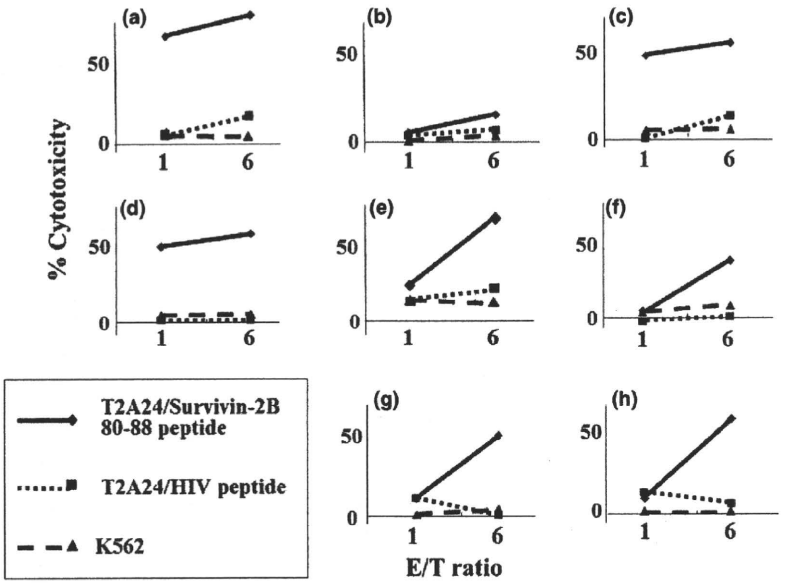

Fig. 4. Analysis of survivin-2B80-88 peptide-specific cytotoxicity. T cells of wells (a) through (h) in Figure 3 were assessed for their peptide-specific cytotoxicity against T2A24 target cells pulsed with the survivin-2B80-88 peptide and HIV peptide, and against control K562 cells. E/T, effector/target; HIV, human immunodeficiency virus.

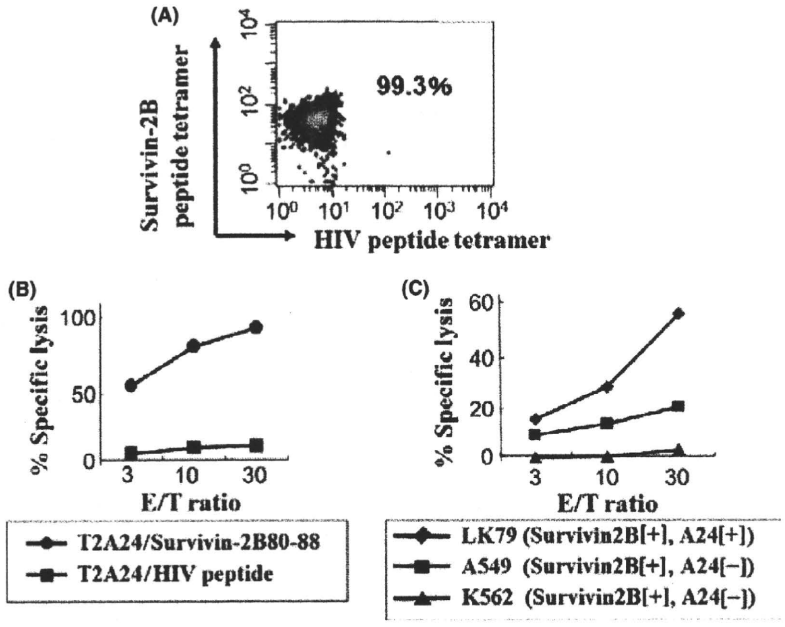

Fig. 5. Clonal analysis of survivin-2B80-88 peptide-specific CD8 T cells. T cells in well (d) in Figure 3 were cultured and CTL clone D-13 was obtained. Subsequently, D-13 was studied for (A) tetramers and (B) cytotoxicity against T2A24 cells pulsed with survivin-2B80-88 and HIV peptides, and (C) cytotoxicity against human tumor cells (LK79, A549 and K562) that express survivin and/or HLA-A24 molecules as indicated. CTL, cytotoxic T lymphocyte; E/T, effector/target; HIV, human immunodeficiency virus; HLA, human leukocyte antigen.

survivin-2B80-88 plus IFA and IFNα appears effective in other cancers such as pancreatic cancer as well, this protocol may be useful as a standard immunotherapy modality.

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Disclosure Statement

The authors have no conflict of interest.

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COMPARISON OF SPEEDY PCR-SSP METHOD AND SEROLOGICAL TYPING OF HLA-A24 FOR JAPANESE CANCER PATIENTS

Munehide Nakatsugawa,[1,2] Yoshihiko Hirohashi,[1] Toshihiko Torigoe,[1] Satoko Inoda,[1] Kenji Kiriyama,[1] Yasuaki Tamura,[1] Eiji Sato,[1] Hiroki Takahashi,[2] and Noriyuki Sato[1]

[1]*Department of Pathology, Sapporo Medical University School of Medicine, Sapporo, Japan*
[2]*Third Department of Internal Medicine, Sapporo Medical University School of Medicine, Sapporo, Japan*

☐ *Human leukocyte antigen (HLA) typing is essential to carry out HLA-class I restricted antigenic peptide-based cancer immunotherapy. To establish a one-step polymerase chain reaction–sequence-specific primer (PCR-SSP) method, we designed two novel HLA-A24-specific primer sets and determined the optimal conditions for specific amplification. Then, we performed HLA-A24 typing of two healthy donors' and 17 cancer patients' peripheral blood with serological typing and PCR-SSP typing. Eleven of the 19 cases were determined HLA-A24-positive by the PCR-SSP method precisely; however, five cases showed false positive with serological analysis. Thus, for HLA-A24 typing in the Japanese population, the PCR-SSP method is faster and more accurate than serological typing.*

Keywords HLA-A24, HLA-typing, monoclonal antibody, PCR-SSP

INTRODUCTION

Human leukocyte antigen (HLA)-class I–restricted antigenic peptides are essential to establish cancer immunotherapy. Previously, we identified several HLA-A24–restricted antigenic peptides encoded within tumor-associated antigens (TAAs).[1–8] We have launched clinical trials of cancer vaccine therapy with HLA-A24–restricted antigenic peptides.[9,10] HLA-A24 typing is essential to carry out cancer vaccine therapy with HLA-A24–restricted peptides. There are several methods of HLA typing, and serological typing is the most common in routine analysis. However, recent

Address correspondence to Yoshihiko Hirohashi, Department of Pathology, Sapporo Medical University School of Medicine, South-1 West-17, chuo-ku, Sapporo 060-8556, Japan. E-mail: hirohash@sapmed.ac.jp

advances in assigning HLA-class I alleles by techniques involving DNA analysis indicate that serological typing may not be sufficiently reliable.[11,12] One of the reasons is the limitations of HLA-A24–specific monoclonal antibodies (mAbs). Monoclonal antibodies, clone A11.1 M and C7709A2.6, are possible candidates for HLA-A24[13,14]; however, A11.1 M is also reactive for HLA-A11, which is also a common allele, and is not suitable for HLA-A24 detection. Thus, we evaluate clone C7709A2.6 for HLA-A24 detection.

In this report, to establish speedy and accurate HLA-A24 typing in Japanese, we compared serological typing with mAb C7709A2.6 and polymerase chain reaction (PCR)-sequence specific primer (SSP) with double HLA-A24-specific primer sets. mAb C7709A2.6 was positive for HLA-A24-positive cases' peripheral blood mononuclear cells (PBMCs). On the other hand, C7709A2.6 was partially false positive for HLA-A24-negative cases. On the other hand, the results of PCR-SSP analysis with two primer sets were completely identical with whole HLA-class I genotyping. These data suggest that the PCR-SSP method with two novel primers is a quick and accurate method for HLA-A24 screening.

EXPERIMENTAL

Flow Cytometric (FCM) Analysis and Monoclonal Antibodies (mAbs)

Peripheral blood mononuclear cells (PBMCs) were isolated by standard density gradient centrifugation on Lymphoprep (Nycomed, Oslo, Norway). The cells were incubated with a mouse anti-HLA-A24 monoclonal antibody (C7709A2.6, kind gift from Dr. P. G. Coulie) at saturating concentration for 30 min on ice, washed with phosphate-buffered saline (PBS), and stained with a polyclonal goat anti-mouse antibody coupled with FITC for 30 min. Samples were analyzed using a FACSCalibur analyzer (Becton Dickinson, Mountain View, CA, USA). An HLA-A24 positive EB virus-transformed B cell line, LG2-EBV (a kind gift from Dr. B. J. van den Eynde), was used as a positive control. HLA-class I negative leukemic cell line K562 was purchased from ATCC and used as a negative control.

