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# A Strategy to Identify Genes Associated with Circulating Solid Tumor Cell Survival in Peripheral Blood

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## Abstract

Efforts in metastasis research have centered on the phenotypic and genetic differences between primary site and metastatic site tumors. However, genes that may be used as molecular markers of metastasis in circulating tumor cells remain unidentified. Genes regulating the dissemination and survival of solid tumor cells in the blood, as well as their adaptation to new environments, could be candidates for unique metastatic tumor markers. Differential display (DD) was conducted to compare the blood of tumor-free individuals with the blood of patients with lung, breast, and colon cancers. Twenty-one up-expressed genes in the tumor patient blood samples but none in the tumor-free donor blood samples were identified. Nine of these samples were isolated, amplified, and directly sequenced. A gene AB-1 homologous to a Bcl-2 family member, which might function as an apoptosis inhibitor, was identified. The overexpression of an apoptosis inhibitor in blood from patients with metastatic tumors might be correlated with the capability of solid tumor cells to survive in peripheral blood. This is the first demonstration of the usefulness of comparing control and patient blood samples by DD to find novel potential genetic markers identifying metastasis in the blood.

## Introduction

Cancer recurrence and metastasis continue to pose major problems in clinical management (1). Advances in diagnostic techniques and technology may allow cancer detection at earlier stages, when the tumor burden is smaller and potentially more curable. The relationship between circulating tumor cells and the development of secondary disease is not fully understood and a method to detect small numbers of such cells may provide a tool with which to evaluate their role in the disease process. However, very little is known about the molecular mechanism that regulates circulating tumor cells' survival and metastasis. Genes regulating the dissemination and survival of solid tumor cells in the blood, as well as their adaptation to new environments, could be candidates for unique metastatic tumor markers.

Systemic spread of tumor cells in peripheral blood is an essential step for hematogenous metastasis (2). The vast majority of tumor cells that enter the circulation are rapidly elim-

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inated by factors such as blood turbulence (3), natural killer cells (4), and macrophages (5). Nitric oxide secretion by activated macrophages and endothelial cells is a major cytotoxic mediator responsible for the destruction of tumor cells passing through capillary beds (6). In addition, activation of apoptosis also contributes to eliminate metastatic cells (7,8). In contrast, fibrin deposits, platelet aggregation, and adhesion around the tumor emboli may protect circulating cells from mechanical trauma, facilitate their arrest in capillary beds, and protect tumor emboli from destruction by host immunity (9).

To find novel genetic markers for solid tumor cells circulating in blood, differential display (DD) (10) was conducted to compare RNA isolated from the blood of tumor-free individuals with RNA from the blood of patients with lung, breast, and colon cancers.

## Materials and Methods

### *Patients' Samples*

Eleven blood samples from patients with histologically documented nonhematological cancer, with either localized or metastatic disease, were analyzed. All lung cancer blood samples analyzed in this study were collected before anti-tumor chemotherapy treatment or surgery. For negative controls, three blood samples from healthy donors with non-neoplastic disease were used. The Scientific Committee of University Hospital-UFRJ, Brazil and Dana-Farber Cancer Institute, Boston, MA approved this investigation.

### *RNA Preparation and DD*

Three milliliters of venous blood was obtained with a standard venipuncture technique using anticoagulant. Whole blood was centrifuged at 1800 × *g* for 40 min in a clinical centrifuge. The cells present in the buffy coat were collected and washed with 1 ml of buffer containing 10 mM Tris-HCl, pH 7.6, 5 mM $MgCl_2$, and 10 mM NaCl. They were centrifuged at 1800 × *g* for 1 min and this step was repeated three times. The pellet containing nucleated cells was incubated for 5 min with 1 ml of Holmes Boner buffer (11). RNA was extracted with phenol:chloroform (1:1), pH 6.0 (11). The pellet containing RNA was resuspended in 300 μl of sterile water, and DNAse I treated in TE buffer, pH 7.5, containing 100 mM $MgCl_2$ and 10 mM DTT, and 40 U RNAse inhibitor (12). DD was performed using the RNA Image® kit (GenHunter Corp., Nashville, TN). Table 1 describes sequences of primers not included in the GenHunter kit (13,14). Polymerase chain reaction (PCR)-amplified cDNA products were resolved on a 6% DNA sequencing gel (Genomyx Corp., Foster City, CA). The bands of interest were excised from the gel (10,14,15).

