Cell-Free Nucleic Acids Circulating in the Plasma of Colorectal Cancer Patients Induce the Oncogenic Transformation of Susceptible Cultured Cells

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Abstract

It has been proposed that cell-free nucleic acids in the plasma participate in tumorigenesis and the development of metastases via transfection-like uptake of such nucleic acids by susceptible cells. This putative phenomenon is tentatively referred to as “genometastasis.” In this study, we examined the effects on cultured cells of plasma from healthy individuals and from patients with colon cancer. Cultures of NIH-3T3 cells and human adipose-derived stem cells (hASC) were supplemented with samples of plasma from patients with K-ras-mutated colorectal tumors or from healthy subjects using two different protocols: direct addition of plasma to cultures in standard plates and addition in the absence of contact between plasma and cells, which were separated by a membrane with 0.4-μm pores. In plasma-treated hASCs, no K-ras-mutated sequences were detected by real-time PCR. In contrast, in most cultures of plasma-treated NIH-3T3 cells (murine cells), the transfer of human DNA occurred, as verified by the detection of human K-ras sequences, p53 sequences, and ß-globin–encoding sequences. Moreover, NIH-3T3 cells that had been cultured with plasma from patients with colon cancer were oncogenically transformed, as shown by the development of carcinomas in nonobese diabetic–severe combined immunodeficient mice after the injection of such cells. Microscopic analysis of membranes that had separated plasma from cultured cells confirmed the complete absence of cells in the plasma. We only observed noncell particles, having diameters of <0.4 μm. Our results indicate that plasma from cancer patients is able to transform cultured cells oncogenically, supporting the previously proposed hypothesis of genometastasis. Cancer Res; 70(2); 560–7. ©2010 AACR.

Introduction

The accepted paradigm of tumor progression, which involves the development of the tumorigenicity and of the invasive capacity of cells at the site of the primary tumor, with subsequent dissemination and metastasis, has been sometimes challenged. Nearly 3 decades ago, some authors encouraged to reevaluate the accepted view of the origin and progression of malignant conditions, proposing new exciting theories, such as the horizontal transmission of malignancy (1). However, those studies were not continued enough. In recent years, complementary and alternative theories have been proposed and we still do not fully understand the processes of invasion and metastasis (2, 3).

The detection of cell-free nucleic acids in the serum and plasma of cancer patients has aroused considerable interest. The phenomenon was first reported some decades ago and has been verified for a variety of tumors (4, 5). In addition, it has been shown repeatedly that some of the circulating nucleic acids originate in tumors (4–6), although the mechanism whereby nucleic acids are released from tumor cells is not yet fully understood. It has been suggested that such nucleic acids are liberated from cells as a result of necrosis and apoptosis (7), but other active mechanisms are also feasible (8). Many researchers have tried to determine the clinical value of the detection and quantitation of nucleic acids in the plasma of cancer patients, in particular with respect to the management of such patients, with successful results in some cases (5). However, to our knowledge, there are no conclusive data about the role of such nucleic acids in tumor progression.

In 1999, it was proposed that cell-free nucleic acids in plasma might be directly involved in the development of metastases through, probably, transfection-like uptake by susceptible cells (9, 10). This putative phenomenon is referred to as “genometastasis.” Subsequent studies have provided evidence that supports this hypothetical mechanism (11, 12). Moreover, biological mechanisms of the horizontal transfer of genes from cancer cells have been described (13, 14) and seem to be compatible with the hypothesis of genometastasis.
The present study was performed to show the biological feasibility of gene transfer and of the transformation of cells by cell-free tumor-derived nucleic acids in the plasma of cancer patients. In our analysis, we examined the effects on cultured cells of plasma from healthy individuals and from patients with colon cancer.

Materials and Methods

Patients and healthy subjects. We recruited 13 patients with colon cancer who had undergone resection of primary colorectal cancer in the Department of General Surgery at La Paz University Hospital (Madrid, Spain) according to a protocol approved by the Ethics Committee of the hospital. Clinical profiles are shown in Table 1.