One-Step PCR Analysis of Genomic DNA

Genomic DNA from 200 μL of peripheral blood was isolated with a QIAamp DNA mini kit (QIAGEN) as described in the manufacturer's protocol. Then, PCR amplification was performed in 20 μl of PCR mixture containing 1 μl of genomic DNA, 0.1 μl of Taq DNA polymerase (QIAGEN), and 12 pmol of primers. The PCR mixture was initially incubated at 94°C

for 2 min, followed by 35 cycles of denaturation at 94°C for 15 sec, annealing at 68°C for 30 sec, and extension at 72°C for 30 sec. Primer pairs used for PCR analysis were as follows. The primer 1 set for HLA-A24-specific detection was composed of 5′-ACTGACCGAGAGAACCTGCGGAT-3′ and 5′-ACTTGCGCTTGGTGATCTGAGCC-3′ as sense and anti-sense with an expected PCR product size of 464 base pairs (bps). The primer 2 set for HLA-A24-specific detection consisted of 5′-ACAGACTGACCGAGAGAACC TGC-3′ and 5′-ACTTGCGCTTGGTGATCTGAGCC-3′ as sense and anti-sense with an expected PCR product size of 468 bps. As an internal control, an HLA common sequence was detected with the primer 3 set, 5′-ACGTGGACGACACGCAGTTCGTG-3′ and 5′-TTCCCGTTCTCCAGGTAT CTGCG-3′ as sense and anti-sense with an expected PCR product size of 713 bps. The PCR products were visualized with ethidium bromide staining under UV light after electrophoresis on 1.2% agarose gel. Nucleotide sequences of the PCR products were confirmed by direct sequencing using an ABI Genetic Analyzer PRISM 310 and an AmpliCycle sequencing kit (Perkin-Elmer, Foster City, CA, USA).

RESULTS

Novel HLA-A24–Specific Primer Sets and Determination of a Suitable Annealing Temperature for PCR-SSP

To establish the one-step PCR-SSP method, we designed two novel HLA-A24–specific primer sets 23 bps in length (Table 1 and Figure 1). To determine the appropriate annealing temperature, we performed PCR amplification with serial annealing temperatures to amplify the HLA-A*2402-positive and HLA-A*2402-negative genomic DNAs (Case #5 and #3). As shown in Figure 2, PCR bands were observed in both HLA-A*2402-positive and HLA-A*2402-negative samples with annealing temperatures of 60–66°C, suggesting that gene amplifications were not specific for HLA-A*2402. On the other hand, the PCR band could be observed in only the HLA-A*2402-positive sample with annealing temperatures of 68°C and

TABLE 1 PCR Primer Pairs

Primer	Orientation	Oligonucleotide Sequence (5′—3′)
Primer 1	sense	ACTGACCGAGAGAACCTGCGGAT
	antisense	ACTTGCGCTTGGTGATCTGAGCC
Primer 2	sense	ACAGACTGACCGAGAGAACCTGC
	antisense	ACTTGCGCTTGGTGATCTGAGCC
Primer 3	sense	ACGTGGACGACACGCAGTTCGTG
	antisense	TTCCCGTTCTCCAGGTATCTGCG

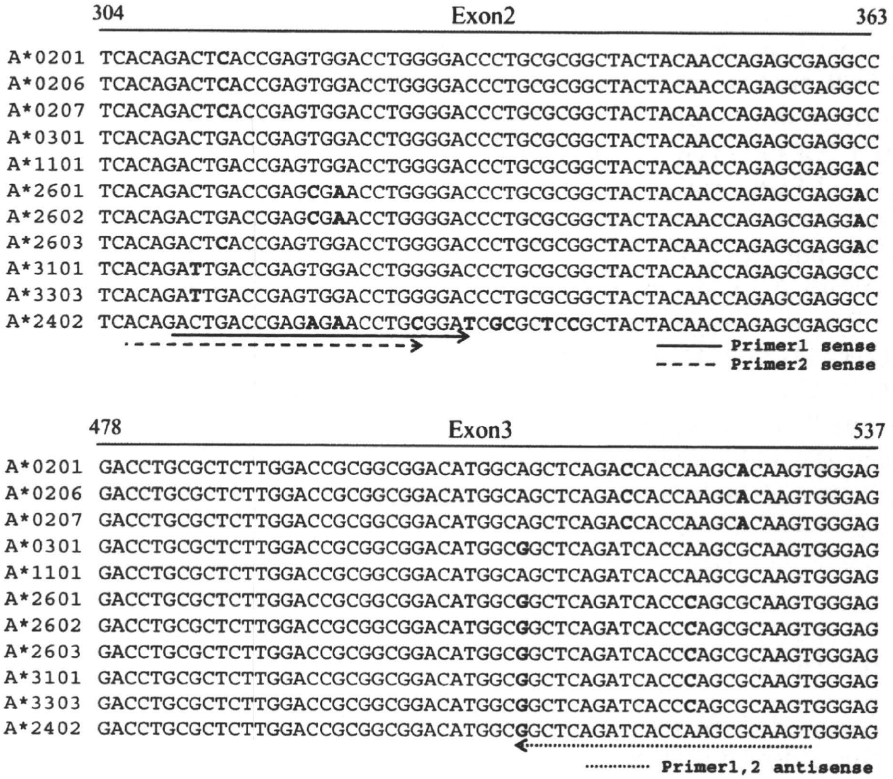

FIGURE 1 Gene specific primers for HLA-A*2402. Gene sequence alignments of HLA-class I molecules frequent in Japanese populations are shown. Bolds show the allele specific alignments. Numbers indicate numbers from the start codon. HLA-A24 gene specific primers were designed based on HLA-A24 specific alignments (primer 1 and primer 2).

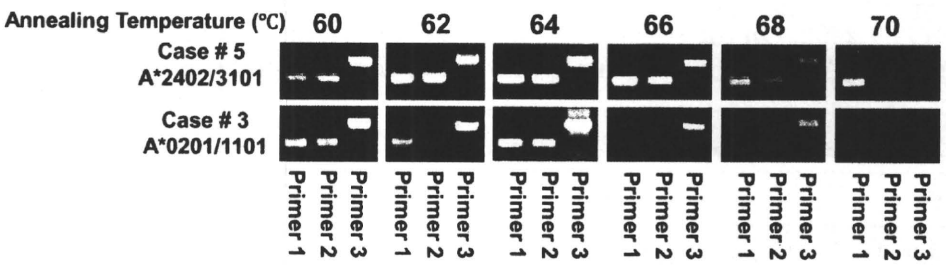

FIGURE 2 Determination of adequate annealing temperature for specific HLA-A24 detection. HLA-A24–positive and HLA-A24–negative genomic DNAs were amplified with HLA-A24-specific primer sets (primer 1 and primer 2). Primer 3 for a common HLA sequence was used as a positive control. Annealing temperatures were settled from 60°C to 70°C. Case #5 (HLA-A*2402/3101) and case #3 (HLA-A*0201/1101) genomic DNAs were used as positive and negative controls, respectively.

70°C. The PCR bands were confirmed to be identical with HLA-A*2402 sequence by DNA direct sequencing (data not shown). Thus, HLA-A24–specific amplification could be attained with annealing temperatures of 68°C and 70°C. Therefore, we have chosen 68°C as the annealing temperature for HLA-A24–specific amplification with these primer sets.

Detection of HLA-A24 with the PCR-SSP and Serological Methods

To compare the serological typing of HLA-A24 with the one-step PCR-SSP method, samples from two healthy donors and 17 cancer patients were evaluated. PBMCs from donors were isolated, and analyzed by FCM to detect the cell surface HLA-A24 molecules. The whole genomic DNA was isolated from whole blood and analyzed with one-step PCR-SSP method with 68°C for the annealing temperature. Figure 3 shows a representative HLA-A24-positive and -negative cases. HLA-A24 could be detected by both serological typing and PCR-SSP typing with HLA-A*2402-positive case (Case #8) specifically, suggesting that both PCR-SSP and serological typing work well.