**Table 1. Primer sequences**

| | |
|---|---|
| Anchor primers | LHT11G: 5'-TGCCGAAGCT11G |
| | LHT11A: 5'-TGCCGAAGCT11A |
| | LHT11C: 5'-TGCCGAAGCT11C |
| Arbitrary primers | OPA2: 5'-CGTGAATTCGTGCCGAGCTG |
| | OPA4: 5'-TGCCGAAGCTTAATCGGGCTG |

### *PCR and Direct Sequencing*

PCR reactions of isolated cDNA fragments were performed with 2.5 U/μl AmpliTaq DNA polymerase (Perkin Elmer, Branchburg, NJ), 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$, and 250 μM each of dNTP and 50 nM of each primer in 50 μl reaction mix. The PCR reaction was programmed as follows: 95°C for 36 sec, 53°C for 36 sec, 72°C for 90 sec for 35 cycles; elongation was at 72°C for 5 min, and refrigeration at 4°C. A second round of the PCR reaction was performed when necessary. The PCR products of cDNA fragments observed were mostly single bands (data not shown). The nucleotide sequences of cDNA fragments were determined using the Circum Vent Sequencing Kit (New England BioLabs, Beverly, MA) with [$\gamma$-$^{32}$P] rATP 5′ end-labeled primers, as described in the supplier's instructions. The DNA template was purified from an agarose gel (QIAquick). The samples were run on a 6% polyacrylamide gel (Genomyx) at 60°C, 3000 V, 125 W and/or sequenced using AmpliTaq® DNA polymerase, FS dye-terminator, modified from Applied Biosystems by the Molecular Biology Core Facility, Dana-Farber Cancer Institute.

### *RT-PCR and Northern Blot*

The reverse-transcription (RT) reaction was performed with Superscript II per the manufacturer's instructions (Gibco-BRL, Life Technologies, Gaith-

