Sensitive detection of tumor cells in peripheral blood of carcinoma patients by a reverse transcription PCR method

Application

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Abstract

Occurrence of tumor cells in the peripheral blood of individuals suffering from cancer may serve as an early indication that the primary tumor has dispersed from its tissue of origin. Despite defence mechanisms of the organism, individual cancer cells may attach in distant regions and form colonies as an initial step of metastasis formation. Hence, early detection of metastatic potential can be estimated by detecting tumor cells in the blood circulation. Sensitivity and specificity are the main objectives of any method applied for this purpose. Most highly sensitive techniques, encounter an increasing problem of specificity due to background signaling from illegitimate transcription. AdnaGen established a method of tumor cell selection, their specific identification and analysis with a lower limit of detection of at least 2 tumor cells in 5 mL blood (10^{10} cells). To achieve this we combined the enrichment of tumor cells using a specifically designed antibody mixture with RT-multiplex PCR techniques for the detection of mRNAs encoding for tumor associated markers. Preliminary results of case studies showed the occurrence of tumor cells in blood of carcinoma patients indicating a potential tumor relapse, in some cases several months prior to an elevation of serum tumor markers. AdnaGen provides a sensitive and specific method to detect disseminated tumor cells in peripheral blood of testicular, breast and colorectal carcinoma patients. This innovative method is an option for clinicians as a predictive tool with respect to metastasis formation and may result in an appropriate selection of patients for adjuvant therapy.

Introduction

Metastases are the major cause of death in cancer patients. Metastases occur as a result of the interaction between the tumor cells and the host, and this interaction adds to the extreme complexity of the events associated with tumor dissemination. Spreading of tumor cells into the blood circulation from either primary tumors or subsequently from the lymphatic origin may be a result of dissemination of the primary tumor or a secondary event during tumor progression. Many of these cells reaching the circulation may be killed by an individual response of the immune system but the metastatic potential of remaining tumor cells cannot be ruled out. The presence of disseminated tumor cells in systemic circulation is an indication for tumor relapse. Their early detection followed by an immediate and possibly even individual therapy may prevent formation of metastases[1]. Specificity and maximal sensitivity are the main objectives for any method in this field. A very high sensitivity is possible by means of expression markers that are detected and amplified specifically on transcription level. However, due to background from illegitimate transcription in nucleated blood cells, most techniques described encounter the problem of specificity. This can be overcome by specific enrichment of non-blood cells[2-6]. This Application Note describes a sensitive and specific method to detect testicular, breast and colorectal carcinoma cells from blood. The tumor cell selection

and analyses are developed and provided by AdnaGen, using an Agilent 2100 bioanalyzer for detection.

Material and methods

AdnaGen developed a diagnostic system which is based on a two-step procedure:

AdnaTest CancerSelect

A combined method based on immunomagnetic enrichment of tumor cells directly from whole EDTA-blood with an antibody mixture, which was especially designed to isolate breast, testicular and colorectal carcinoma cells. mRNA was extracted with magnetic oligo-(dT) beads (DYNAL) followed by a high sensitive reverse transcription (QIAGEN).

AdnaTest CancerDetect

Expression analysis of tumor cells with multiplex PCR (up to five markers per assay) for the detection of mRNAs encoding for tumor markers. Multiplex PCR reactions were prepared using the cDNA from above as template, the primer mixture provided by AdnaGen and the HotStar PCR-kit (QIAGEN). PCR samples were analyzed using the Agilent 2100 bioanalyzer and the DNA 500 LabChip® kit.

Inoculation system

Cell spiking experiments with different carcinoma cell lines for each kind of investigated cancer were used to test the potential lower detection limit of this technique. Defined numbers of 2-100 cells were added to 5-mL aliquots of whole blood from healthy donors, an unspiked blood sample was used as negative control, cancer cell lines served as positive controls. The specificity of the selected antibodies was determined by flow cytometry.

Results and discussion

AdnaGen developed a diagnostic system for early detection of disseminated cells in peripheral blood of testicular, breast and colorectal carcinoma patients. This diagnostic approach is designed to optimize enrichment and analysis of tumor cells. The specificity of the method is shown by immunofluorescence staining (figures 1 and 2) and the lower detection limit by RT-PCR (figure 3).

