# The Hematopoietic System–specific Minor Histocompatibility Antigen HA-1 Shows Aberrant Expression in Epithelial Cancer Cells

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## Abstract

Allogeneic stem cell transplantation (SCT) can induce curative graft-versus-tumor reactions in patients with hematological malignancies and solid tumors. The graft-versus-tumor reaction after human histocompatibility leukocyte antigen (HLA)-identical SCT is mediated by alloimmune donor T cells specific for polymorphic minor histocompatibility antigens (mHags). Among these, the mHag HA-1 was found to be restricted to the hematopoietic system. Here, we report on the HA-1 ribonucleic acid expression by microdissected carcinoma tissues and by single disseminated tumor cells isolated from patients with various epithelial tumors. The HA-1 peptide is molecularly defined, as it forms an immunogenic peptide ligand with HLA-A2 on the cell membrane of carcinoma cell lines. HA-1–specific cytotoxic T cells lyse epithelial tumor cell lines in vitro, whereas normal epithelial cells are not recognized. Thus, HA-1–specific immunotherapy combined with HLA-identical allogeneic SCT may now be feasible for patients with HA-1$^+$ carcinomas.

Key words: stem cell transplantation • graft-versus-tumor • carcinomas • cytotoxic T cells • minimal residual disease

## Introduction

Overall mortality from solid cancers has only slightly decreased despite earlier diagnosis, improved surgical techniques, and novel therapy regimens (1). One of the promising novel therapies is the application of stem cell transplantation (SCT).* Clinical and experimental data indicate that allogeneic SCT not only reconstitutes the patient's hematopoietic system, but also mediates a powerful curative effect in patients transplanted for hematological malignancies or solid tumors (2–8). These alloimmune graft-versus-host reactions generally lack tumor specificity and are often accompanied by severe GVHD. Therefore, definition of novel target structures for systemic therapy of carcinomas is needed. The prerequisites of target antigens for successful immunotherapy of cancers are tissue specificity, functional membrane expression on the tumor cells, and the capacity of inducing alloimmune T cell responses. The graft-versus-host reactions after HLA-identical SCT are attributed by antigens encoded by genes other than the MHC, which are generally referred to as minor histocompatibility antigens (mHags; reference 9). mHags are peptides from polymorphic intracellular proteins that are encoded by genes on the Y chromosome and autosomal genes. Their immunogenicity arises as a result of their expression on the plasma membrane where they are recognized by alloreactive MHC-restricted T cells (10). We demonstrated earlier that mHags either show ubiquitous or restricted tissue expression (11). The tissue expression of the mHag HA-1 is limited to the hematopoietic cells. Functional studies with HA-1–specific CTLs demonstrated the efficient lysis of hematopoietic cells, including leukemic cells (11, 12) and the inhibition of leukemic progenitor cell outgrowth (13), whereas no CTL recognition was ob-

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**Abbreviations used in this paper:* CGH, comparative genomic hybridization; LCL, lymphocyte cell line; mHag, minor histocompatibility antigen; PBGD, *porphobilinogen deaminase*; SCT, stem cell transplantation.

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served when nonhematopoietic cells were used as target cells (11). The mHag HA-1, with its tissue distribution restricted to hematopoietic cells, fulfills the prerequisites for successful immunotherapy of hematological tumors. Therefore, we developed ex vivo protocols to treat leukemia relapse after HLA-matched mHag HA-1–mismatched SCT with low risk of GVHD. Here, donor-derived CTLs specific for the hematopoietic system–specific mHag HA-1 are generated (14). Upon transfusion, these SCT donor–derived HA-1–specific CTLs will eliminate the HA-$1^+$ patients' hematopoietic and leukemic cells, while HA-$1^-$ nonhematopoietic cells and normal tissues will be spared.

Here, we report that HA-1, in addition to its exclusive expression on the hematopoietic cell lineage, is aberrantly expressed on epithelial tumor cells with no expression on normal epithelial cells. Our observation of HA-1 expression on various types of nonhematological tumors offers a novel target molecule for the treatment of epithelial tumors. Similar to the immunotherapy protocol for the treatment of relapsed leukemia as described above, the aberrant expression of HA-1 on carcinoma cells may be exploited for the treatment of HA-$1^+$ carcinoma patients in combination with HLA-identical HA-$1^-$ SCT.

## Materials and Methods

*mRNA Levels by Quantitative Real-time PCR.* Total RNA was prepared from subconfluent layers of the adherent cell cultures using the RNAzol method (Cinaa/Biotecx Laboratories, Inc.) according to the manufacturer's instructions. cDNA was synthesized using 2 μg RNA and random hexameric primers. PCR amplification and quantification were performed using the Taqman PCR assay (7700 Sequence Detector; Applied Biosystems). We used comparative quantification that normalized the HA-1 and CD45 gene to an internal standard gene, the ubiquitously expressed housekeeping gene *porphobilinogen deaminase* (PBGD). The relative levels of expression of the HA-1 and CD45 genes in the test samples were calculated as percentages of the levels of expression in the reference cell line KG-1, which expresses both genes. All samples that showed expression levels <10% in the real-time quantitative PCR did not produce detectable PCR fragments in a standard PCR. Therefore, expression levels ≤10% are considered not significant. The relative quantification calculated by the linear calibration function between the threshold cycle ($C_t$) value and the logarithm of the initial starting quantity (N) were $C_t = -3.31 \log (N) + 26.1$, $C_t = -3.5 \log (N) + 21.6$, and $C_t = -3.41 \log (N) + 25.6$ for HA-1, CD45, and PBGD, respectively. The HA-1, CD45, and PBGD expression were quantified in all test samples using these calibration functions.

*Preparation of Cryosections.* 5-μm sections from freshly snap frozen primary tumors were placed on a polyethylene membrane on a glass slide, stained with Meyer's hematoxylin, and dehydrated in 70%, 90%, and 100% ethanol. The PALM Microbeam system (Bernried) was used for microdissection and catapulting. The PALM laser microdissection microscope was provided by Altana Pharma.