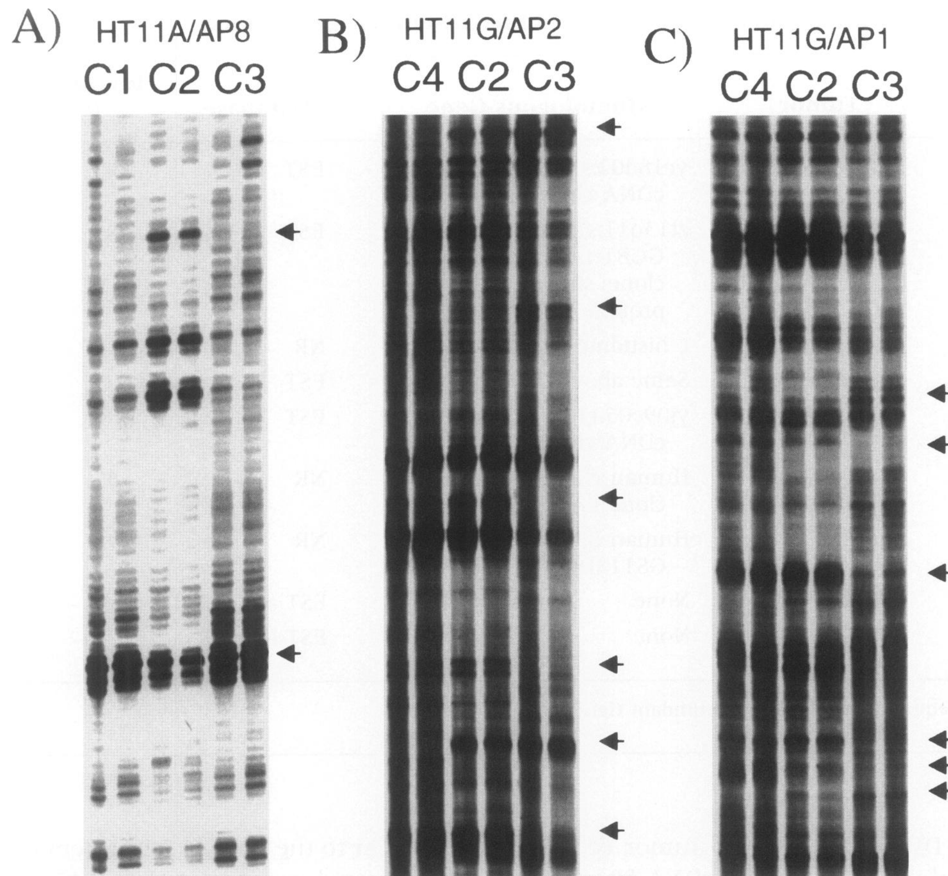


**Fig. 1. Individual variation among donor blood RNA samples compared by DD.** RNA from tumor-free donor blood samples C1–C4 were compared in duplicate by DD using RNA Image kit primer sets (A) HT11A/AP8, (B) HT11G/AP2, and (C) HT11G/AP1. Arrows show bands differentially expressed among samples.

ersburg, MD). The AB-1 gene was amplified using primers 5′ CTCTGGAAGGTCAAGTTACATCATC 3′ and 5′ AGAGTTTCATTCTGTCGCCCAGGC 3′. Radioactive PCR reactions for probe preparations were performed with 2.5 U/$\mu$l AmpliTaq (Perkin Elmer), 100 mM Tris-HCl, pH 8.3, 500 mM KCl, 1.5 mM $MgCl_2$, and 200 $\mu$M each of dNTP, 50 nm of each primer, and 2.5 $\mu$l of [$\alpha^{32}$P] dCTP (3000 Ci/mmol) in 50 $\mu$l reaction mix. The PCR reaction was programmed as follows: initial denaturation was at 94°C for 5 min, then 94°C for 1 min, 53°C for 1 min, 72°C for 1 min for 35 cycles; elongation was at 72°C for 5 min, and refrigeration at 4°C. Northern blots were performed with Express-Hyb™ hybridization solution following the manufacturer's instructions (Clontech Laboratories, Palo Alto, CA). Densitometry analysis using Model GS-700 Imaging Densitometer (Bio-Rad Laboratories, Hercules, CA) and standard normalization procedures were performed to determine mRNA relative expression.

## Results and Discussion

The gene expression pattern variation between individual samples must not distort the ability of DD to distinguish genes related to metastasis. To test the extent of individual variation between individual blood samples by DD, mRNA from blood of control donors were compared. The variability between control donors differed depending on the primer combination used. Figure 1A shows one of the primer sets that resulted in very few differences among controls, as indicated by arrows, which should have little effect on masking genes marking metastatic tumors. Tremendous variations in cDNA pattern among controls were observed using the anchor primer HT11G as indicated by the arrows in Figure 1B and C. Thus, the primer combinations that generated extended variability, such as HT11G, were not used for the subsequent DD experiments.