Then, we compared several cases with PCR-SSP and FCM methods. Serologically HLA-A24-positive cases (Figure 4, upper panel) and serologically HLA-A24-negative cases (Figure 4, lower panel) were completely identical with the results of PCR-SSP analysis. On the other hand, some cases showed

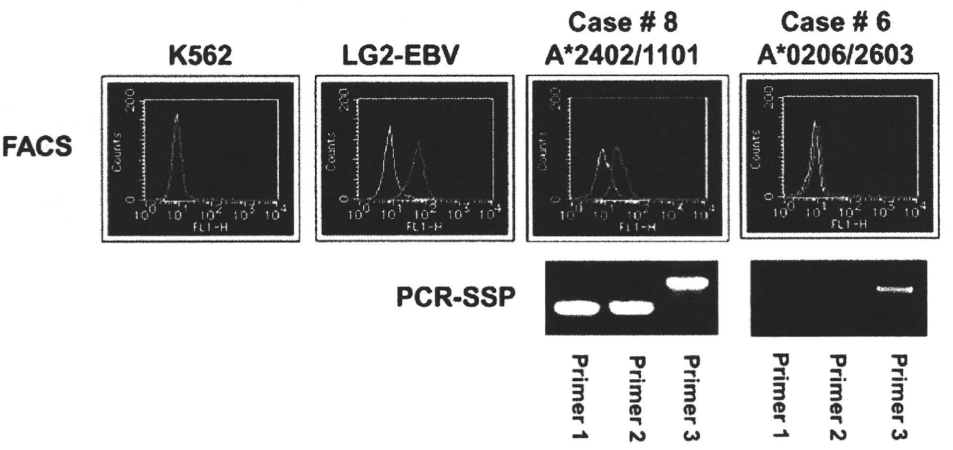

FIGURE 3 Specific detection of HLA-A24 with the serological method and PCR-SSP method. HLA-A24 molecule was detected with FCM analysis using mAb C7709A2.6 and PCR-SSP analysis using gene-specific primers. Cell surface HLA-A24 molecules were detected by flow cytometry. The PCR-SSP method was carried out with an annealing temperature of 68°C. K562 is an HLA-A24-negative cell line, and LG2-EBV is an HLA-A24-positive cell line. Case #8 HLA-A genotype was HLA-A*2402/1101 and case #6 was HLA-A*0206/2603.

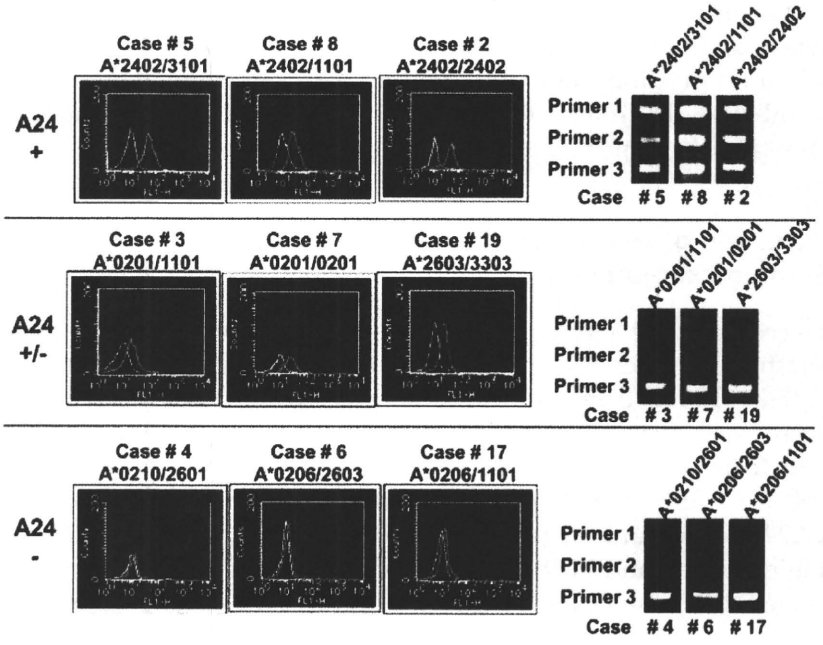

FIGURE 4 Serological HLA-A24–positive, weakly positive, and negative cases. Representative serological HLA-A24–positive, weakly positive, and negative cases are shown (upper, middle, and lower panel). PCR-SSP analysis data are shown in the right column. Corresponded HLA-A genotypes are shown.

TABLE 2 Summary of Flow Cytometry and PCR-SSP Analysis of HLA-A24

Case	Sex	Donor Type	Flow Cytometry	PCR-SSP	Genotype (HLA-A)
1	Female	Healthy donor	+	+	**2402**/3303
2	Male	Healthy donor	+	+	**2402/2402**
3	Male	Lung cancer	+/−	−	0201/1101
4	Male	Colon cancer	−	−	0210/2601
5	Female	Lung cancer	+	+	**2402**/3101
6	Male	Lung cancer	−	−	0206/2603
7	Female	Breast cancer	+/−	−	0201/0201
8	Male	Lung cancer	+	+	**2402**/1101
9	Male	Lung cancer	+	+	**2402**/0207
10	Female	Breast cancer	+	+	**2402**/0206
11	Female	Breast cancer	+	+	**2402/2402**
12	Male	Lung cancer	n.d.	+	**2402**/0206
13	Male	Lung cancer	n.d.	+	**2402**/3303
14	Male	Lung cancer	+/−	−	0207/1101
15	Female	Breast cancer	+/−	+	**2402/2402**
16	Male	Colon cancer	+/−	−	**2402/2402**
17	Male	Lung cancer	−	−	0206/1101
18	Female	Head and neck cancer	+/−	−	0201/0201
19	Male	Lung cancer	+/−	−	2603/3303

n.d.: not detected.

serological HLA-A24–weakly positive as compared with serologically HLA-A24–positive cases with mAb C7709A2.6 (Figure 4, middle panel). Part of these serological weakly positive cases were proved to be HLA-A24-negative (false positive) by one-step PCR-SSP analysis. The whole HLA-class I typing was completely consistent with the results obtained using the one-step PCR-SSP method. Five of the 19 samples were proved to be serological false positives (Table 2). With one-step PCR-SSP analysis, all 19 cases were analyzed appropriately. These data suggested that the one-step PCR-SSP method was suitable for routine HLA-A24 typing in the Japanese population.

DISCUSSION

Recently, a large number of cancer-related antigenic peptides, including HLA-A24–restricted peptides, has been reported, and cancer immunotherapy with such antigenic peptides has been initiated all over the world.[15] Some cases were reported to show clinical responses, including tumor regression and decrease in markers. These data will accelerate the use of cancer immunotherapy as a new treatment modality. Because HLA binding affinities of antigenic peptides depend on HLA-types, HLA-typing is essential to carry out cancer immunotherapy with HLA-restricted antigenic peptides. The PCR-SSP method and serological typing are candidates to detect specific HLA alleles. Several reports have shown that there were discrepancies between DNA typing and serological typing.[11,12] There are already several reports of methods for DNA typing of specific alleles.[16–19] However, there is no report about the typing of HLA-A24, which is the most frequent allele in Japanese and Asian populations and is also frequent in other ethnicities. Thus, the PCR-SSP method for HLA-A24 is required.

In this article, we report the establishment of a PCR-SSP method with novel HLA-A24-specific primers within exon 2 and exon3. Exon2 and exon 3 encode the alpha-1 and alpha-2 domains of the HLA molecule, which then bind to antigenic peptides and are highly polymorphic domains. Thus, exon 2 and exon 3 are suitable to design primers for PCR-SSP. As shown in Figure 1, there are a couple of additional HLA-A*2402 specific sequences in exon 2, which are potentially suitable for design of sense primers. Actually, prior to this study, we evaluated two more HLA-A*2402 specific primers (5′-AGAGAACCTGCGGATCGCGCTCC-3′ and 5′-GACCGA GAGAACCTGCGGATCGC-3′) as sense primers; however both of those primers did not show specific amplification, even with 70°C for annealing. Thus, we decided to use two primers (primer 1 and primer 2), and we think these two primers are optimized for specific detection. This method is

speedy, taking only 2 h to carry out. Furthermore, this PCR-SSP method has high specificity compared with serological typing with mAb C7709A2.6. This suggests that PCR-SSP analysis is more suitable for daily screening of HLA-A24. However, since the PCR-SSP method detects only several HLA-A24–specific DNA sequences, it cannot assure the presence of functional HLA-A24 molecules on the cell surface. Ishikawa et al. showed that point mutations within the HLA-A2 coding region caused a stop codon, which resulted in immature and nonfunctional HLA molecules.[20] Laforet et al. showed that an intronic mutation caused lower expression of the HLA-A24 allele.[21] Thus, we might not be able to detect such variants with the PCR-SSP method, and the functional cell surface expression of the HLA-A24 molecule is essential to carry out antigenic peptide–based cancer immunotherapy. Therefore, it might be important to perform FCM analysis post–PCR-SSP screening to assure the presence of functional HLA-A24 molecules.