*HA-1 and CD45 Expression Analysis on Primary and Single Disseminated Epithelial Cancer Cells.* The detection of disseminated cells and the global amplification of microdissected areas and of single cells from bone marrow and lymph nodes were performed as previously described (15). For the detection of HA-1 and CD-45 message by gene-specific PCR, we used 1 μl of the primary product after global PCR. For the highest sensitivity, 50 cycles for all primer pairs were run. All samples were analyzed by two primer pairs for HA-1: (I) forward 5′-GAC GTC GTC GAG GAC ATC TCC CAT-3′ and reverse 5′-GAA GGC CAC AGC AAT CGT CTC CAG-3′ and (II) forward 5′-ACA CTG CTG TCG TGT GAA GTC-3′ and reverse 5′-TCA GGC CCT GCT GTA CTG CA-3′, and by one primer pair for CD45: forward 5′-CTG AAG GAG ACC ATT GGT GA and reverse 5′-GGT ACT GGT ACA CAG TTC GA-3′ primer. Amplification products of the HA-1 (I) primers were digested with the restriction enzyme BstUI and amplification products of the HA-1 (II) primers with HinfI. Southern blot was performed according to standard protocols.

*CTL Recognition Studies.* Tumor cell lines were used as target cells in a standard 4-hr $^{51}Cr$ release assay. The tumor cells from subconfluent cultures were harvested and dispensed at 2,500 cells/well in 96-well flat-bottomed microtiter plates and allowed to attach either in the presence or absence of 250 U/ml rIFNγ and 250 U/ml TNFα (both from Genentech, Inc.) for 48 h. The tumor cells were labeled with $^{51}Cr$ for 1hr. The experiments were performed in sixplicates. The percentage-specific lysis was calculated as follows: % specific lysis = (experimental release − spontaneous release) / (maximal release − spontaneous release) × 100.

*Primary Cultures and Cell Lines Used.* The following primary cell cultures were provided: proximal tubular epithelial cells by M. Daha, melanocytes by N. Schmitt, Langerhans cells by M. Mommaas, and keratinocytes and fibroblasts by Mrs. J. Kempenaar (all from Leiden University Medical Center, Leiden, Netherlands). The following cell lines were provided: MDA-MB 231, 734 B, MCF-7, and ZR75-1 by B. Eibl (University Hospital of Internal Medicine, Innsbruck, Austria); HBL-100 cell line, Mel 93.04, LB 33, MZ 1851, MZ 1752, and MZ 1774 by S. Osanto (Leiden University Medical Center, Leiden, Netherlands); SW 707, SW 2219, SW620, Col 205, and SW 948 by H.W. Verspaget (Leiden University Medical Center, Leiden, Netherlands); BT-20, BT, MEWO, E9, BT, MNT, and BA by G.C. de Gast (University Medical Center, Utrecht, Netherlands); GLC2, GLC 8, and GLC 36 by L. de Leij (Academisch Ziekenhuis Groningen, Groningen, Netherlands); BB74/2940, KUL 68/3636, and BB 49/1413 by F. Brasseur (Ludwig Institute for Cancer Research, Brussels, Belgium); HuH7 and HepG2 by B.J. Scholte (Erasmus University, Rotterdam, Netherlands); and SW 480 by G. Eisner (University Regensburg, Regensburg, Germany). Mast cell lines and HT29 (American Type Culture Collection [ATCC]: HTB-37) and Caco-2 (ATCC: HTB-37), which are ATCC cell lines, were provided by B. Henz (Charité-Virchow Klinikum, Berlin, Germany).

## Results

*HA-1 Transcription Is Restricted to Cells of the Hematopoietic Lineage.* Earlier, functional studies with mHag HA-1–specific CTL clones demonstrated the hematopoietic restricted tissue distribution of the mHag HA-1 (11). To confirm the latter restricted tissue distribution on the transcriptional level, HA-1 mRNA levels were analyzed by quantitative real-time PCR in eight different hematopoietic and six different nonhematopoietic cell types. Only cells of hematopoietic origin expressed significant levels of the

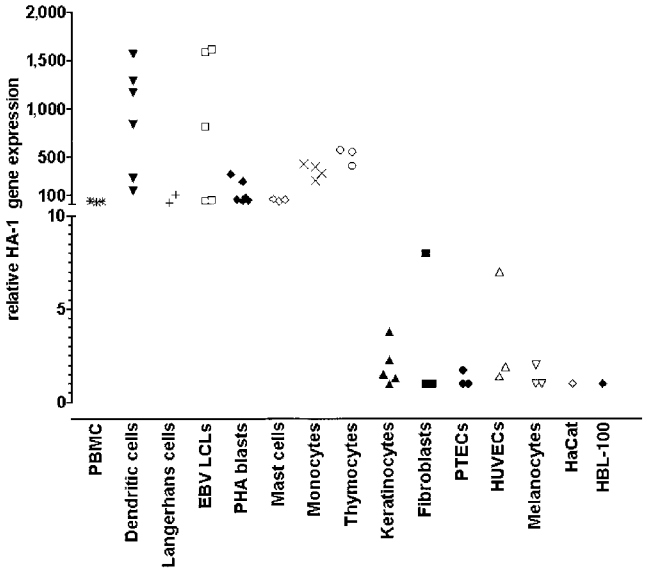



**Figure 1.** HA-1 gene expression is restricted to hematopoietic cells. The relative HA-1 gene expression levels were determined in cells of hematopoietic and nonhematopoietic origin. Hematopoietic cells tested were: *, PBMCs ($n = 3$); ▼, dendritic cells ($n = 6$); +, Langerhans cells ($n = 2$); □, EBV-LCLs ($n = 5$); ◆, PHA blasts ($n = 6$); ◇, mast cell lines ($n = 3$); ×, monocytes ($n = 4$); and ○, thymocytes ($n = 3$). Nonhematopoietic cells tested were: ▲, keratinocytes ($n = 5$); ■, fibroblasts ($n = 2$); ●, proximal tubular epithelial cells (PTECs; $n = 3$); △, umbilical vein endothelial cells (HUVECs; $n = 3$); ▽, melanocytes ($n = 3$); and two SV40 immortalized breast cell lines: ◇, HaCat and ◆, HBL-100.

HA-1 gene (Fig. 1). No significant HA-1 gene expression was detected in cells of nonhematopoietic origin, i.e., keratinocytes, dermal fibroblasts, proximal tubular epithelial cells, umbilical vein endothelial cells, melanocytes, and normal breast cell lines HaCaT and HBL 100 (Fig. 1). CD45 mRNA expression was analyzed in parallel. The transcription levels of HA-1 and CD45 were very similar with significant levels of expression in hematopoietic cells and insignificant levels (i.e., <10%) in nonhematopoietic cells (unpublished data).