A limit dilution analysis to determine the

**Table 2. Potential unique markers for solid tumor dissemination in blood**

| cDNA | Tumor | Homologous Gene | Database | Identities (%) | GenBank (ID) |
|---|---|---|---|---|---|
| 104b1 | Lung, breast, colon | yc18d02.s1 *Homo sapiens* cDNA clone | EST | 94 | T70191 |
| 104b2 (AB-1) | Lung, breast | zt13q11.s1 NCI CGAP-GCB1 *Homo sapiens* cDNA clone: similar to A1 protein | EST | 97 | AA282294 |
| 104b4 | Lung, breast | L-histidine decarboxylase | NR | 94 | D16583 |
| 104b9 (AB-1) | Lung, breast | Same above: 104b2 | EST | 97 | AA282294 |
| 110b1 | Lung, breast | yj09e05.r1 *Homo sapiens* cDNA clone | EST | 100 | H13749 |
| GP1 | Lung, breast, colon | Human chromosome 10 clone LA10NC01 | NR | 87 | U82212 |
| L1 | Lung | Human BAC clone GS113D04 | NR | 98 | AC002087 |
| B1 | Breast | None | EST and NR | — | — |
| B2 | Breast | None | EST and NR | — | — |

EST, expressed sequence tags; NR, nonredundant GenBank.

sensitivity of DD to detect a few tumor cells in blood was performed. A 200-bp cDNA fragment specific to HeLa cells displayed differential expression in a 3-ml blood sample to which only 100 HeLa cells were added (data not shown).

To begin identifying metastatic genes, the RNA of two individual control donor blood samples, representing the greatest variation between individuals, and up to five metastatic cancer patient blood samples were compared by DD. As potential markers, we considered RNA up-regulated in at least two patient blood samples and low or absent in normal blood samples. Differentially expressed cDNA fragments of interest were isolated and reamplified by PCR using DD primer (14). A total of 15 up-regulated bands identified by this strategy were isolated. Five of these were overexpressed in blood samples from lung and breast cancer and were considered candidates for general molecular markers for tumor dissemination (Table 2). One of the bands was also detected in the blood sample of a colon cancer case.

Figure 2A shows a comparison of blood from tumor-free donors with that from two metastatic lung cancer patients, P1 and P2, respectively. A cDNA fragment differentially expressed in patient blood denominated AB-1 (*A*poptosis in *B*lood-1) that bears 95% homology with a gene similar to the human A1 protein in a database of expressed sequences tags (EST) was identified (GenBank ID AA282294). Human A1 protein is a member of the bcl-2 family, described as an apoptotic suppressor (16). The identification of an apoptosis inhibitor homologue gene in blood from patients with metastatic tumors might be correlated with the capability of solid tumor cells to survive in peripheral blood.

In an attempt to identify tumor-specific metastatic markers, we next analyzed a pool containing RNA from blood of four individual normal donors (CP) compared with pools of RNA from blood of three metastatic lung cancer patients (LP) and two metastatic breast cancer patients (BP) (Fig. 2B). The comparison of these samples by DD identified one lung-specific gene (L1), two breast-specific genes (B1 and B2), and one general gene (GP1). The comparison of the control donor pool (CP) with a pool of metastatic tumor blood RNA consisting of LP, BP, and one metastatic colon cancer blood sample by DD generated very few differences in cDNA pattern and was not a useful strategy for investigation of general molecular markers (data not shown).

Table 2 summarizes the results of GenBank analysis of nine cDNA fragments of interest. We required that a match include 95% identical bases over a stretch of at least 30 bases. The two