In all cases, histopathologic analysis revealed that tumors were adenocarcinomas. Healthy subjects were recruited according to normal protocols for such studies.

Detection of K-ras mutations in tumors. In this study, mutated K-ras sequences were used for detection of gene transfer phenomena. Therefore, we selected only those patients whose tumors had such mutations. We examined samples of individual tumors by real-time PCR and later confirmed our results by sequencing. The purpose of the first test was to obtain results in a short time so that plasma from the identified patients could be added to cell cultures within 24 h of collection.

After the extraction of DNA with a DNeasy Tissue kit (Qiagen), samples were analyzed by real-time PCR with fluorescent hybridization probes to examine the presence of mutations at codon 12 of the exon 1 of the K-ras gene (Gly to Asp, to Cys, or to Val). Briefly, in addition to specific primers for amplification of a 164-bp fragment, we used two hybridization probes for each amplification. One of the two probes (the sensor probe) was designed to be complementary to a point mutation of interest and labeled at its 5’ ends with LC-Red705 dye and phosphorylated at its 3’ ends. The anchor probe was labeled at its 3’ end with fluorescein. Probes were manufactured by Tib Mobiol. In addition, we added a peptide nucleic acid (PNA-oligomer) probe, as a PCR-clamp, to suppress the amplification of the wild-type K-ras sequence such that only mutated K-ras sequences would be amplified. PNA-oligomer encompassed codons 10 to 14 and was obtained from Panagene.

We performed all PCRs with the LightCycler System (Roche Diagnostics) using LightCycler Software (version 3.5).

When a tumor yielded positive results that corresponded to one of the mutations of interest, the result was validated by sequencing, and simultaneously, a sample of the corresponding plasma was added to cultures of human adipose-derived stem cells (hASC) and NIH-3T3 cells, as noted below.

For sequencing, we first amplified a 286-bp sequence of the K-ras gene. Then, we prepared the reaction mixtures for sequencing of the products of PCR using the BigDye Terminator Cycle Sequencing v3.1 Ready Reaction kit (Applied Biosystems) following the manufacturer’s instructions. Sequences were analyzed with a Genetic Analyzer (model 3130xl) and Sequencing Analysis Software v5.2 from Applied Biosystems.

Isolation of human plasma. Samples of blood were collected, in tubes with EDTA, in the operating room immediately before surgical resection of tumors. We obtained 15 mL of blood from each patient. All samples were subjected to centrifugation immediately after extraction at 1,800 × g for 10 min. Plasma was collected and subjected to a second centrifugation at 3,000 × g for 10 min.

Detection of K-ras mutations in plasma. DNA was extracted from plasma with a QIAamp DNA Blood Mini kit

<p>| Table 1. Main clinical characteristics of the patients with colorectal cancer whose plasma was added to cultures of nontumor cells |
|---------------------------------|-----------|----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Code</th>
<th>Age (y)</th>
<th>Gender</th>
<th>Tumor location</th>
<th>TNM</th>
<th>Differentiation</th>
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<tr>
<td>342</td>
<td>78</td>
<td>Male</td>
<td>Sigmoid colon</td>
<td>Not analyzed (irresectable tumor)</td>
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<tr>
<td>346</td>
<td>64</td>
<td>Male</td>
<td>Rectum</td>
<td>T3N2M0</td>
<td>Moderate</td>
</tr>
<tr>
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<td>Sigmoid colon</td>
<td>T4N1M0</td>
<td>Moderate</td>
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<tr>
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<td>Female</td>
<td>Right colon</td>
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<tr>
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</tr>
<tr>
<td>364</td>
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<td>Left colon</td>
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<tr>
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<tr>
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<td>T4N1M0</td>
<td>Moderate</td>
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</table>

Abbreviations: TNM, tumor-node-metastasis; RT+CHT, radiotherapy and chemotherapy.
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(Qiagen) according to the manufacturer’s protocol for a sample volume of 1 mL. Samples were analyzed by two real-time PCRs with fluorescent hybridization probes by the same protocol as described for the analysis of DNA from tumors.