*HA-1 Transcription in Tumor Cell Lines.* The HA-1 gene transcription levels were analyzed in 35 nonhematopoietic epithelial tumor cell lines derived from different types of carcinomas (Table I). The HA-1 gene transcription, analyzed by quantitative real-time RT-PCR, revealed significant HA-1 mRNA in 26 out of the 35 cell lines of various nonhematopoietic malignant origins. HA-1 mRNA has also been recently demonstrated on 17 tumor cell lines of nonhematopoietic origin by Fujii et al. (16). To justify the HA-1 expression on the cell lines, we executed the CD45 gene expression in parallel. None of the tumor cell lines we analyzed showed significant CD45 gene expression, demonstrating that HA-1 transcription observed in the tumor cell lines is not due to contaminating hematopoietic cells (Table I).

*HA-1–specific Lysis of Tumor Cell Lines.* Functional recognition by HA-1–specific CTLs is a prerequisite for tumor-specific targeting in immunotherapeutical settings. The mHag HA-1 locus encodes two alleles, the HA-$1^H$ and the HA-$1^R$ allele. The HA-$1^H$ allele is the T cell epitope that is recognized by HLA-A2–restricted HA-1–specific

**Table I.** *HA-1 Gene Expression in Tumor Cell Lines*

| Tumor type | Cell line | HA-1 percentage[a] |
|---|---|---|
| Breast cancer | ZR75-1 | 54 |
| | BT-20 | 40 |
| | 734B | 27 |
| | T47 D | 17 |
| | MDA-MB231 | 15 |
| | MCF-7 | ≤10 |
| | BT 474 | ≤10 |
| Melanoma | Mel 93.04 | 68 |
| | KUL 68/3636 | 67 |
| | BB 74/2940 | 57 |
| | MNT | 27 |
| | LB33 | 24 |
| | BT | 15 |
| | 453 Ao | 12 |
| | 518A | ≤10 |
| | E9 | ≤10 |
| | MEWO | ≤10 |
| Lung carcinoma | GLC 36 | 22 |
| | GLC 8 | ≤10 |
| | GLC 2 | ≤10 |
| Renal cell carcinoma | MZ 1851 | 29 |
| | MZ 1752 | 13 |
| | MZ 1774 | ≤10 |
| | BA | ≤10 |
| Hepatoma | HuH7 | 37 |
| | HepG2 | 35 |
| Colon carcinoma | SW 707 | 147 |
| | CaCo-2 | 81 |
| | SW 480 | 70 |
| | SW 2219 | 48 |
| | SW 620 | 28 |
| | Col 205 | 21 |
| | SW 948 | 12 |
| | HT29 | 11 |
| Head and neck cancer | BB 49/1413 | 54 |

[a]The percentage of CD45 gene expression was ≤10 for all of the samples tested.

**Table II.** *HA-1–specific Lysis of Nonhematopoietic Tumor Cell Lines*

| | | | Percent-specific lysis by allo HLA-A2 CTLs | | Percent-specific lysis by HLA-A2/HA-1 CTLs | | | |
|---|---|---|---|---|---|---|---|---|
| | | | 1E2 IFNγ/TNFα | | 5W38 IFNγ/TNFα | | 3HA15 IFNγ/TNFα | |
| Tumor type | Target cell designation | E/T ratio | no | yes | no | yes | no | yes |
| A2 HA-$1^H$ | | | | | | | | |
| Breast cancer | MDA-MB231 | 2:1 | 10 | 13 | 8 | 15 | 8 | 13 |
| | | 10:1 | 50 | 64 | 31 | 47 | 25 | 39 |
| Melanoma | MEL 93.04 | 2:1 | 10 | 14 | 1 | 13 | −2 | 10 |
| | | 10:1 | 54 | 64 | 12 | 37 | 17 | 40 |
| Melanoma | 453 AO | 2:1 | 7 | 24 | 1 | 10 | 1 | 18 |
| | | 10:1 | 25 | 43 | 5 | 21 | 2 | 22 |
| | | 20:1 | 35 | 45 | 7 | 24 | 7 | 21 |
| Lung carcinoma | GLC 36 | 1:1 | 33 | 35 | 6 | 12 | 0 | 8 |
| | | 10:1 | 59 | 80 | 8 | 25 | 17 | 25 |
| Colon carcinoma | CaCo-2 | 1.6:1 | 20 | 22 | 1 | 2 | 6 | 7 |
| | | 16:1 | 29 | 49 | 4 | 4 | 11 | 17 |
| A2 HA-$1^R$ | | | | | | | | |
| Breast cancer | 734B | 2:1 | 29 | 41 | 3 | 1 | 4 | 0 |
| | | 10:1 | 32 | 67 | 5 | 1 | 0 | 0 |
| Melanoma | MNT | 1:1 | 33 | 51 | 0 | 0 | 1 | 1 |
| | | 10:1 | 62 | 84 | 3 | 3 | 0 | 0 |
| Melanoma | BT | 1:1 | 33 | 36 | 0 | 0 | 0 | 4 |
| | | 10:1 | 76 | 79 | 0 | 0 | 0 | 9 |
| A2-ve | | | | | | | | |
| Breast cancer | ZR75-1 | 1:1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | | 10:1 | 0 | 2 | 1 | 6 | 0 | 0 |
| Controls | | | | | | | | |
| EBV-LCLs | A2 HA-$1^H$ | 1:1 | 60 | ND | 35 | ND | 24 | ND |
| | | 10:1 | 87 | ND | 43 | ND | 43 | ND |
| | A2 HA-$1^H$ | 1:1 | 69 | ND | 51 | ND | 32 | ND |
| | | 10:1 | 83 | ND | 41 | ND | 68 | ND |
| | A2 HA-$1^R$ | 1:1 | 54 | ND | 6 | ND | 4 | ND |
| | | 10:1 | 81 | ND | 3 | ND | 0 | ND |
| | A2 HA-$1^R$ | 1:1 | 55 | ND | 0 | ND | 1 | ND |
| | | 10:1 | 83 | ND | 1 | ND | 0 | ND |