CONCLUSION

We established a one-step PCR-SSP method to detect HLA-A*2402 in the Japanese population. This method can be completed in about two hours, and is not expensive per test; thus it is suitable for daily screening for HLA-A24.

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Stem Cells, Tissue Engineering, and Hematopoetic Elements

Cytotoxic T Lymphocytes Efficiently Recognize Human Colon Cancer Stem-Like Cells

Satoko Inoda,*†‡ Yoshihiko Hirohashi,* Toshihiko Torigoe,* Rena Morita,* Akari Takahashi,* Hiroko Asanuma,§ Munehide Nakatsugawa,* Satoshi Nishizawa,* Yasuaki Tamura,* Tetsuhiro Tsuruma,† Takeshi Terui,‡ Toru Kondo,¶ Kunihiko Ishitani,‡ Tadashi Hasegawa,§ Koichi Hirata,† and Noriyuki Sato*

From the Department of Pathology, First Department of Surgery,† and Division of Clinical Pathology,§ Sapporo Medical University School of Medicine, Sapporo; the Higashi Sapporo Hospital,‡ Sapporo; and the Team for Cell Lineage Modulation,¶ RIKEN Center for Developmental Biology, Kobe, Japan*

Cancer stem-like cells (CSCs) and tumor-initiating cells (TICs) are a small population of cancer cells that share three properties: tumor initiating ability, self-renewal, and differentiation. These properties suggest that CSCs/TICs are essential for tumor maintenance, recurrence, and distant metastasis. Here, we show that cytotoxic T lymphocytes (CTLs) specific for the tumor-associated antigen CEP55 can efficiently recognize colon CSCs/TICs both *in vitro* and *in vivo*. Using Hoechst 33342 dye staining, we isolated CSCs/TICs as side population (SP) cells from colon cancer cell lines SW480, HT29, and HCT15. The SP cells expressed high levels of the stem cell markers SOX2, POU5F1, LGR5, and ALDH1A1 and showed resistance to chemotherapeutic agents such as irinotecan or etoposide.To evaluate the susceptibility of SP cells to CTLs, we used CTL clone 41, which is specific for the CEP55-derived antigenic peptide Cep55/c10orf3_193 (10) (VYVKGLLAKI). The SP cells expressed HLA class I and CEP55 at the same level as the main population cells. The SP cells were susceptible to CTL clone 41 at the same level as main population cells. Furthermore, adoptive transfer of CTL clone 41 inhibited tumor growth of SW480 SP cells *in vivo*. These observations suggest that Cep55/c10orf3_193(10) peptide-based cancer vaccine therapy or adoptive cell transfer of the CTL clone is a possible approach for targeting chemotherapy-resistant colon CSCs/TICs. *(Am J Pathol 2011, 178:1805–1813; DOI: 10.1016/j.ajpath.2011.01.004)*

Colon cancer is one of the most common malignancies worldwide. With recent progress in treatment, the prognosis has improved to some extent. In advanced disease, however, the prognosis remains unfavorable, because of recurrence, distant metastasis, and resistance to treatment. Thus, novel treatment modalities are needed.

Cancers contain morphologically heterogeneous populations. This fact has led to the cancer stem cell theory,[1] the idea that cancers are composed of several types of cells, and that only a small population of cancer cells that can regenerate cancer tissues, much as normal tissue can be regenerated only by a small population of stem-like cells. Recently, cancer stem-like cells and tumor-initiating cells (CSCs/TICs) have been isolated from various types of malignancies, including colon cancer.[2–6] In colon cancer, CSCs/TICs can reinitiate tumors that resemble mother colon cancer tissues morphologically when transplanted into immunodeficient mice.[3] Furthermore, these CSCs/TICs have higher tumorigenic potential than do non-CSCs/TICs. Previous reports have shown that CSCs/TICs are resistant to a variety of treatments, including chemotherapy and radiotherapy, with varied mechanisms of resistance, including high expression of drug transporters, relative cell cycle quiescence, high levels of DNA repair machinery, and resistance to apoptosis.[7] These reports[3–6] support the hypothesis that malignant cancers comprise heterogeneous populations that organize in a hierarchical differentiation model. The CSCs/TICs are located at the top of this hierarchy, and targeting CSCs/TICs is essential to achieve efficient effects for treatment of malignant diseases. Recently, some trials targeting CSCs/TICs have been reported for hema-

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Address reprint requests to Yoshihiko Hirohashi, M.D., Ph.D., Department of Pathology, Sapporo Medical University, School of Medicine, South-1 West-17, Chuo-ku, Sapporo 060-8556, Japan. E-mail: hirohash@sapmed.ac.jp.

topoietic malignancies.[8] Hedgehog signaling is essential for maintenance of myeloid leukemia stem cells, and inhibition of hedgehog signaling by cyclopamine is effective for imatinib-resistant myeloid leukemia.[9] To date, however, no such CSC/TIC targeting approach has been reported for colon cancer.

In the present study, we evaluated the efficiency of CTL-based immunotherapy targeting colon CSCs/TICs. Using Hoechst 33342 dye, we isolated colon CSCs/TICs as side population (SP) cells from six colon cancer cell lines. The SP cells derived from SW480, HT29, and HCT15 showed higher tumorigenicity than did main population (MP) cells. On the other hand, SP cells from KM12LM, Lovo, and Colo320 did not show any increase in tumorigenicity, compared with MP cells. This suggests that SW480, HT29, and HCT15 SP cells (but not KM12LM, Lovo, and Colo320 SP cells) were enriched with CSCs/TICs. In RT-PCR analysis the SW480, HT29, and HCT15 SP cells showed a stem cell-like gene expression signature, including SOX2, POU5F1, LGR5, and ALDH1A1. Furthermore, these SP cells also showed resistance to chemotherapeutic agents, including irinotecan and etoposide. These observations support the idea that these SP cells had stem cell-like features. To assess the immunogenicity of SP cells, we evaluated the expression of HLA class I and of CEP55, which is a tumor-rejection antigen of breast and colon cancer.[10,11] The SP cells expressed HLA class I (and also HLA-A24) at the same level as MP cells. The SP cells also expressed CEP55 messenger RNA (mRNA) at the same level as MP cells in RT-PCR. To confirm the susceptibility of SP cells to cytotoxic T lymphocytes (CTLs), we used CTL clone 41, which recognizes CEP55 in an HLA-A24-restricted manner.[10] CTL clone 41 killed SW480, HT29, and HCT15 SP cells at the same level as it killed MP cells and presorted cells. These observations suggest that colon CSCs/TICs are also sensitive to CTLs, as non-CSC/TIC populations are. Furthermore, adoptive transfer of CTL clone 41 inhibited the tumor growth of SW480 SP cells in immunodeficient mice. These observations suggest that CTL-based colon cancer immunotherapy is efficient for colon CSCs/TICs. To our knowledge, the present study provides the first direct evidence that colon CSCs/TICs are susceptible to CTLs and thus opens possibilities for future applications in immunotherapy using CSC/TIC-specific vaccines.

Materials and Methods

Cell Lines

Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402), HCT15 (HLA-A*0201/2402), HT29 (HLA-A1/24), Lovo, and Colo320 were kind gifts of Dr. K. Imai (Sapporo, Japan), and KM12LM was a kind gift of Dr. K. Itoh (Kurume, Japan). All cell lines except K562 were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). K562 was cultured in RPMI-1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum. HCT15-B2M, a stable transfectant of HCT15 cells with B2M (β2 microglobulin) cDNA, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 10 μg/mL puromycin (Sigma-Aldrich).[11]

Side Population Analysis

Side population analysis was performed as described previously, with some modifications.[12] Trypsinized cultured cells were washed with PBS and were resuspended at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. After 10 minutes preincubation, the cells were labeled with Hoechst 33342 dye (Lonza, Walkersville, MD) for 90 minutes at concentrations of 3.75 μg/mL for Colo320, 5 μg/mL for SW480 and Lovo, 7.5 μg/mL for HT29 and KM12LM, and 10 μg/mL for HCT15, with or without verapamil (Sigma-Aldrich), which is an inhibitor of ABC transporters, at concentrations of 50 μmol/L for SW480, HCT15, and Colo320, 75 μmol/L for Lovo, and 100 μmol/L for HT29. Cells were counterstained with 1 μg/mL propidium iodide to label dead cells. Next, 1×10^6 viable cells were analyzed and sorted using a BD FACSAria II fluorescence-activated cell sorting system (BD Biosciences, Franklin Lakes, NJ). The Hoechst dye was excited at 355 nm, and its fluorescence was measured at two wavelengths using optical filters 405 DF20 [450/20 nm bandpass filter O (Hoechst Blue)] and 635LP [635 nm longpass edge filter (Hoechst Red)]. Propidium iodide labeling was measured through a 630/BP30 filter for discrimination of dead cells.