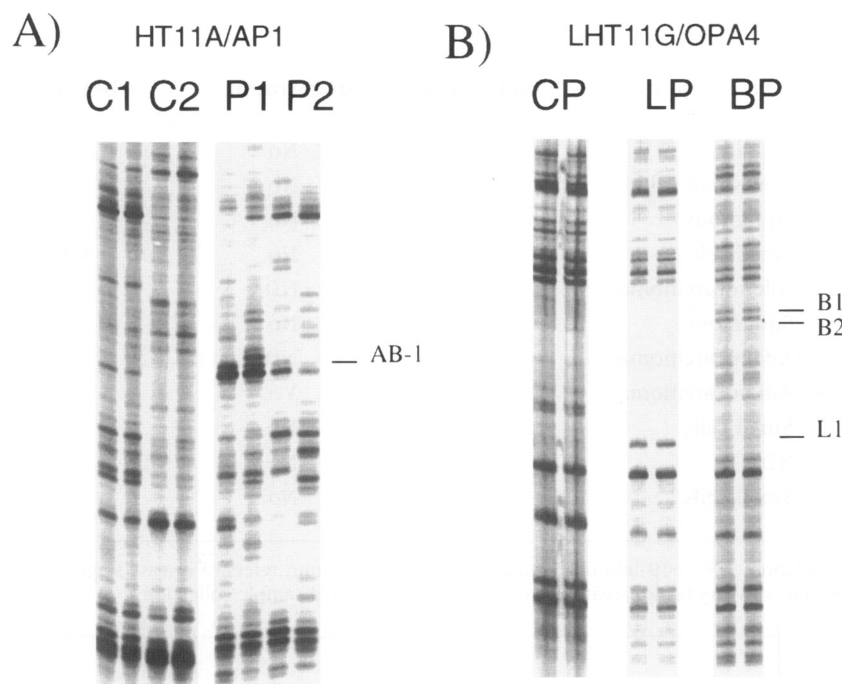


**Fig. 2. Identification of potential metastatic markers in peripheral blood samples by DD.** Sections of DD gel demonstrating differential expression of AB-1, L1, B1, and B2 cDNAs in patient blood samples. (A) DD using RNA Image kit primer set HT11A/AP1. Individual control blood RNA samples (C1, C2) were compared with individual blood RNA samples of metastatic cancer patients (P1, P2). (B) DD using long primer set LHT11G/OPA4 (Table 1). Comparison of tumor-free blood RNA pool (CP) with a metastatic lung cancer blood RNA pool (LP) and a metastatic breast cancer blood RNA pool (BP).

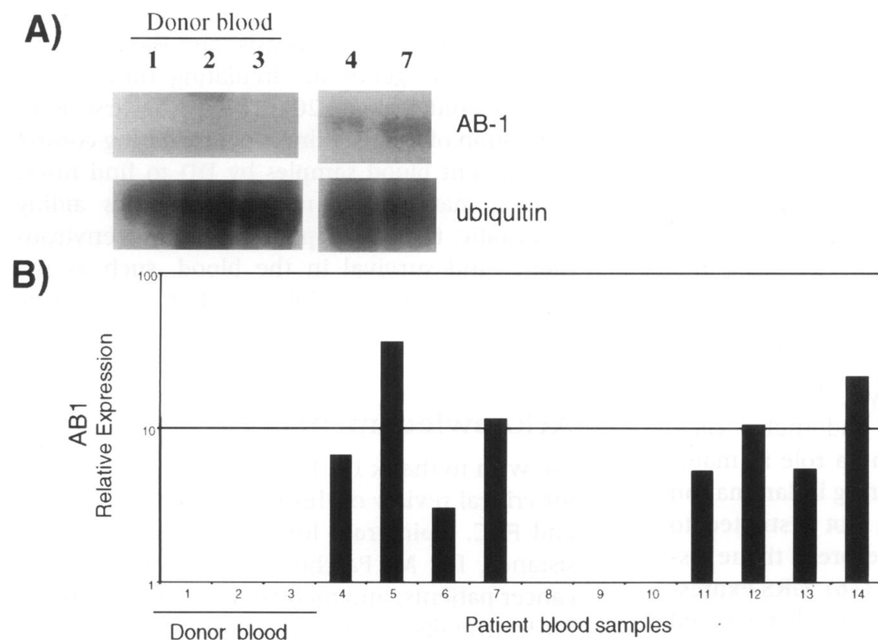


**Fig. 3. Expression of AB-1 mRNA in blood of solid tumor patient.** (A) Northern blot analysis confirming the low expression of AB-1 in tumor-free donor blood samples (lanes 1–3) versus cancer patient blood samples (lanes 4 and 7). The blot was stripped and reprobed with ubiquitin for normalization. (B) Quantitative representation of Northern blot analysis of tumor-free donor blood samples (lanes 1–3) and 11 tumor patient blood samples (lanes 4–14).