The percentage of specific lysis by one allo HLA-A2 and two HLA-A2/HA-1 CTLs was determined with at least two effector to target cell (E:T) ratios. The lysis of HLA-A2/HA-$1^H$ tumor cell lines by the allo HLA-A2 and HLA-A2/HA-1 CTLs, and the lysis on HLA-$A2^+$ and HA-$1^R$ tumor cell lines by allo HLA-A2 CTLs, but not by the HLA-A2/HA-1 CTLs, is shown. The absence of lysis on an HLA-$A2^-$ tumor cell line by the allo HLA-A2 CTLs and the HLA-A2/HA-1 CTLs is also shown. The control target cells used in the same experiments, HLA-A2/HA-$1^H$–typed EBV-LCLs, are lysed by the allo HLA-A2 and HLA-A2/HA-1 CTLs. HLA-$A2^+$ and HA-$1^R$–typed EBV-LCLs are lysed by the allo HLA-A2 CTLs, but not by the HLA-A2/HA-1 CTLs.

CTLs (17). The exclusive specificity of the CTLs for the HLA-A2/HA-$1^H$ ligand has been extensively demonstrated earlier in immunogenetic and segregation analyses and on the molecular level (17–19). Thus, the conditions for CTL recognition studies are the expression of both the HLA-A2 restriction molecule and the HA-$1^H$ T cell epitope on the tumor cell lines. Here, all the tumor cell lines (Table I) with significant HA-1 gene expression levels, but no CD45 RNA levels, were HLA and HA-1 genotyped (20). Those cell lines positive for both the HLA-A2 and HA-$1^H$ alleles were selected for functional CTL analyses. Table II shows significant lysis of the five HLA-A2/HA-$1^{H+}$ cell lines by two HA-1–specific clones that could be enhanced in all cases by IFNγ and TNFα treatment of the target cells. The colon carcinoma cell line CaCo-2 was recognized by one of the two HA-1–specific CTL clones and only upon

IFNγ and/or TNFα treatment of the cell line. All carcinoma cell lines were also recognized by the allo HLA-A2 CTLs. The various control target cells are analyzed in parallel to the same experiments (Table II). The results show the specificity of the HLA-A2/HA-$1^H$ CTLs, i.e., they only show lysis on target cells expressing both the HLA-A2 molecule and the HA-$1^H$ peptide. These data are in agreement with the exclusive HLA-A2/HA-$1^H$ specificity of these CTLs as previously demonstrated (11–14, 17–20).

*HA-1 Transcripts in Primary Epithelial Tumors.* Next, we aimed at analyzing the HA-1 expression by epithelial tumors in vivo. However, given the expression of HA-1 by hematopoietic cells and in view of the virtual omnipresence of hematopoietic cells in tumors, positive results of a PCR analysis caused by contaminating hematopoietic cells should be avoided. To this end, we applied laser-mediated microdissection to cryosections of fresh frozen cancer samples (Fig. 2 A). This enables the isolation of a selected area by a laser beam and directly catapults it into the reaction tube. Hence, contamination by surrounding tissue is practically excluded. However, infiltration of the tissues by a single or a few hematopoietic cells, or contamination by invisible mRNA molecules that result from the generation of the cryosections, cannot entirely be ruled out. Therefore, various controls were performed. First, the CD45 gene-specific PCR was run in parallel to all tumor samples to test whether HA-1 expression might be attributed to single infiltrating leukocytes or intravascular cells. The absence of CD45 mRNA will provide strong evidence that the HA-1 signal originates from the epithelial tumor cells in vivo, whereas the coamplification of HA-1 and CD45 of microdissected areas would not provide evidence of the HA-1 expression by tumor cells. Second, the sensitivity and reliability of the amplification protocol was tested using primary lymph nodes. Tiny areas from 5,000 to 40,000 $\mu m^2$ were microdissected and the isolated mRNA was globally amplified (15). From the primary product, 1 out of 50 was used in the secondary PCRs for the housekeeping gene EF-1α, HA-1, and CD45 (Fig. 2 B). Although all samples were positive for EF-1α, HA-1 and CD45 could only be detected in areas larger than 5,000 $\mu m^2$, indicating the detection limit of the approach for these samples. As expected, HA-1 and CD45 were strictly coexpressed in the positive samples, demonstrating similar expression levels in these lymph nodes. Third, we investigated normal breast tissues for the presence of both HA-1 and CD45 messages. We microdissected areas from 10,000 to 40,000 $\mu m^2$ of normal breast glands from three patients who underwent breast reduction surgery. From eight out of nine areas, mRNA was successfully isolated as judged by the amplification of EF-1α. Except for one area (NB-3.1), which was positive for HA-1, all samples were negative for both HA-1 and CD45 messages. The results on lymph nodes and normal breast tissues indicate that the applied approach reliably detected infiltrating lymphocytes via the coexpression of HA-1 and CD45, whereby the latter always appears more