Xenograft Model

The SP cells, MP cells, and presorted cells from colon cancer cell lines were mixed 1:1 by volume with Matrigel (BD Biosciences) and were injected subcutaneously into the backs of female 4- to 8-week-old nonobese diabetic/severe combined immunodeficiency (NOD/SCID) mice. Tumor size in cubic millimeters was assessed weekly with calipers and was calculated as Tumor Size = (Longest Diameter $\times$ Shortest Diameter^2)/2.

RT-PCR Analysis of SP and MP Cells

RT-PCR analysis was performed as described previously.[10] Total RNAs were isolated from both SP cells and MP cells using an RNeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was synthesized from 2 μg of total RNA by reverse transcription using SuperScript III reverse transcriptase (Invitrogen). The PCR amplification was performed in 20 μL of PCR mixture containing 1 μL of cDNA mixture, 0.5 μL of Taq DNA polymerase (Qiagen) and 4 pmol of primers. The PCR mixture was initially incubated at 98°C for 2 minutes, followed by 30 cycles of denaturation at 98°C for 15 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The following primer pairs were used for RT-PCR analysis (forward and reverse, respectively): 5′-CATGATG-

GAGACGGAGCTGA-3′ and 5′-ACCCCGCTCGCCATGCTATT-3′ for SOX2, with an expected PCR product size of 410 bp; 5′-TGGAGAAGGAGAAGCTGGAGCAAAA-3′ and 5′-GGCAGATGGTCGTTTGGCTGAATA-3′ for POU5F1, with an expected PCR product size of 163 bp; 5′-CTCTTCCTCAAACCGTCTGC-3′ and 5′-GATCGGAGGCTAAGCAACTG-3′ for LGR5, with an expected PCR product size of 181 bp; 5′-TGTTAGCTGATGCCGACTTG-3′ and 5′-TTCTTAGCCCGCTCAACACT-3′ for ALDH1A1, with an expected PCR product size of 154 bp; 5′-TGAGTTTGCCATCACAGAGC-3′ and 5′-TTGCTTGCTGGTGCATTAAC-3′ for CEP55, with an expected PCR product size of 521 bp; and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase (GAPDH), with an expected product size of 452 bp. GAPDH was used as an internal control.

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR was performed using an ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Primers and probes were designed by the manufacturer (TaqMan gene expression assays; Applied Biosystems). Thermal cycling was performed using 40 cycles of 95°C for 15 seconds followed by 60°C for 1 minute. Each experiment was done in triplicate, with normalization to the *GAPDH* gene as an internal control.

Flow Cytometric Analysis and Monoclonal Antibodies

Cells were incubated with mouse monoclonal antibodies at saturation concentration for 30 minutes on ice, washed with PBS, and stained with a polyclonal goat anti-mouse antibody coupled with fluorescein isothiocyanate for 30 minutes. Samples were analyzed using a BD FACSCalibur flow cytometry system (Becton Dickinson, Mountain View, CA). Anti-pan HLA class I (W6/32) and anti-HLA-A24 monoclonal antibodies (C7709A2.6 hybridoma, a kind gift from Dr. P.G. Coulie, Brussels, Belgium) were prepared from hybridomas.

Survival Studies for Etoposide and Irinotecan

We isolated SP and MP cells of SW480 and HCT15 and seeded them into 96-well culture plates at 1×10^4 cells per well for each population of cells. The cells in both populations were treated with etoposide (1 and 5 μg/mL) or irinotecan (40 and 400 μg/mL for SW480, 10 and 100 μg/mL for HCT15). After 72 hours of exposure to the chemotherapeutic agents, viability of the cells was determined using the SOD assay kit WST-1, which was performed according to the manufacturer's protocol (Dojindo Molecular Technologies, Kumamoto, Japan; Rockville, MD).

Cytotoxicity Assay for SP Cells with CTL Clone 41

We had previously established CTL clone 41, which recognizes an HLA-A24 restricted antigenic peptide (VYVKGLLAKI) termed Cep55/c10orf3_193(10), from an HLA-A24-positive breast cancer patient's peripheral blood mononuclear cells.[8] The lytic activity of CTL clone 41 for SP cells, MP cells, and presorted cells was evaluated by ^{51}Cr release assay. Briefly, SP cells, MP cells and presorted cells were labeled with 100 μCi of ^{51}Cr for 1 hour at 37°C, washed four times with PBS, and resuspended in AIM-V medium (Invitrogen). The ^{51}Cr-labeled target cells (2000 cells/well) were then incubated with various numbers of effector cells for 6 hours at 37°C in 96-well culture plates. Radioactivity of the culture supernatant was measured with a gamma counter. The percentage of cytotoxicity was calculated as follows: % Specific Lysis = (Experimental Release − Spontaneous Release) × 100/(Maximum Release − Spontaneous Release). Target cells were treated with 100 units/mL interferon-γ for 48 hours before the assay.

Winn Assay

SW480 SP cells were mixed with CTL clone 41 at a ratio of 1 SP cell to 10 CTL cells. The resulting mixture (200 μL with 1×10^6 CTL clone 41 and 1×10^5 SP cells) was injected subcutaneously into the backs of NOD/SCID mice. A control group of five mice was injected with SP cells alone. Tumor size was assessed weekly.

CTL Adoptive Transfer

NOD/SCID mice were inoculated subcutaneously on the back with 1×10^3 SW480 SP cells. Three weeks later, when the tumor started to be palpable, 5×10^4 Cep55/c10orf3_193(10)-specific CTL clone cells or PBS was injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical Analysis

In the xenograft model, survival studies using chemotherapeutic agents, cytotoxicity assay, Winn assay, and adoptive transfer model, the data were analyzed using the Mann-Whitney *U*-test, with $P < 0.05$ conferring statistical significance.

Results

Isolation of Colon CSCs/TICs as SP Cells

Several methods to isolate colon cancer CSCs/TICs has been reported, including cell surface markers such as CD44 or PROM1 (CD133), SP cells, and the Aldefluor assay.[3–6,13] In the present study, we isolated colon CSCs/TICs using SP cell analysis. Several colon cancer cell lines were dyed with Hoechst 33342 and then analyzed with a BD FACSAria II flow cytometer as