First, we amplified the total (mutated and nonmutated) K-ras sequences. For the second PCR, we added the PNA-oligomer probe so that only mutated K-ras sequences were amplified. When no mutated K-ras was detected, we assumed that amplification of K-ras corresponded exclusively to the amplification of nonmutated DNA.

Determination of human β-globin–encoding sequences by PCR. A PCR amplification test was performed by a previously described protocol to detect human β-globin–encoding sequences (17) in murine cells that had been cultured with human plasma. Amplifications by PCR of a 249-bp fragment were performed with a minimum of 20 ng of template DNA in a total volume of 50 μL. The products of amplification were subjected to electrophoresis on a 2% agarose gel, as described above.

Detection of mutated murine k-ras sequences by mutant allele-specific amplification. We performed mutant allele-specific amplification (MASA) by PCR, as previously described for rat sequences (11) and modified for the detection of mouse DNA. Using this technique, we examined the presence of the Gly12Asp mutation in exon 1 of the k-ras oncogene. We amplified a fragment of 140 bp. In brief, the reaction mixture for PCR, with a total volume of 50 μL, contained a minimum of 20 ng of template DNA. To avoid unspecific reactions (false-positive results), the amount of DNA did not exceed 250 ng. The products of amplification were subjected to electrophoresis on a 3% agarose gel, as described above.

To ensure the validity of the technique, all samples of DNA were also amplified to examine the presence of nonmutated k-ras under the same conditions.

Electron microscopy. Inserts of Transwells were fixed in 3.5% glutaraldehyde for 30 min at 37°C, postfixed in 2% osmium tetroxide for 2 h, rinsed, dehydrated, and embedded in Araldite (Durcupan, Fluka BioChemika). We cut serial 1.5-μm semithin sections with a diamond knife and stained them with 1% toluidine blue for localization of pores. For the identification of cells and particles within membranes, we cut ultrathin (0.05 μm) sections with a diamond knife, stained them with lead citrate, and examined them under an FEI Tecnai spirit electron microscope.

Assessment in vivo of the tumorigenicity of cells that had been cultured with human plasma. Nine-week-old male nonobese diabetic–severe combined immunodeficient (NOD-SCID) mice (Charles River Laboratories) were used. Breeding and care of animals were undertaken in compliance with European Community Directive 86/609/CEE for the use of laboratory animals and with Spanish law (Real Decreto 1201/2005).

Two million cells were injected s.c. into each mouse. Four animals were injected with untreated NIH-3T3 cells; two with NIH-3T3 cells that had previously been directly cultured with plasma from healthy subjects; four with NIH-3T3 cells that had previously been directly cultured with plasma from cancer patients; and two with NIH-3T3 cells that had previously been cultured with plasma from a cancer patient and a healthy subject, respectively, in Transwell plates. We also injected three mice with human colon adenocarcinoma cells (SW-480 cells) as a positive control. The SW-480 cells have a point mutation (Gly to Val) at codon 12 of exon 1 of the K-ras oncogene.

The growth of s.c. tumors was monitored in all animals and recorded weekly. We measured the greatest diameter of each tumor with electronic calipers. Animals were sacrificed 32 to
injected with SW-480 cells and in all four mice that had been injected with plasma from patients with colon cancer by injection of cells into NOD-SCID mice. Five days after injection, we detected human mutated sequences from the first to the third dispersion until the end of the experiment (Fig. 1). In 4 of the 12 cultures, cells died before the first dispersion. We repeated this last culture with the same result. NIH-3T3 cells that had been cultured with plasma from healthy subjects were positive, in all cases, for human K-ras sequences and negative for mutated K-ras sequences. Thus, they harbored only nonmutated K-ras sequences. No culture of NIH-3T3 cells was positive for mutated murine K-ras sequences when examined by the MASA technique.

We detected human p53 sequences and human β-globin-encoding sequences in six of eight samples of NIH-3T3 cells that had been cultured with plasma from cancer patients. We investigated the presence of p53 sequences in untreated hASCs and found only the ARG-encoding triplet at codon 72 of exon 4. Thus, we were able to examine whether the PRO-encoding sequence, which was present in the DNA in three of the samples of plasma added to cells, had been transferred to cells. We performed PCR to analyze the DNA from hASCs that had been cultured with patients’ plasma and we failed to detect any PRO-encoding sequence.

