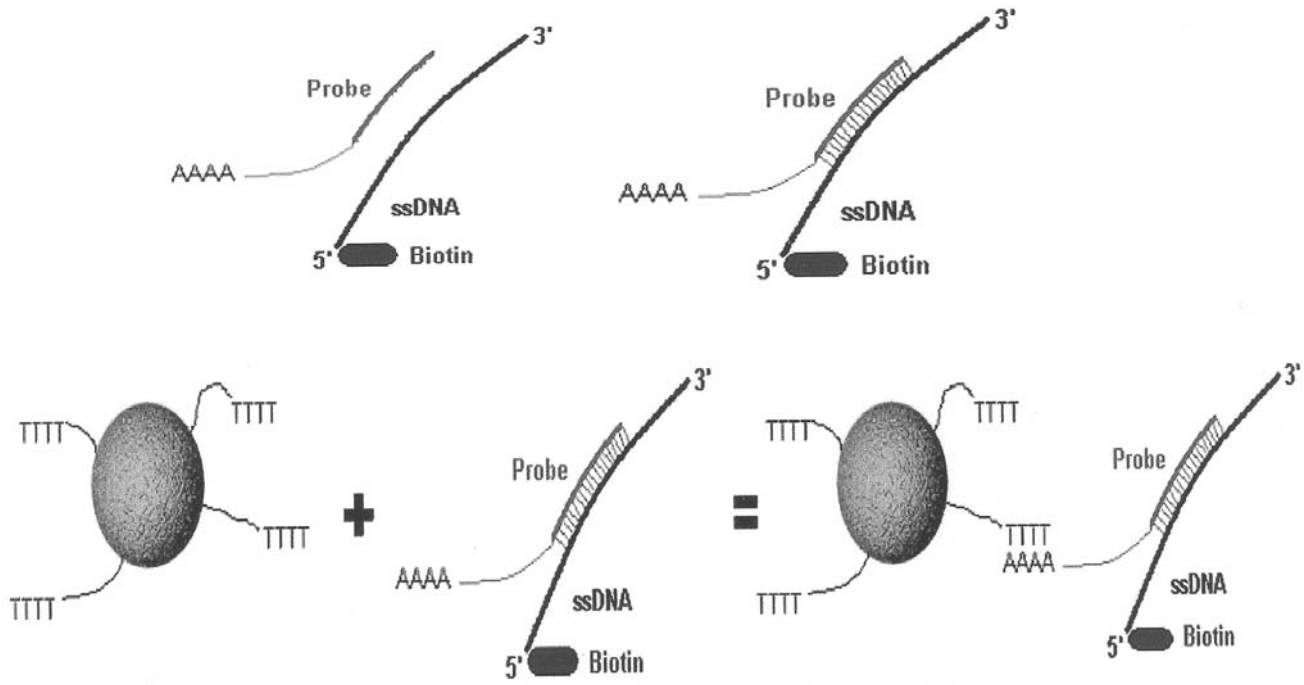


Figure 1. *Biotin-labelled PCR product is hybridized with specific oligonucleotide, the internal probe. A Poly-A tail is attached to the 3' end of the probe.*

bone marrow samples, were positive for Ep-CAM. All 4 patients who provided blood samples were found positive for Ep-CAM. In total 19 out of 26 bone marrow samples (73%) and 26 out of 27 blood samples (96%) were found positive for Ep-CAM expression using the strip detection method and the standard 1.5% agarose gel electrophoresis. Using the same methods of Ep-CAM detection, all 20 healthy controls were found negative for the above-mentioned antigen.

## Discussion

Numerous studies have reported the use of reverse transcriptase polymerase chain reaction (RT-PCR) technology to detect circulating tumor cells from peripheral blood and bone marrow of cancer patients. The main technique for the detection of occult tumor cells involves amplification of tissue-specific mRNA by RT-PCR. It is well known that colorectal carcinoma tissue universally expresses high levels of Ep-CAM (12). Since disseminated tumor cells are considered to be the origin of metastasis, it has been suggested that Ep-CAM mRNA could serve as a target for the detection of single tumor cells (13).

The present study examined the presence of Ep-CAM in peripheral whole blood and bone marrow of patients with metastatic colorectal cancer, using two methods for the detection of the PCR product, the standard method of agarose gel electrophoresis and a novel chromatographic molecular method. All patients had disseminated disease and heavy tumor load. Ep-CAM was detected in 26 of the 27 blood samples (96%) and in 19 of the 26 bone marrow samples (73%). Lower detection rates for circulating tumor cells in bone marrow have also been described in other studies using different molecular targets. Notably, all Ep-CAM-positive bone marrow samples were associated with Ep-CAM detection in peripheral blood. In contrast, none of the 20 normal control blood samples expressed epithelial cell adhesion molecules.