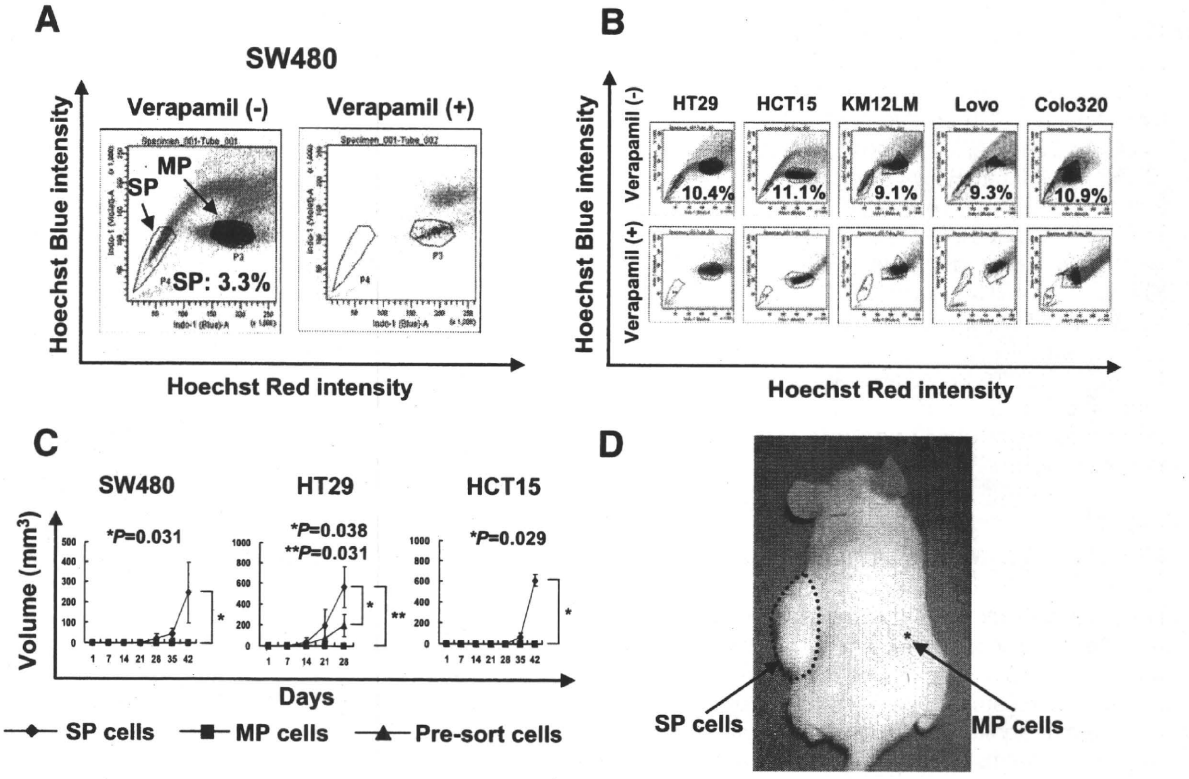

Figure 1. Isolation of colon CSCs/TICs from colon cancer cell lines and tumor growth of the SP cells. **A:** Colon cancer cell line SW480 was stained with Hoechst 33342 dye as described under *Materials and Methods*, with or without verapamil. Stained cells were analyzed using a BD FACSAria II fluorescence-activated cell sorting system. Frequency of SP cells was 3.3%. **B:** Colon cancer cell lines (HT29, HCT15, KM12LM, Lovo, and Colo320) were stained with Hoechst 33342 dye with or without verapamil. Stained cells were analyzed using a BD FACSAria II system. Frequencies of SP cells ranged from 9.1% for KM12LM cells to 11.1% for HCT15 cells. **C:** SP cells, MP cells, and presorted cells of colon cancer cell lines SW480, HT29, and HCT15 were inoculated subcutaneously into the backs of NOD/SCID mice (1×10^3 cells injected). Data are reported as means ± SD. *P* values indicate differences between cell types according to a Mann-Whitney *U*-test. **D:** Representative tumor growth in NOD/SCID mice at the SP cell injection site (1×10^3 cells injected). SP cells and MP cells were inoculated subcutaneously into the left and right side of the back, respectively.

described under *Materials and Methods*. Side population cells could be detected in all six colon cancer cell lines analyzed (ie, SW480, HT29, HCT15, Colo320, Lovo, and KM12LM) (Figure 1, A and B). The frequency of SP cells ranged from 3.3% for SW480 to 11.1% for HCT15 cells. All these SP cells were specifically inhibited by verapamil, as has been shown previously,[14] suggesting that these SP cells were specific for ABC transporter expression. Because previous studies showed that some colon cancer SP cells were not enriched with a CSC/TIC population,[15] it was essential to confirm the presence of CSCs/TICs in SP cells for further analysis. We inoculated these SP cells subcutaneously into the back of immunodeficient NOD/SCID mice using serial dilution. The SP cells derived from SW480, HCT15, and HT29 showed higher tumor initiating ability, compared with MP cells (Table 1). Furthermore, SW480, HT29, and HCT15 SP cells showed faster tumor growth, compared with MP cells (Figure 1, C and D), suggesting the presence of CSCs/TICs in these SP cells. In contrast, the SP cells derived from Colo320, Lovo, and KM12LM did not show any difference in tumorigenicity or tumor growth, compared with MP cells. We therefore restricted further analysis to the SW480, HT29, and HCT15 SP cells as colon cancer CSCs/TICs.

RT-PCR Analysis of Colon Cancer SP Cells

To examine the molecular properties of SP cells, we performed RT-PCR analysis. SOX2 and POU5F1 are representative markers for embryonal stem cells and CSCs/TICs.[16] The SP cells derived from SW480, HT29, and HCT15 showed higher expression of both SOX2 and POU5F1, compared with MP cells (Figure 2A). ALDH1A1, a colon CSC/TIC marker,[6] was expressed at a higher level in SP cells of HCT15 than in MP cells, but SP cells of SW480 and HT29 did not show any difference in comparison with MP cells. SW480 and HT29 SP cells also showed higher expression of LGR5, which is known as a normal colon stem cell marker.[17] To confirm the expression of stem cell markers, we also performed real-time PCR. The SW480 SP cells expressed 90 times higher SOX2, 7 times higher POU5F1, 153 times higher LGR5, and 6.1 times higher ALDH1A1, compared with MP cells (Figure 2B). These findings indicate that these SP cells had molecular properties similar to those of embryonal stem cells.

Resistance to Chemotherapeutic Reagents

Although SP cells derived from liver cancer cell line HuH7 have showed resistance to chemotherapy,[13] we know of no conclusive previous studies of such resistance in co-

Table 1. Tumor Initiating Ability of Colon Cancer SP Cells

Cell line (% SP cells)	Tumor initiating ability*		
	$1 \times 10^{4\dagger}$	$1 \times 10^{3\dagger}$	$1 \times 10^{2\dagger}$
SW480 (3.3)			
SP cells	4/4	4/6	4/4
MP cells	2/4	3/5	0/4
HT29 (10.4)			
SP cells	3/3	2/3	3/3
MP cells	3/3	0/3	0/3
HCT15 (11.1)			
SP cells	3/3	3/4	3/3
MP cells	1/3	1/4	0/3
Colo320 (10.9)			
SP cells	2/2	1/2	1/2
MP cells	2/2	2/2	1/2
Lovo (9.3)			
SP cells	0/1	1/1	0/1
MP cells	1/1	0/1	0/1
KM12LM (9.1)			
SP cells	1/2	2/2	1/1
MP cells	1/2	2/2	1/1

MP, main population; SP, side population.
*Tumor initiating ability is expressed as the ratio of tumor-initiation to injection.
†The tumor initiation abilities were evaluated at day 42 after injection of the indicated number of cells.

lon SP cells. We performed a cell survival study of colon cancer SP cells using the chemotherapeutic agents irinotecan and etoposide. The SW480 and HCT15 SP cells were more resistant to both irinotecan and etoposide than were MP cells (Figure 3, A and B). This finding is consistent with findings for CSCs/TICs derived from other organs.[22,24]

Expression of HLA and Tumor-Associated Antigens in SP Cells

Because CTLs recognize tumor-associated antigen (TAA)-derived antigenic peptides presented by HLA class I molecules, expression of HLA class I molecules is essential for activation of CTLs. Several types of malignancies have been reported to lose the expression of HLA class I molecules through various mechanisms and so escape CTL attack.[18] We therefore evaluated the expression of HLA class I molecules and TAA. We assessed the differences of HLA class I and HLA-A24 expression between SP cells and MP cells by flow cytometry. Because ELISA study has revealed that HCT15 cells lack B2M because of gene mutations of *B2M*,[19] we transduced wild-type B2M cDNA into HCT15 cells and so established HCT15-B2M cells. The SW480, HT29, and HCT15-B2M SP cells showed HLA class I and HLA-A24 expression at the same level as MP cells (Figure 4, A and B). Furthermore, we assessed the expression of one of the colon cancer TAAs, CEP55, by both RT-PCR and real-time PCR (Figure 2, A and B). Both SP cells and MP cells derived from SW480, HT29, and HCT15-B2M expressed CEP55 mRNA at the same level. These data raised the possibility that SP cells are also sensitive to CTLs specific for the CEP55-derived antigenic peptide. Because both SP cells and MP cells expressed CEP55 mRNA at the same level, this appeared to be an ideal target for comparing the susceptibilities of SP cells and MP cells to CTLs.

Susceptibility of CSCs/TICs to CTLs, in Vitro *and* in Vivo

We had previously established CTL clone 41, which is specific for the cancer-related, antigen-derived, HLA-A24-restricted peptide Cep55/c10orf3_193(10).[10] CTL clone 41 recognized CEP55-positive and HLA-A24-positive cancer cells, as described previously.[10,11] In the present study, we used a ^{51}Cr release assay to examine whether CTL clone 41 can recognize SP cells. All SP cells derived from SW480, HT29, and HCT15-B2M showed susceptibility to CTL clone 41 at the same level as the MP cells and the presorted bulk cell lines (Figure 4C). This indicates that the colon CSCs/TICs were sensitive to CTLs.