fragments specific for metastatic breast cancer blood samples had the same sequence but did not match with any EST sequence. With the exception of AB-1 and a cDNA fragment that is partially similar to the human gene for L-histidine decarboxylase, the remaining fragments analyzed were unknown genes.

To confirm DD data showing the presence of AB-1 in patient blood but not in tumor-free donor blood samples, Northern blot analyses were conducted on this gene. Figure 3A shows that AB-1 was not expressed in three tumor-free donor blood samples. AB-1 was up-regulated in 72% of cancer patient blood samples tested (Fig. 3B). More than 10-fold overexpression was observed in four out of eight metastatic cancer blood samples (Fig. 3B, lanes 5, 7, 12, and 14). Three of them were cases of small-cell lung can-

**Table 3. Patient background**

| Sample Number | Tumor | Histology | Metastatic | Treatment | AB-1 RNA |
|---|---|---|---|---|---|
| 4 | Lung | Small cells | No | No | + |
| 5 | Lung | Non-small cells | Yes | No | ++ |
| 6 | Lung | Squamous | No | No | + |
| 7 | Lung | Small cells | Yes | No | ++ |
| 8 | Colon | Adenocarcinoma | Yes | ND | — |
| 9 | Lung | Squamous | Yes | No | — |
| 10 | Colon | Adenocarcinoma | Yes | ND | — |
| 11 | Breast | Adenocarcinoma | Yes | Yes | + |
| 12 | Lung | Small cells | Yes | No | ++ |
| 13 | Lung | ND | ND | No | + |
| 14 | Lung | Small cells | Yes | No | ++ |

ND, not determined; +, 1- to 10-fold up-regulation; ++, >10-fold up-regulation. Metastatic column refers to disease stage at the time of blood collection. Treatment column refers to any tumor treatment or surgery before blood sample collection.

cer carcinoma (SCLC), which is a very aggressive and metastatic form of lung cancer (17). Whether AB-1 is correlated with tumor prognosis is still unknown. Table 3 describes patient information. The poor detection of AB-1 in donor blood samples suggests that the AB-1 gene may be correlated with solid-tumor cell survival in peripheral blood. More investigation is needed to evaluate the application of this gene as a molecular marker for metastatic cancers.

The AB-1 gene is highly homologous to the human A1 gene as well as to GRS and Bfl-1 cDNA. Human A1 is induced by IL-1$\beta$ and tumor necrosis factor $\alpha$ (TNF-$\alpha$) in endothelial cells, which suggests that it may play a role in maintaining endothelial survival during inflammation (18). However, human A1 is not restricted to endothelial cells and has a widespread tissue distribution, including leukocytes (16). GRS expression in normal human tissues is largely restricted to the hematopoietic compartment as well as in hematopoietic malignancies and melanoma cell lines (19). Our data support that AB-1, in contrast with its homologues A1 and GRS, is not expressed in normal leukocytes and is present in blood samples from solid-tumor patients.

Efforts in metastasis research have centered on the phenotypic and genetic differences between primary site and metastatic site tumors. They focus on differences in angiogenesis, signal transduction pathways, cell communication, migration, and adhesion. However, genes that could be used as molecular markers of tumor dissemination and metastasis, and the biological role of these genes in circulating tumor cells, remain unidentified (20). This is the first demonstration of the usefulness of comparing control and patient blood samples by DD to find novel genetic markers for metastasis. Genes aiding metastatic tumor adaptation to new environments and survival in the blood, such as the potential apoptotic inhibitor AB-1, need to be further characterized for clinical use.

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