Analysis of human K-ras sequences in cells cultured with human plasma in Transwell plates yielded similar results to those described above for direct cultures. Specifically, we detected mutated K-ras sequences in NIH-3T3 cells but not in hASCs.

**Microscopic analysis of membranes of Transwell that had contained plasma.** Microscopic analysis of the inserts of Transwell plates that had contained plasma confirmed the complete absence of cells. We observed only particles (Fig. 2), many of which seemed to be passing through the pores of the membrane of the insert. Thus, such particles had diameters of <0.4 μm.

**Oncogenic potential of cells in mice.** We examined the oncogenic potential of cells that had been cultured directly with plasma from patients with colon cancer by injection of such cells into NOD-SCID mice. Five days after injection, we found palpable masses in the three mice that had been injected with SW-480 cells and in all four mice that had been injected with NIH-3T3 cells that had previously been cultured with plasma from cancer patients (Fig. 3). At sacrifice, only the seven mice had s.c. masses, with greatest diameters that ranged from 1.1 to 2.7 cm (Fig. 3). No masses were found in the two mice injected with NIH-3T3 cells that had been cultured with plasma from healthy subjects, and none were found in mice injected with untreated NIH-3T3 cells.

Histologic examination of excised masses, after staining with H&E, revealed that they were undifferentiated carcinomas (Fig. 3). We also examined samples of liver, lungs, and mesenteric lymph nodes from all animals but found neither macroscopic nor microscopic tumors.

We detected human K-ras sequences by real-time PCR in all samples of tissues from all animals, with the exception of the mice injected with untreated NIH-3T3 cells. In the animals injected with NIH-3T3 cells that had previously been cultured with plasma from cancer patients or SW-480 cells, we detected mutated human K-ras sequences in all tissues examined, including tumors, but we did not find such sequences in any samples from the animals injected with NIH-3T3 cells that had previously been cultured with plasma from healthy subjects, in which only nonmutated K-ras sequences were found.

![Figure 1. Detection, by real-time PCR, of a point mutation in codon 12 of exon 1 of the human K-ras oncogene (GGT to GAT; Gly to Asp) in cells cultured with human plasma. In this PCR, we examined samples from three plates of NIH-3T3 cells and three plates of hASCs that had been cultured with plasma from patients with colorectal cancer, from three plates of NIH-3T3 cells and three plates of hASCs that had been cultured with plasma from healthy subjects, from a tumor known to have the mutation of interest (positive control), and water. We detected the mutated human sequences only in the murine cells that had been cultured with plasma from patients with colorectal cancer (dotted lines) and in the positive control (continuous line).](image-url)
As described for tissue samples, we detected human sequences in the buffy coat from all animals, with the exception of the animals injected with untreated NIH-3T3 cells. Moreover, mutated K-ras sequences (to derived, presumably, from circulating tumor cells) were found only in the blood from mice that had been injected with SW-480 cells.

In an analysis of plasma samples, we detected mutated human K-ras sequences in the mice that had been injected with SW-480 cells and in the mice that had been injected with NIH-3T3 cells that had previously been cultured with plasma from cancer patients (Fig. 4). No human sequences were detected in the plasma from the animals that had been injected with cells cultured with plasma from healthy subjects.

We also found a tumor in the mouse that had been injected with NIH-3T3 cells that had been cultured with plasma from a cancer patient in a Transwell plate. This tumor, with a final maximum diameter of 1 cm, was positive for mutated human K-ras sequences. No mass was found in the mouse injected with NIH-3T3 cells that had been cultured with plasma from a healthy subject in a Transwell plate. Identical to above-described results, we detected human K-ras sequences in all samples of tissues from the two animals, but mutated K-ras sequences were only detected in the samples obtained from the mouse that was injected with cells that had previously been cultured with plasma from a cancer patient.