To analyze the cytotoxic activity of the CTL clone *in vivo*, we performed a Winn assay. SW480 SP cells with or without CTL clone 41 were injected into the backs of NOD/SCID mice subcutaneously. CTL clone 41 significantly inhibited the tumorigenicity of SW480 SP cells (Figure 4D). Because CTLs were injected at the same time and place as the SP cells in the Winn assay, we could not rule out the possibility that SP cells were killed *in vitro*. We

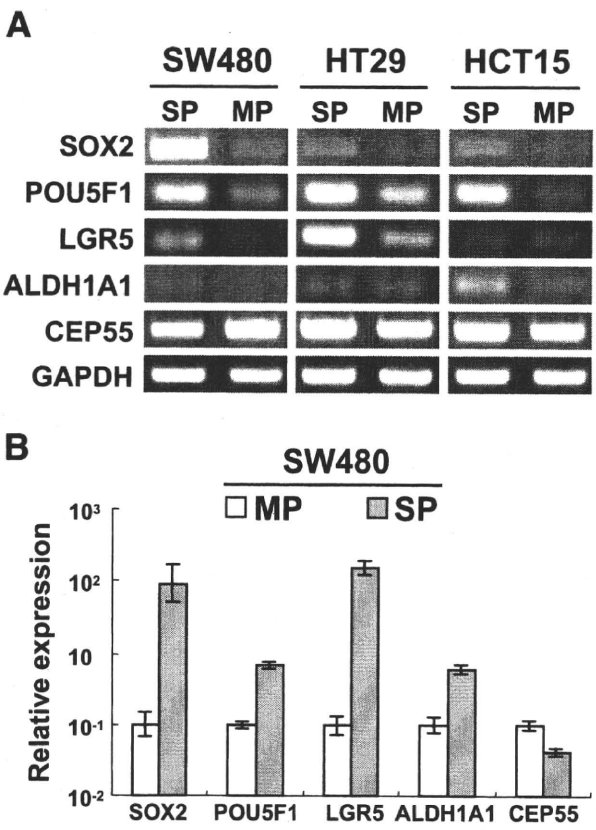

Figure 2. Expression of stem cell markers in SP and MP cells. **A:** mRNAs purified from SW480, HT29, and HCT15 SP and MP cells were analyzed by RT-PCR. **B:** mRNA purified from SW480 SP cells and MP cells were analyzed with real-time PCR. mRNA expression level is relative to MP cells. Data are reported as means ± SD.

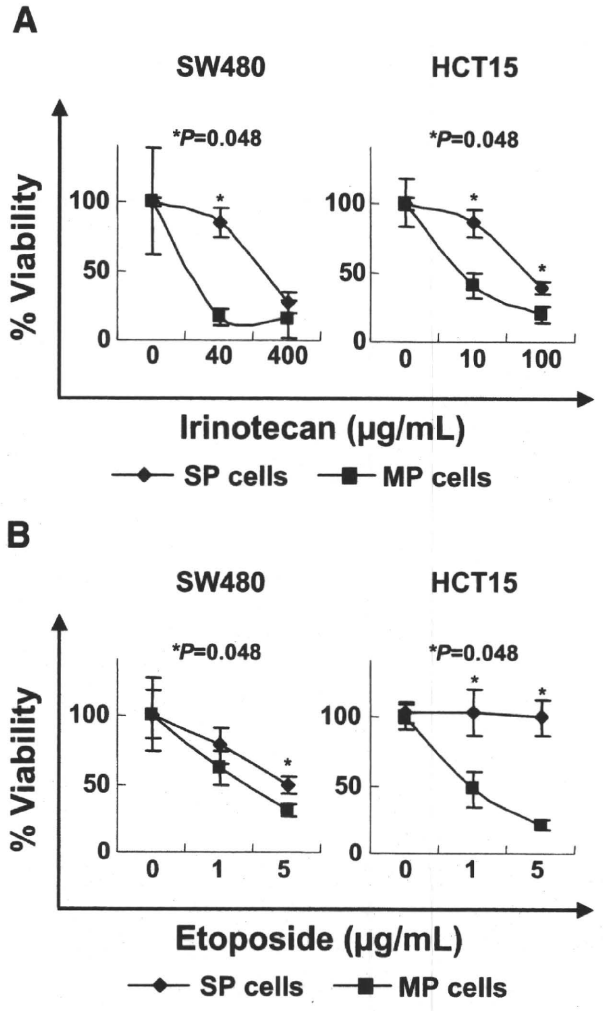

Figure 3. Sensitivity to chemotherapeutic agents. SP cells and MP cells derived from SW480 and HCT15 were incubated in the presence of irinotecan (CPT-11) (**A**) or etoposide (VP-16) (**B**) for 3 days. After incubation, the cell viabilities were measured by WST-1 assay. Data are reported as means ± SD. Differences between SP cells and MP cells were examined for statistical significance using the Mann-Whitney *U*-test.

therefore used an adoptive transfer model, as described under *Materials and Methods*. SW480 SP cells were inoculated into the back of NOD/SCID mice subcutaneously. Three weeks later, after confirmation of palpable tumors, CTLs were injected intravenously. Tumors of CTL-injected mice were significantly inhibited in growth, compared with tumors of control mice (Figure 4E). These data indicate that CTLs could recognize CSCs/TICs both *in vitro* and *in vivo*.

Discussion

In the present study, we successfully isolated colon cancer CSCs/TICs as SP cells, using Hoechst 33342 staining. Side population cells were first described by Goodell et al,[12] and CSCs/TICs of several types of malignancies were successfully isolated as SP cells in subsequent studies.[14,20–24] Haraguchi et al[13] isolated SP cells from gastrointestinal cancer cell lines; they reported the gene expression profiles and resistance to chemotherapeutic agents of SP cells derived from liver cancer cell line Huh7, but did not determine their tumorigenicity. Burkert et al[15] found that SP cells derived from gastrointestinal cancers cell lines HT29, HGT101, Caco2, and HRA19a1.1 were not enriched with a CSC/TIC population. In the present study, we were able to isolate SP cells from all six colon cancer cell lines studied (SW480, HT29, HCT15, KM12LM, Lovo, and Colo320). However, in only three of the six cell lines did the SP cells show higher tumorigenicity than MP cells, suggesting that these SP cells were enriched with CSC/TIC populations. Thus, SP cells might not be the definitive phenotype of CSCs/TICs, and confirmation of tumorigenicity in immunodeficient mice is essential for validation of SP cells as a source of CSCs/TICs. In the present study, the SP cells derived from SW480, HCT15, and HT29 cells were confirmed to be enriched with CSCs/TICs. Furthermore, these SP cells expressed stem cell markers, including SOX2, POU5F1 and LGR5, at higher levels than MP cells, suggesting correspondence with CSCs/TICs. Thus, these SP cells would be a useful tool for analysis of colon CSCs/TICs.

In the present study, we evaluated the immunogenicity of colon CSCs/TICs. Colon cancer CSCs/TICs expressed HLA class I molecules, and also CEP55, which is one of the TAAs. Furthermore, colon CSCs/TICs expressed several other TAA-encoding genes (data not shown), including BIRC5 (encoding apoptosis inhibitor survivin), BIRC7 (encoding livin), WT1, CTAG1B (alias *NY-ESO-1*), and MAGEA4. As a novel finding, colon cancer CSCs/TICs were sensitive to CTLs both *in vitro* and *in vivo*. Recently, Todaro et al[25] showed that colon CSCs/TICs were sensitive to γδT cells. Because both CTLs and γδT cells kill target cells through secretion of perforin (encoded by the *PRF1* gene) and granzyme B (encoded by *GZMB*), these observations strongly suggest that CSCs/TICs are sensitive to PRF1- and GZMB-dependent apoptosis. Todaro et al[26] had earlier reported that PROM1-positive ($CD133^+$) colon cancer CSCs/TICs secrete IL-4 in an autocrine manner and upregulate the antiapoptotic proteins CFLAR (c-FLIP), BCL2L1 (Bcl-xL), and PEA15 (PED), thereby gaining resistance to chemotherapeutic agents. Saigusa et al[27] reported that distant recurrence of rectal cancer after chemotherapy was related to the expression of CSC/TIC markers such as PROM1 (CD133), POU5F1 (Oct3/4), and SOX2. These reports support the idea that colon CSCs/TICs are resistant to apoptotic cell death. The fact that immunocytes induce apoptosis in their target cells raises the question of whether colon CSCs/TICs are also sensitive to immunotherapy.