Several studies have reported the use of RT-PCR to detect colorectal carcinoma-specific markers in peripheral blood, bone marrow, lymph nodes and peritoneal washings with conflicting findings (14-17). Carcinoembryonic Antigen Messenger RNA (CEA mRNA) and cytokeratins (CK's) 19 and 20 have been proposed as particularly useful molecular targets for identification of circulating colorectal cancer cells (18-20). Interestingly, the molecular detection of Ep-CAM has not been performed extensively in colorectal cancer, even though it has been used in a number of carcinoma-directed immunotherapeutic approaches (21).

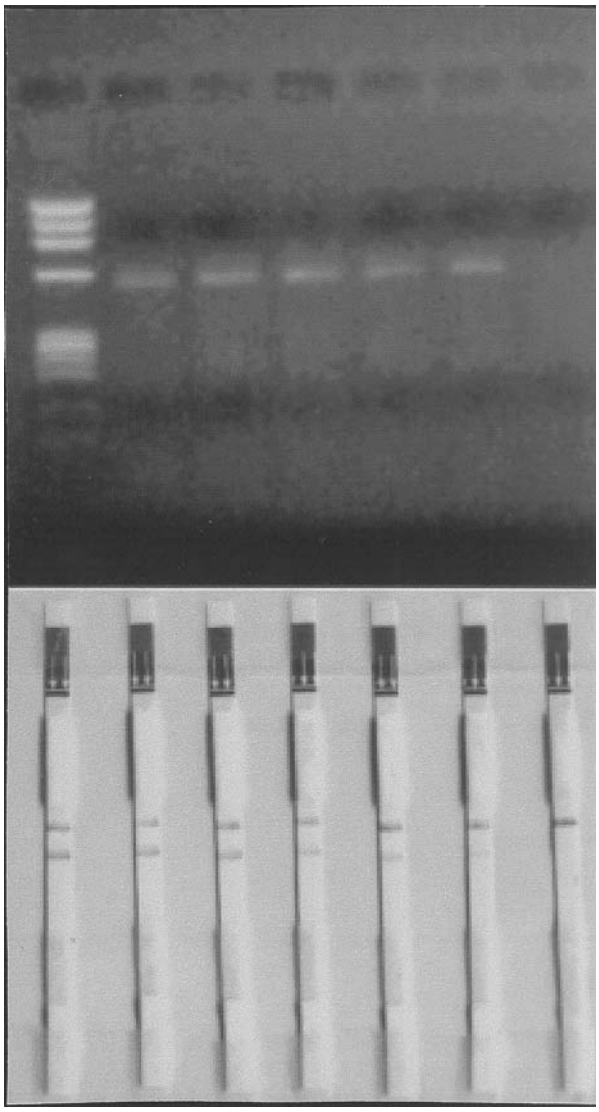

Figure 2. *Ep-CAM mRNA detection using the classic method of agarose gel electrophoresis and the novel chromatographic method with molecular strips.*

The detection rate clearly relates to the sensitivity of the assay used. In the present study, the sensitivity of Ep-CAM mRNA detection by RT-PCR in bone marrow and peripheral blood was particularly high. Concerning the detection of this specific antigen in patients with colorectal carcinoma, no false-positive results for the above marker were observed in control subjects. Moreover, the universal expression of Ep-CAM in peripheral blood of this patient population does not permit any correlation between its presence and the overall survival. Ep-CAM mRNA detection was not performed after the completion of chemotherapy or after the achievement of any response. The reason was that, since complete remission could not be expected in such advanced disease, any PCR conversion from positive to negative seemed to be very unlikely. Thus, Ep-CAM mRNA detection at the time of tumor recurrence has a limited prognostic value.

The new procedure can give rapid and highly reliable results for the detection of specific PCR products utilizing a chromatographic method. According to this method, biotin-labelled products are applied to a strip and their presence can be analyzed chromatographically due to a chromogenic reaction. In our study, we used this product for the detection of the Ep-CAM gene expression and we confirmed the results by the standard method of agarose gel electrophoresis (Figure 2). The new strip detection method is at least as reliable as agarose gel electrophoresis and certainly easier to handle, faster (the result can be obtained in 15 min) and safer for the user (no ethidium bromide staining needed). Also it is highly specific for the PCR product under investigation due to the use of the internal probe. Thus, we recommend the use of the above method for the molecular detection of circulating micrometastatic cancer cells.

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