Discussion

In the present study, two lines of cells (i.e., NIH-3T3 cells and hASCs) were cultured with plasma from patients with colorectal cancer and from healthy subjects. NIH-3T3 cells, derived from mouse embryo fibroblasts, are p16-deficient cells that have frequently been used in studies of oncogenic transformation (18, 19). Disruption of the expression of the p16/Rb pathway in rodent cells is extremely efficient in preventing Ras-induced growth arrest (18). hASCs are well-established lines of pluripotent stem cells that are often used in clinical trials6 as a consequence of their excellent safety profile (20).

In the cultures of hASCs treated with plasma from patients with K-ras–mutated colorectal tumors, we never detected mutated K-ras sequences by real-time PCR. In contrast, in most such cultures of NIH-3T3 cells, we detected mutated human sequences soon after the start of incubation (from the first to the third dispersion) and these sequences were still detectable at the end of the experiment, ~3 weeks after removal of human plasma from the culture medium. NIH-3T3 cells that had been cultured with plasma from healthy subjects were negative, in all cases, for mutated human K-ras sequences and positive for nonmutated K-ras sequences. Thus, it seemed that, whereas hASCs were resistant to transformation by cell-free tumor-derived DNA in patients’ plasma, NIH-3T3 cells (p16-deficient cells) were able to stably incorporate such foreign DNA during simple incubation with plasma from cancer patients.

As positive controls for transfection of cells with tumor DNA, we set up cultures of hASCs and NIH-3T3 cells supplemented with a solution of DNA that had been extracted from K-ras–mutated tumors and CaCl2. The results were similar to those obtained when cells were cultured with plasma: we detected mutated K-ras sequences in NIH-3T3 cells but not in hASCs (data not shown). However, when we quantified the amounts of mutated K-ras sequences in the samples used for the various incubations, we found that the amount was almost 1 order of magnitude higher in the case of DNA from tumors (30.7 ± 19.3 fg; mean ± SD) than in plasma (3.5 ± 2.3 fg). The low concentrations of DNA in the plasma and the fact that such sequences were incorporated into cells as a result of the simple addition of plasma to cell cultures, without prior extraction of DNA, suggest the considerable efficiency of the transfection phenomenon when the DNA is added and originates in plasma.

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Figure 2. Electron micrographs of the insert of a Transwell plate. Plasma was placed above the insert, and NIH-3T3 cells were placed in the bottom of the plate so that there was a membrane with 0.4-μm pores between the plasma and the cells. Ten days later, the insert was removed, fixed, and sectioned for electron microscopy. No cells were detected in the plasma, but many unidentified particles of <0.4 μm in diameter were observed within the pores. Scale bars, 10 μm (A), 1 μm (B), and 500 nm (C).

When we incubated six plates of NIH-3T3 cells with DNA that had been extracted from buffy coat samples from healthy subjects, we detected human \( K\text{-ras} \) sequences in only three of the cultures (data not shown). Thus, the rate of transfection with the purified DNA seemed to be lower than after the addition of human plasma.

We detected the \( p53 \) human sequences and human \( \beta\)-globin–encoding sequences in five of six samples of cells that had been cultured with plasma from cancer patients. In contrast, DNA from none of the genes examined was transferred to hASCs during culture with plasma from cancer patients. Thus, the transfer of DNA to murine NIH-3T3 cells was not restricted to tumor-related DNA sequences. Moreover, it seemed that “humanization” of murine cells had occurred during incubation with plasma from cancer patients. These results are coherent with those of a previous study in which authors showed that mouse fibroblasts, which were transformed by transfection with human tumor DNA, acquired not only the transformation genes but also a large array of other cotransfected human DNA fragments (21). This similarity also supports the idea that transfection provoked by the simple addition of plasma to cell cultures is strongly similar, although more efficient, to that obtained by transfection with DNA extracted from tumors.

Having shown the apparent transfer of human DNA to NIH-3T3 cells, we examined whether this DNA could recombine with the DNA of the host cell to generate a similar mutation within the DNA of the host. Using a MASA technique (11), we examined the presence of mutated murine \( K\text{-ras} \) sequences in the NIH-3T3 cells that had been cultured with plasma from \( K\text{-ras} \)-mutated cancer patients. No cultures were positive for such \( K\text{-ras} \) mutation.