Specificity

Figure 1 shows the specific recognition of tumor cells by the selected antibodies coupled to magnetic beads (AdnaTest CancerSelect) by microscopy. The immunofluorescence assay (figure 2) documents the specificity of the antibody mixture (AdnaTest CancerSelect) used for immunomagnetic enrichment.

Lower detection limit

Cell spiking experiments with different cell lines were performed to test the potential sensitivity of the assay. After inoculation and immunomagnetic enrichment, two carcinoma cells were detected in 5 mL blood samples (10^{10} cells) using the AdnaTest CancerDetect kit (figure 3). In addition specificities of up to 100 % are obtained for each marker of the multiplex PCR. We demonstrated this using blood of healthy donors, patients with different non-malign diseases (e.g. gastrointestinal diseases) and inoculated non-malignant epithelial cells. No signals were detected in the PCR analysis (data not shown). The combination of the two steps above provides high specificity and sensitivity for the diagnostic approach.

RNA stabilization

Due to the high instability of cellular RNA in vitro, preserving the RNA expression profile is essential for reliable analysis of gene

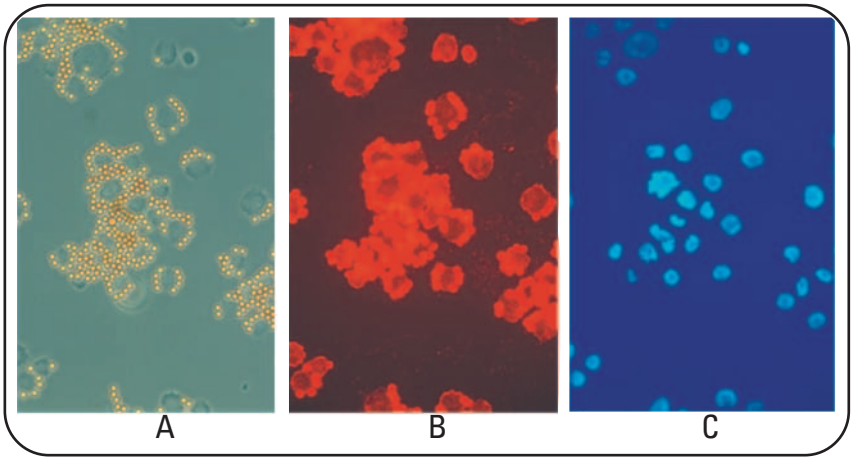

Figure 1
Inoculated tumor cells isolated using AdnaTest CancerSelect
A. Detection of antibody coupled beads (yellow) and tumor cells (phase contrast microscopy). B. Cells were stained (red fluorescence) with a secondary antibody against the selecting antibodies (AdnaTest CancerSelect). C. Nuclei were visualized by Hoechst 33342 (blue fluorescence).

expression. To achieve this we developed a reagent, which stabilizes cellular RNA for up to 2 days at 5 °C and 45 °C, and up to 8 days at room temperature (figure 4). Storage at 45 °C caused a reduction in expression level especially of tumor marker 2, but the tumor marker could still be detected. Thus we are able to overcome the critical aspect of degradation of mRNA during storage and transport of patient samples.

Clinical validation

At present, the clinical validity of the diagnostic system is being investigated in several studies for each tumor entity (colorectal, testicular and breast cancer). Exemplary we investigated 50 colorectal carcinoma patients at the time of primary diagnosis and in follow-ups over a period of six months. In several cases tumor cells were detected by multiplex PCR (AdnaTest Colon CancerDetect) prior to the standard CEA-ELISA (table 1). These colorectal carcinoma patients showed negative ELISA values after surgery and in follow-ups over a period of six months. In two cases (patient 1 and 3) the multiplex PCR showed positive results after six months and in the case of patient 2 after three months and in all following samples. Until now, all three cases characterize the efficiency of surgery and chemotherapy by the detection of tumor cells: In the case of patient 2 tumor cells in the blood could be detected even during chemotherapy, indicating an ineffectual drug treatment. In all three cases tumor cells could be detected after surgery prior to the standard CEA-ELISAs, indicating a potentially tumor relapse.