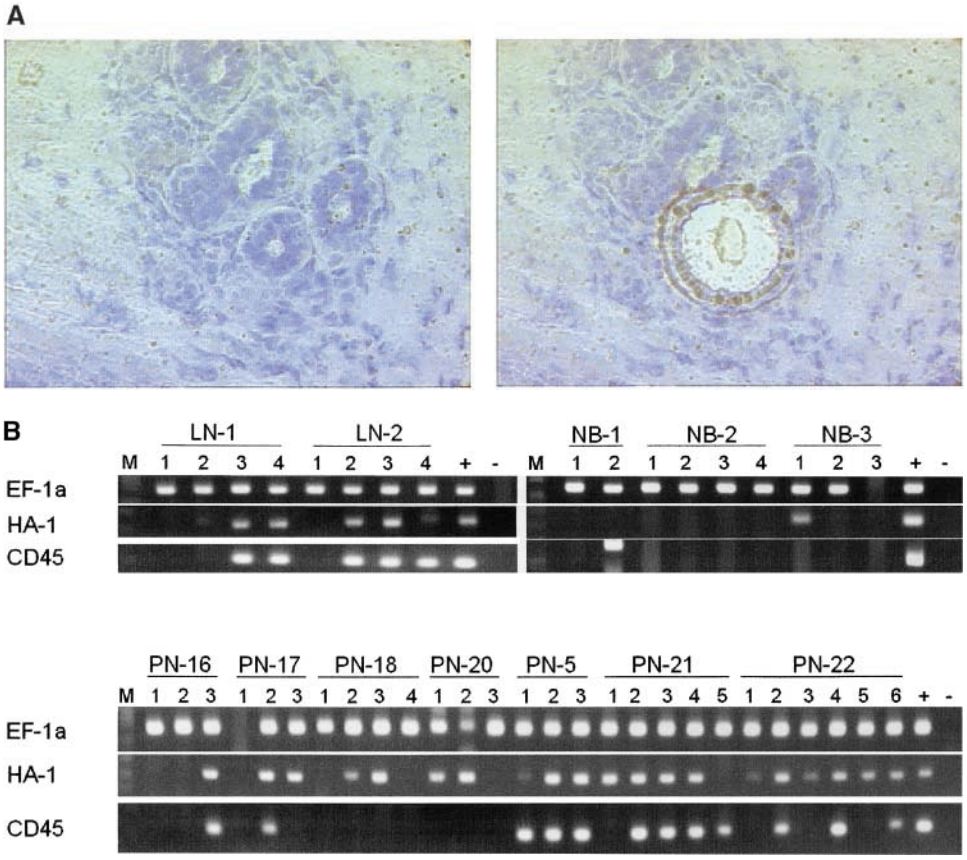



**Figure 2.** HA-1 and CD45 expression of microdissected tissue samples. (A) Laser microdissection of a normal breast gland, from which mRNA was subsequently isolated. The cryosection with the clearly visible glandular structure is shown on the left. On the right, the mammary cells have been microdissected and catapulted into the reaction tube using a laser beam. (B) Gene-specific PCR of microdissected lymph nodes (LN), normal breast (NB), and tumor patients (PN) for EF-1α, HA-1, and CD45 using amplicons after global amplification of the cDNA that was obtained from the microdissected areas. Areas from 5,000 to 60,000 $\mu m^2$ were individually analyzed. M, size marker; +, HT29 for HA-1 and normal bone marrow for CD45; −, water control.

intense. This further underlines our decision that only in the absence of CD45 mRNA, a positive judgment on the HA-1 expression, is allowed. The results on the normal tissues also demonstrate that contamination by invisible nucleic acids from the process of tissue preparation would very rarely lead to spurious results.

Next, we investigated primary cancers. From the tumors of seven patients with breast and lung cancers, we isolated 27 areas from 10,000 to 60,000 $\mu m^2$ comprising ~30–200 cells. Except for one area (PN-17.1), mRNA could be successfully isolated from all samples. 21 samples displayed PCR bands of HA-1, whose identity was confirmed by Southern blotting (unpublished data). 11 of the 26 samples contained hematopoietic cell infiltrates as demonstrated by the CD45 PCR band. Consequently, HA-1 expression of these tumors could not be assessed. Of the 15 remaining samples, 5 were negative for both CD45 and HA-1 (33%). In one sample (PN-21.5), CD45 amplification in the absence of HA-1 was observed. However, in the remaining nine samples (60%) that could be evaluated, HA-1 expression without CD45 expression was detected, providing strong evidence that HA-1 is indeed expressed by primary tumor cells. To test whether the lack of CD45 was just due to spurious amplification of its message, we analyzed six samples by array analysis. The labeled cDNAs were hybridized to a small array consisting of informative histogenetic marker molecules (Fig. 3 A). Three of the six hybridized samples (PN-18.3, PN-21.1, and PN-22.3) had been positive for HA-1 and negative for CD45 by gene-specific PCR. The other three samples of PN-5 were positive for both the HA-1 and CD45 genes (Fig. 2 B). On the array, all six samples displayed signals for genes consistent with their malignant epithelial origin, such as the epithelial-restricted cytokeratins or the tumor-specific MAGE genes (Fig. 3 A). Strikingly, although the $CD45^+$ samples coexpressed several other hematopoietic markers, such as CD16, CD33, CD34, CD37, and CD38, no hybridization signals for any of these genes was seen in the three samples that were solely positive for HA-1. To finally prove that the DNA of the microdissected areas that was hybridized was isolated from tumor tissue, we performed comparative genomic hybridization (CGH) on five of the six samples from which genomic DNA had simultaneously been isolated. The aberrant karyotypes showed that the areas were isolated from tumor tissue (Fig. 3 B).

*HA-1 Transcripts in Single Disseminated Tumor Cells.* Because of the strong evidence of HA-1 expression by primary tumors, we analyzed the HA-1 expression on disseminated tumor cells. Single disseminated cancer cells or defined cell clusters were prepared from bone marrow and lymph node samples using a fluorescent-labeled monoclonal antibody against the epithelial cell adhesion molecule as marker (15). In total, 27 single tumor cells or small cell clusters from 15 cancer patients were isolated by micromanipulation (Fig. 4 A). For cDNA analysis, the same global amplification technique was applied that was used for the microdissected tumor areas, enabling faithful detection of expressed transcripts in viable single cells (15). The labeled cDNAs were hybridized to an array including specific epithelial marker genes, such as the cytokeratin family members mammaglobin and prolactin-induced protein as markers for breast-derived cells, and the transcription factor ELF3. Additional evidence of epithelial origin was provided by claudin 7 and desmoplakin I, which are both involved in epithelial cell adhesion. Expression of cytokeratins and other epithelial markers indicated their epithelial origin (Fig. 4 B). As an indicator of malignancy, the MAGE genes were analyzed. In addition, we evaluated the cells for markers of hematopoietic cells such as the T cell receptor, CD45, CD33, CD34, CD37, CD38, and CD16. None of the isolated cells expressed one of the latter hematopoietic markers. All cells were then tested for HA-1 and CD45 expression by gene-specific PCR. The HA-1 amplification products were subsequently confirmed by restriction enzyme digest and Southern blotting. 6 of the 27 single disseminated cells (22%) expressed the HA-1 gene, whereas none of them expressed the CD45 gene (Fig. 5 A). The HA-1–signifi-