NIH-3T3 cells that had previously been cultured with plasma from cancer patients generated undifferentiated carcinomas when injected into NOD-SCID mice (Figs. 3 and 4). Moreover, mutated human \( K\text{-ras} \) sequences were detected in distant parenchyma (liver and lungs) and in plasma from such mice, as also observed when human colon adenocarcinoma cells (SW-480 cells), as positive controls, were injected into NOD-SCID mice. Thus, our results indicated that, when NIH-3T3 cells were cultured with plasma from patients with colon cancer, the cells acquired the potential for oncogenic activity in vivo. Moreover, this activity was similar to that of a standard line of colon cancer cells. NIH-3T3 cells are normally unable to produce tumors spontaneously in NOD-SCID mice (as confirmed here), but they do have a genetic alteration (p16 deficiency) that confers elevated susceptibility to transformation by \( ras \), as reported previously (18). Incubation with plasma from cancer patients provoked a definitive change in the cells, mediated perhaps by gene transfer, which resulted in oncogenic activity.

**Figure 3.** Photographs of a mouse that had been injected s.c. with NIH-3T3 cells that had been cultured with plasma from a cancer patient. The mouse was sacrificed 36 d after inoculation (when the photographs A and B were taken). C and D, sections of the tumor were stained with H&E and examined by light microscopy. Original magnification, ×10 (C) and ×40 (D). Light micrographs show that the tumor was an epithelial carcinoma in which undifferentiated regions were mixed with necrotic tissue.
transformation. Thus, previous reports that plasma from cancer patients can transform susceptible cells (9, 10) were confirmed.

The oncogenic transformation of NIH-3T3 cells occurred in an identical manner no matter whether cells were in direct contact with plasma or were separated from the plasma by a membrane with 0.4-μm pores. Moreover, electron microscopy failed to reveal any cells on or in the membrane of the insert. We observed only particles (Fig. 2), many of which seemed to be passing through the pores of the membrane of the insert and had, therefore, diameters of <0.4 μm. This result excludes the possibility that the plasma might have contained tumor cells that had contaminated cultures and suggests that nucleic acid–containing particles might have been responsible for oncogenic transfection.

Nearly 30 years ago, it was suggested that horizontal transmission of malignancy from already established neoplastic cells to normal cells might be a biological event involved in cancer progression (1), although there were scarce data about the mechanism of this phenomenon. In 1994, it was proposed that tumor cells might spontaneously release particles that might transform nontumor cells in culture (22). In 2001, it was suggested that tumor cell–derived particles are able to mediate the lateral transfer of DNA to eukaryotic cells and, moreover, that this transfer might result in aneuploidy and the accumulation of genetic changes that are necessary for tumorigenesis (13). There have been reports of cell-derived particles that contain nucleic acids, with diameters of the order that we observed, for example, apoptotic bodies, exosomes, and microvesicles (23). Transfer of nucleic acids by such particles has also been reported (13, 24, 25). The spontaneous release of nucleoprotein complexes by cells has also been described, and the powerful transformational activity of such complexes has been shown (22, 26). Further studies are clearly needed to characterize the particles in the plasma that might be responsible for the transfer of genes and the transformation of nontumor cells, as observed in the present study.

It has been argued that a putative transformation pathway, mediated by gene transfer, cannot explain the organ-specific tropism of metastases (3). It is possible that an analysis of the above-mentioned particles might provide an explanation because, for example, it has been suggested that the communication between mammalian cells that is mediated by exosomes might be cell type specific (24). In addition, further studies are needed to identify the cells that might be the target of this phenomenon (“susceptible” cells).

To conclude, in the present study, we obtained strongly suggestive evidence that plasma from cancer patients can transform cultured cells oncogenically, supporting the concept of genometastasis that we proposed a decade ago (9, 10).

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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Susceptible Cultured Cells Cancer Patients Induce the Oncogenic Transformation of Cell-Free Nucleic Acids Circulating in the Plasma of Colorectal