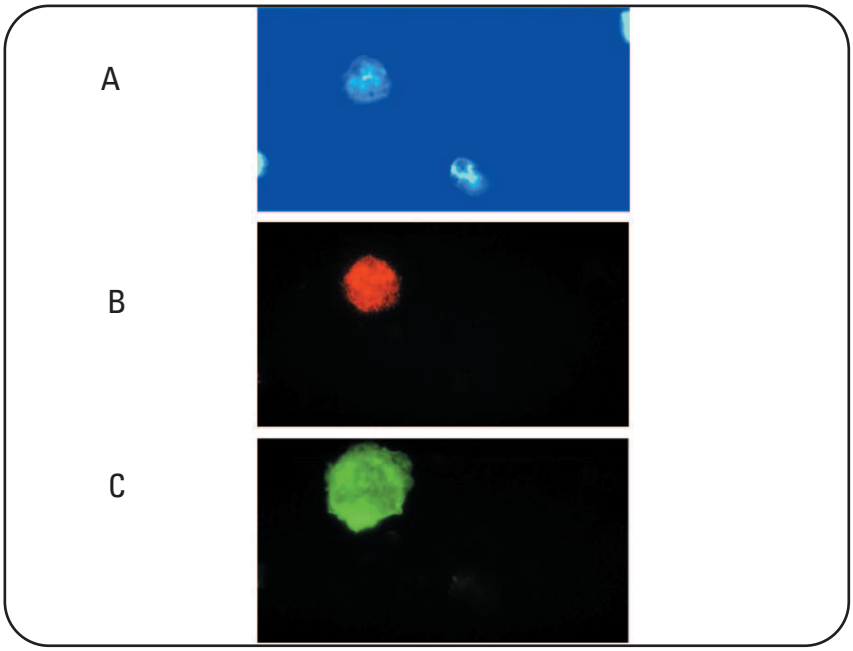

Figure 2
Cytospin preparation of tumor cells in erythrocyte-lysed blood (immunofluorescence images) A. Nuclei of leukocytes and tumor cells were visualized by Hoechst 33342 (blue fluorescence). B. Tumor cell (red fluorescence) stained with proliferation marker Ki-67 co-labelled with secondary antibody coupled to CY3. C. Tumor cell (green fluorescence) recognized by antibodies from AdnaTest CancerSelect co-labelled with secondary antibody coupled to FITC.