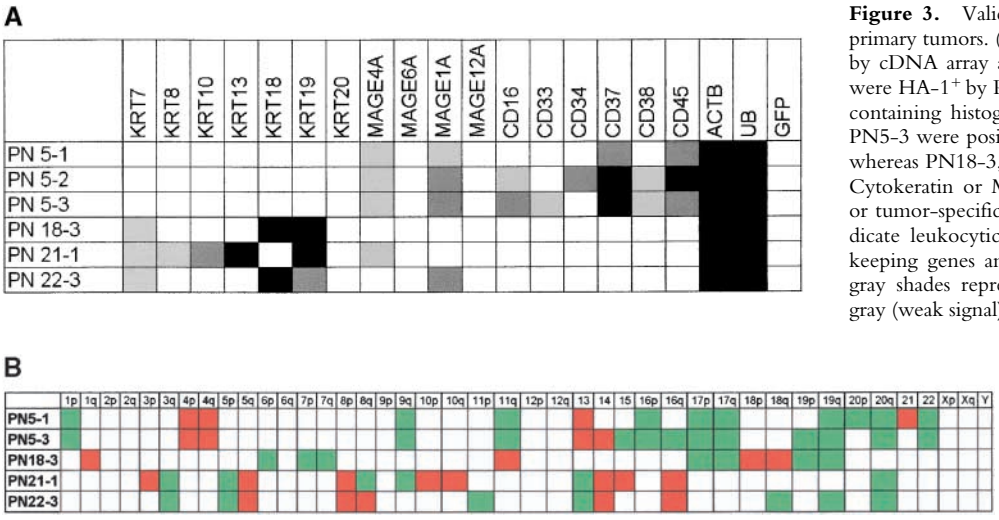
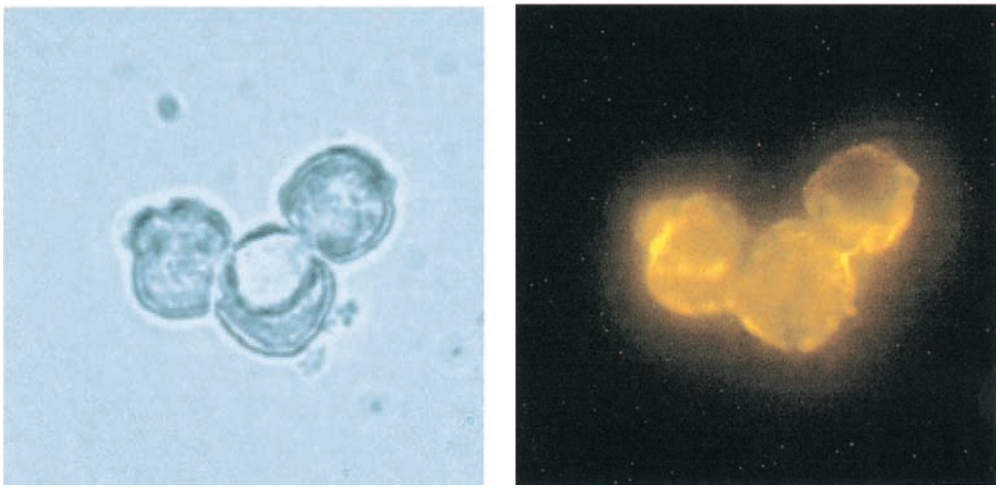



**Figure 3.** Validation of HA-1 expression in human primary tumors. (A) Evaluation of histogenetic markers by cDNA array analysis. Six microdissected areas that were $HA\text{-}1^+$ by PCR were hybridized on a small array containing histogenetic markers. PN5-1, PN5-2, and PN5-3 were positive for CD45 in gene-specific PCR, whereas PN18-3, PN21-1, and PN22-3 were $CD45^-$. Cytokeratin or MAGE transcripts represent epithelial or tumor-specific transcripts, whereas CD markers indicate leukocytic origin. ACTB and UB are housekeeping genes and GFP is the negative control. The gray shades represent the signal intensity, from light gray (weak signal) to black (strong signal). (B) CGH results of five of the six areas shown in A. The chromosome arms are given with green indicating a chromosomal gain and red indicating a chromosomal loss. The aberrations prove that the isolated areas contained at least 50% tumor cells.

A



B

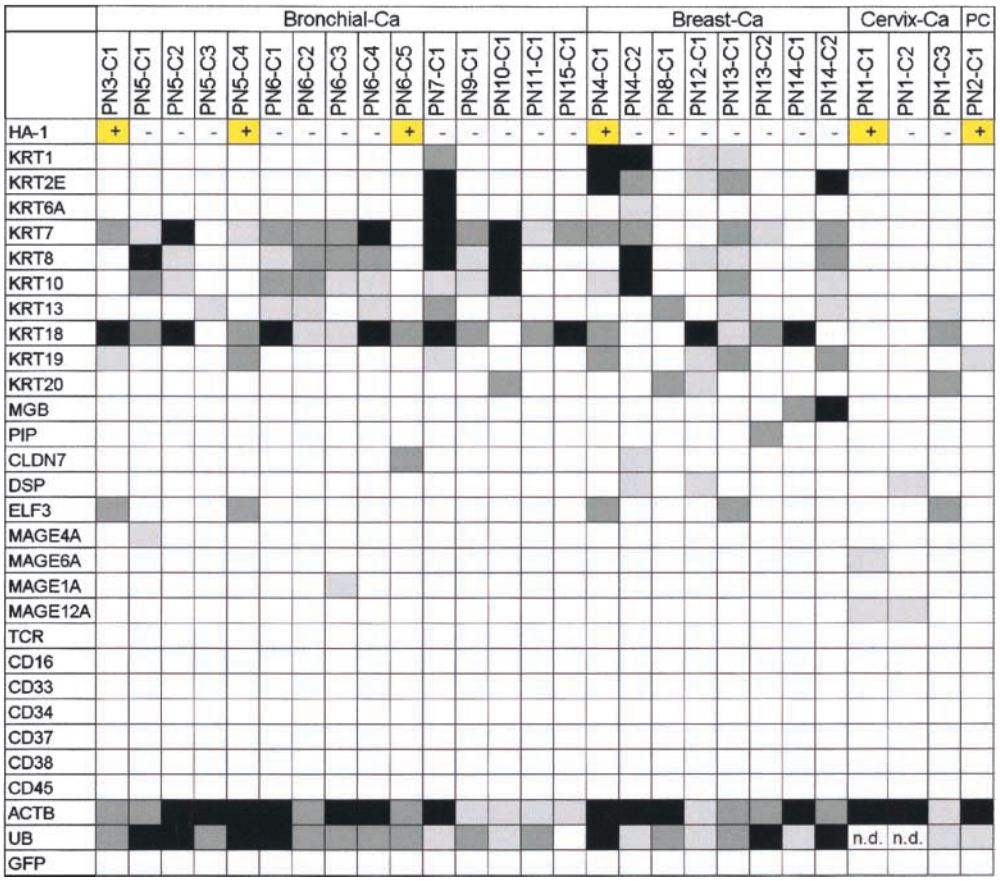



**Figure 4.** Isolation and HA-1 expression analysis of single disseminated cancer cells or small tumor cell clusters. (A) Three-cell cluster (PN5-C4) after micromanipulator-assisted isolation from a cell suspension of a lymph node preparation. All cells of the cluster are intensively stained by the epithelial cell adhesion molecule antibody. (B) Gene expression profiling on cDNA array of isolated tumor cells. HA-1 expression after standard RT-PCR is given in the first line. The gray shades represent the signal intensity, from light gray (weak signal) to black (strong signal).

cant transcripts were observed in samples derived from bronchial carcinoma (PN3-C1, PN5-C4, and PN6-C5), breast cancer (PN4-C1), cervical cancer (PN1-C1), and prostate cancer (PN2-C1). From two of the HA-$1^+$ cells (PN5-C4 and PN3-C1) we also evaluated their genomic DNA by a recently described method (21). The isolated DNA was subjected to whole genome amplification and CGH. Both cells harbored multiple genomic alterations, confirming their malignant nature as exemplified for PN3-C1 in Fig. 5 B.

## Discussion

We show HA-1 RNA transcription in tumor cell lines of a wide array of epithelial neoplastic cells: breast, melanomas, lung, renal cell and colon carcinomas, hepatocellular

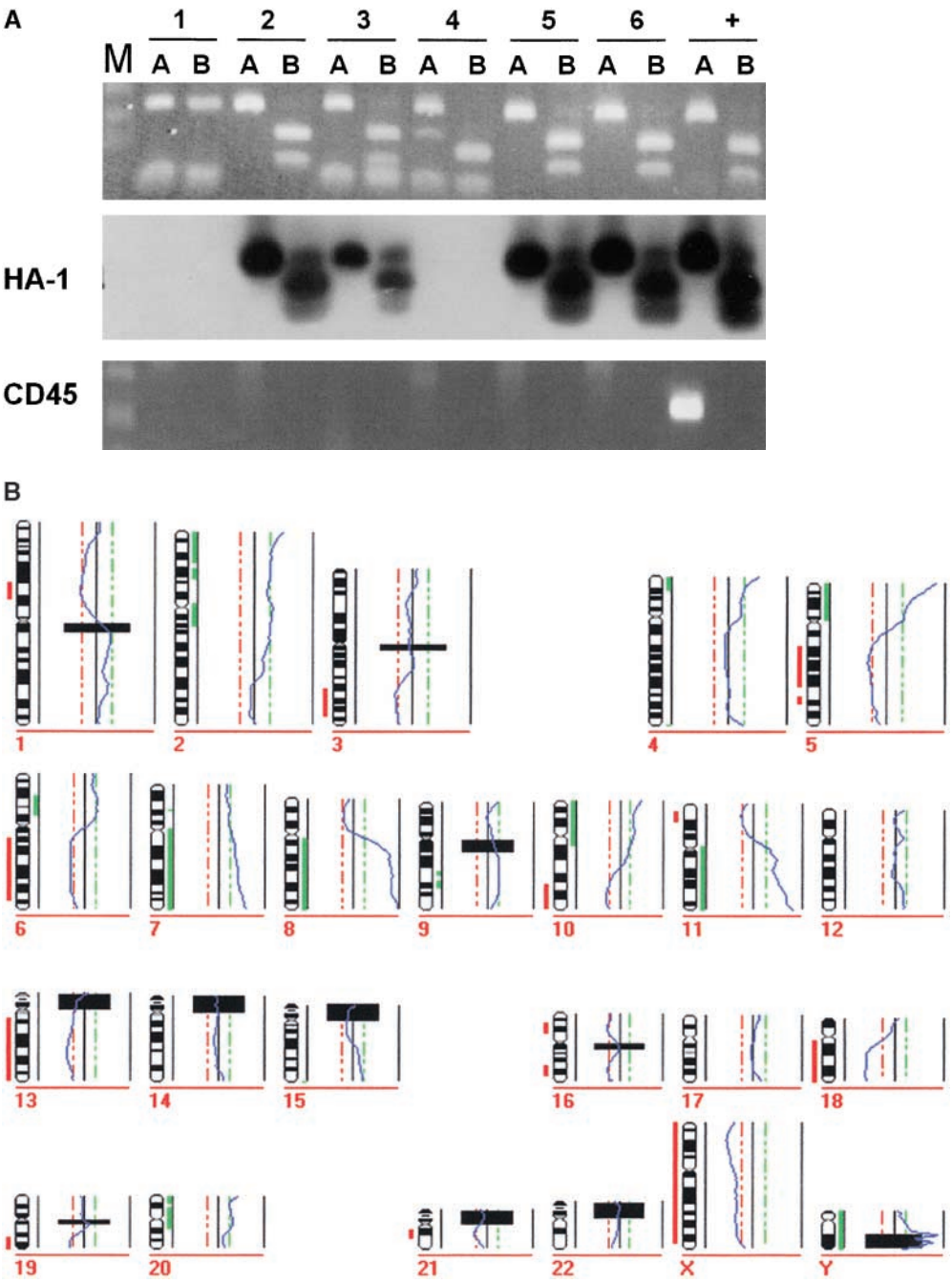



**Figure 5.** HA-1 expression of disseminated cancer cells. (A) Cells positive for the HA-1 (II) primer pair were digested with HinfI, blotted, and hybridized with the respective probe. M, size marker; A, undigested PCR product; B, HinfI-digested product; lane 1, PN12-C1; lane 2, PN4-C1; lane 3, PN3-C1; lane 4, PN5-C4; lane 5, PN6-C5; lane 6, PN2-C1; +, HT29 for HA-1 and normal bone marrow for CD45. (B) CGH profile of cell PN3-C1. Each chromosome is represented by its ideogram and numbered. Deletions are marked with a red bar (e.g., loss of chromosome 13) to the left and gains are marked with a green bar (e.g., gain of chromosome 8q) to the right of the chromosome symbol.