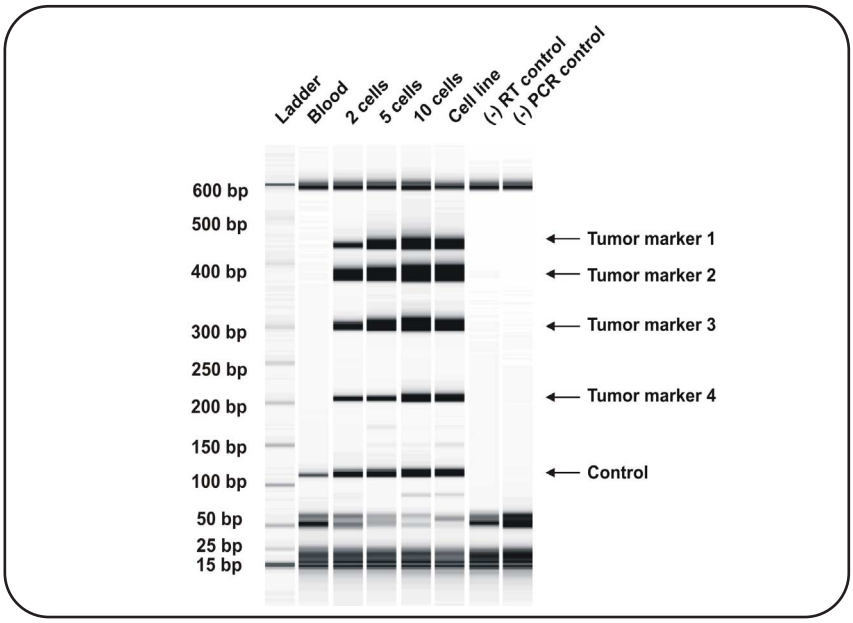

Figure 3
Sensitivity of AdnaTest CancerSelect/CancerDetect kit analyzed with the 2100 bioanalyzer (Agilent Technologies). A defined number of 2-10 carcinoma cells were spiked to 5 mL of blood from healthy donors. Followed by tumor cell enrichment with AdnaTest CancerSelect and a multiplex PCR, using the AdnaTest CancerDetect kit.

Conclusion

AdnaGen developed a combined method of specific tumor cell selection (AdnaTest CancerSelect) and a high sensitivity tumor cell detection (AdnaTest CancerDetect). The Agilent 2100 bioanalyzer provides the performance to detect the PCR products with high sensitivity. The procedure allows a sensitive and specific method to detect disseminated tumor cells in peripheral blood of testicular, colorectal and breast carcinoma patients at an early stage of tumor progression. There is evidence that this procedure enables us to monitor patients during follow-ups with high sensitivity. The relapse free interval could be analyzed more exactly than is possible with current methods. This procedure offers new possibilities for monitoring and prognosis, and may result in an appropriate selection of patients for adjuvant therapy. AdnaGen's solution will set a new standard in tumor diagnostics.

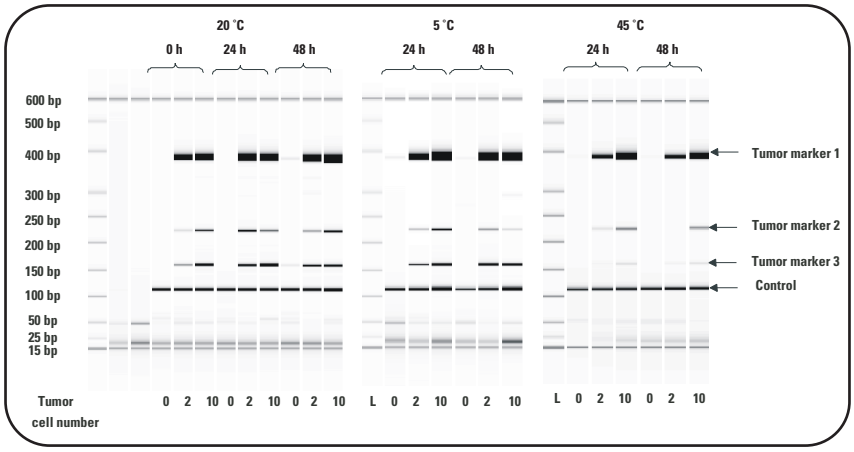

Figure 4

Stabilization of cellular RNA in the range of 5 °C to 45 °C with AdnaGens reagent (2100 bioanalyzer, Agilent Technologies). A defined number of 2 and 10 tumor cells respectively were spiked to 5 mL aliquots of blood from healthy donors, enriched with AdnaTest CancerSelect, stored in stabilization reagent for 24 h and 48 h at the indicated temperatures and analyzed by RT-PCR with AdnaTest CancerDetect.

		Prior surgery	1,5 months	3 months	4,5 months	6 months
Patient 1	ELISA	+	-	-	-	-
	PCR	+	-	-	-	+
Patient 2	ELISA	-	-*	-*	-*	-
	PCR	-	-*	+*	+*	+
Patient 3	ELISA	+	-	-	-	-
	PCR	-	-	-	-	+

Table 1

Case reports: Representative follow-ups of 3 different colorectal carcinoma patients by multiplex PCR and CEA-ELISA. First analysis was performed using blood prior to surgery, followed by analyses at 1.5, 3, 4.5 and 6 months after surgery. We analyzed 3 different colorectal carcinoma patients by multiplex PCR (AdnaTest CancerDetect) and CEA-ELISA. Analyses marked by asterisk are under chemotherapeutical treatment.

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Published June 1, 2003
Publication Number 5988-9341EN

Agilent Technologies