carcinomas, and head and neck cancers. The cell lines that express the HLA-A2 and HA-1 phenotypes, which are required for CTL recognition, were lysed by the HLA-A2–restricted HA-1–specific CTL. We show that HA-1 is expressed by primary solid tumors and single disseminated lung, breast, cervical, and prostatic carcinoma cells. Disseminated cells from 6 out of 15 patients were positive for HA-1. The fact that we could not detect the HA-1 mRNA in all cells from one individual patient either indicates the detection limit of our approach, or reflects tumor cell heterogeneity. Both reasons are equally plausible. In a separate study, using the same amplification method, we noticed that the detection of EMMPRIN mRNA in 60% of single disseminated tumor cells corresponded to 80% expression on the protein level (15). This suggests that HA-1 protein expression might be more frequent than observed in this study. In addition, HA-1 might also be heterogeneously expressed on disseminated tumor cells. Interestingly, we recently observed that genetic instability is the highest before the diagnosis of overt metastasis (unpublished data). As most cells analyzed here were isolated from bone marrow or lymph nodes of patients in the clinical stage without overt metastasis, it is very likely that the heterogeneous expression of HA-1 directly reflects the genetic instability at

the stage of minimal residual disease. The finding of HA-1 expression in the latter patients is of particular clinical importance as this cell population includes the progenitor cells of metachronic metastasis. Therefore, the investigation of whether a more homogeneous HA-1 expression is associated with the progression to overt metastasis is relevant.

The mechanism of the aberrant HA-1 expression in epithelial tumor cells is currently unknown. A variety of gene families such as the MAGE or BAGE family give rise to tumor-specific antigens (22, 23). These genes are both activated in tumors of different histological types and silent in normal cells except for testicular germ cells. DNA methylation has been proposed as the primary mechanism for the inactivation of genes (24). But DNA demethylation is not always sufficient for the activation of genes in nonexpressing cells (25, 26) and therefore the regulation by transcription factors is also required (27). The function of the HA-1 gene, its promoter region, and the relevant transcription factors involved in its activation are presently unknown. Based on computer alignment, a RhoGAP domain was defined within the HA-1–predictive protein (these sequence data are available from Genbank/EMBL/DDBJ under accession no. XM 037574). Therefore, HA-1 might be a GTPase activator protein for Rho-like GTPases. Members of the Rho family are key regulators of the assembly and organization of the actin cytoskeleton. Through their interaction with multiple target proteins, they ensure the control of cellular activities such as gene transcription and adhesion (28). The determination of HA-1 target proteins, functional studies, and analysis of its regulation will reveal the role of HA-1 in cellular mechanisms and might explain the aberrant expression in epithelial tumor cells.

The mHag HA-1 fulfills the prerequisites as target molecule for successful immunotherapy of hematological tumors. Here, we show that HA-1 can also function as a novel target molecule for immunotherapy of carcinomas in combination with HLA-matched HA-1–mismatched allogeneic SCT. In the HLA-identical allogeneic SCT setting for solid tumors, graft-versus-tumor reactivity has been suggested in small cohorts of patients with metastatic cancers, including breast cancer (5, 6, 29), melanomas (7), renal cell carcinomas (4), and ovarian carcinoma (8). One could speculate that this graft-versus-tumor reactivity may be due to specific tumor-associated antigens, tissue- or cell-specific polymorphic mHags such as HA-1. The relevance of the HA-1–specific lysis in vitro on the tumor cell lines restricts itself to the extent that the cell lines reflect tumor cells as they exist in vivo. Yet, at a relatively low effector to target ratio, the lysis can be interpreted as significant. Nonetheless, the HA-1–specific lysis vary among the tumor cell lines and is clearly lower compared with the lysis of the EBV-lymphocyte cell lines (LCLs) in the absence of IFN$\gamma$ and TNF$\alpha$. Evidently, in vivo recognition of tumor cells is crucial. Inappropriate tumor cell lysis would hamper the potential immunotherapy. It is of interest to note that IFN$\gamma$ and TNF$\alpha$ treatment of the tumor cell lines enhances the cytotoxic activity. It is known that these cytokines do up-regulate MHC expression. This might be particularly effective for ligands such as HLA-A2/HA-1 that have low ($\sim$80) HA-1 peptide HLA-A2 complexes expressed per cell (unpublished data). Similar to the immunotherapy protocol for the treatment of relapsed leukemia (14) as described above, adoptive immunotherapy with donor-derived HA-1 CTLs in combination with SCT can also become an attractive treatment of solid tumors. As in the leukemia transplant patients in which residual leukemic tumor cells are present after high dose chemotherapy, HA-1–based immunotherapy might be particularly warranted in cancer patients with minimal residual disease who were shown to confer an increased risk for a later occurring relapse (30). The HA-1–based immunotherapy is as yet limited to HLA-A2$^+$ patients and the HA-1–incompatible SCT patient and/or donor pairs. The HA-1$^H$ phenotype frequency is 69% in the HLA-A2$^+$ population (17). Among the HLA-identical HLA-A2$^+$ patient and/or donor sibling pairs, there is 13% HA-1$^H$ incompatibility. It will be possible to extend the postulated therapy to HLA-B60$^+$ patients. We recently observed an HLA-B60–restricted functional HA-1 T cell epitope within the HA-1 polymorphic locus (unpublished data). Evidently, the HA-1 expression among tumor cells is heterogeneous, which further limits the proposed immunotherapy. Nonetheless, the latter therapy could represent an important proof of principle if it showed that adoptive CTL immunotherapy targeted to specific peptides with restricted tissue expression could be effective. Future studies will also focus on peptide vaccination strategies of stem cell donor and/or recipient in the HLA-matched mHag HA-1–mismatched situation (31). In conclusion, here we describe a constitutive human hematopoietic–specific gene that can function as a tumor-specific antigen for epithelial cancer. The significance of the polymorphic mHag HA-1 for cancer therapy is underscored by its absence of expression on nonmalignant epithelial cells, its known HA-1–immunogenic functional membrane expression, and its adequate CTL recognition.

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