In the present study, and in that of Todaro et al,[25] colon CSCs/TICs were sensitive to perforin- and granzyme B-dependent apoptosis. Thus, both CTLs and γδT cells can be useful tools for colon CSC/TIC targeting therapy. However, because γδT cells do not recognize target cells in an antigen-specific manner, immunotherapy using γδT cells should also recognize the non-CSC/TIC population. Because the number of γδT cells is restricted *in vivo*, it may be in doubt whether γδT cell can recognize colon

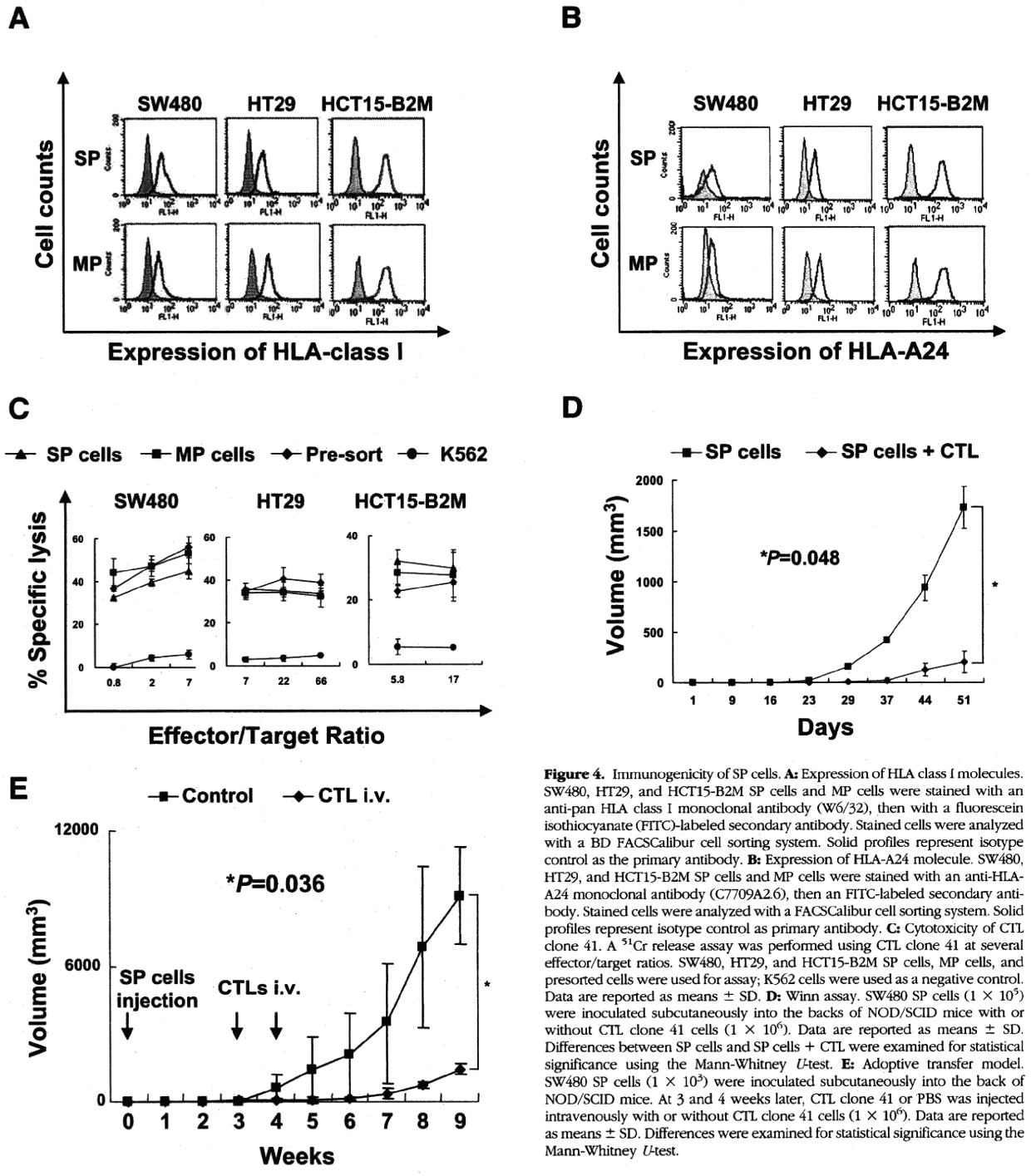

Figure 4. Immunogenicity of SP cells. **A:** Expression of HLA class I molecules. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-pan HLA class I monoclonal antibody (W6/32), then with a fluorescein isothiocyanate (FITC)-labeled secondary antibody. Stained cells were analyzed with a BD FACSCalibur cell sorting system. Solid profiles represent isotype control as the primary antibody. **B:** Expression of HLA-A24 molecule. SW480, HT29, and HCT15-B2M SP cells and MP cells were stained with an anti-HLA-A24 monoclonal antibody (C7709A2.6), then an FITC-labeled secondary antibody. Stained cells were analyzed with a FACSCalibur cell sorting system. Solid profiles represent isotype control as primary antibody. **C:** Cytotoxicity of CTL clone 41. A ^{51}Cr release assay was performed using CTL clone 41 at several effector/target ratios. SW480, HT29, and HCT15-B2M SP cells, MP cells, and presorted cells were used for assay; K562 cells were used as a negative control. Data are reported as means ± SD. **D:** Winn assay. SW480 SP cells (1×10^5) were inoculated subcutaneously into the backs of NOD/SCID mice with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences between SP cells and SP cells + CTL were examined for statistical significance using the Mann-Whitney *U*-test. **E:** Adoptive transfer model. SW480 SP cells (1×10^3) were inoculated subcutaneously into the back of NOD/SCID mice. At 3 and 4 weeks later, CTL clone 41 or PBS was injected intravenously with or without CTL clone 41 cells (1×10^6). Data are reported as means ± SD. Differences were examined for statistical significance using the Mann-Whitney *U*-test.

cancer CSCs/TICs *in vivo* efficiently. Recently, based on a large cohort study, Ogino et al[28] reported that lymphocytic reaction to tumor was associated with longer survival of colorectal cancer patients. They did not analyze the subtypes of infiltrating lymphocytes; however, the findings from this large-scale study strongly support the notion that immune reaction to tumor cells is important for control of the disease.

Wei et al[29] reported recently that glioma-derived CSCs/TICs suppressed T-cell proliferation and activation, and induced T-cell apoptosis through expression of co-stimulatory inhibitory molecule CD274 (B7-H1) and soluble LGALS3 (galectin-3); glioma CSCs/TICs enhance the induction of regulatory T cells. We also observed that SW480 SP cells express higher mRNA of the immunosuppressive cytokine IL-10 than MP cells (data not

shown). Thus, colon CSCs/TICs may have immunosuppressive potential and so inhibit CTL induction. However, colon CSCs/TICs are efficiently killed by CTLs, and colon CSCs/TICs have no influence on the effector phase of CTLs. Thus, adoptive cell transfer of CSC/TIC-specific CTL clones, T-cell-receptor–induced T cells, or peptide vaccination accompanied by an anti-IL-10 monoclonal antibody might be an effective approach for eliminating colon CSCs/TICs.

In the present study, we observed that both colon CSCs/TICs and non-CSCs/TICs were sensitive to CEP55-specific CTLs at the same level. This finding seems reasonable, given that CSCs/TICs express CEP55 mRNA at the same level. Huge numbers of TAAs have already been reported,[30,31] and the next challenge is to identify which TAAs would be the most suitable targets for cancer immunotherapy. According to the manner of expression in CSCs/TICs and non-CSCs/TICs, TAAs can be classified into three categories: i) CSC/TIC-specific antigens, such as SOX2 and ALDH1A1; ii) non-CSC/TIC-specific antigens; and iii) shared antigens, such as CEP55.[32] The frequencies of colon CSCs/TICs are 1% to 10%, and in the present study these cells had 10- to 100-fold higher tumorigenicity than non-CSCs/TICs. It is likely, therefore, that 1% to 10% of colon CSC/TIC populations have almost the same tumorigenic potential as 90% to 99% of the non-CSC/TIC population. To achieve a complete cure of the disease, shared antigens seem to a be reasonable candidate strategy. *In vivo*, however, CTL numbers are limited. Given that 1 L of peripheral blood contains approximately 5×10^9 lymphocytes, there are approximately 5×10^8 CD8 T cells in 1 L of peripheral blood and approximately 3×10^9 CD8 cells in the total volume of peripheral blood in a human adult. If the CTL precursor frequency reaches 0.1% of CD8 T cells in a patient receiving peptide vaccination therapy, then the total peptide-specific CTLs can be calculated as 3×10^6 cells in whole blood. This is not an inconsiderable number. Visible tumors as large as 1 cm diameter contain 1×10^9 tumor cells, and the estimated effector/target ratio (E/T) *in vivo* is 0.003. This ratio may be too low to expect an anti-tumor effect *in vivo*. However, if we focus only on CSCs/TICs, then the effector/target ratio will be improved. For targeting CSCs/TICs with 1% frequency, the effector/target ratio is correspondingly improved (E/T = 0.3). Thus, focusing only on the CSC/TIC population with CSC/TIC-specific antigens seems to be a better approach for advanced cancer cases. For prevention of disease recurrence after treatment, the target cells are likely to be limited, so shared antigens might be a reasonable choice for cancer immunotherapy.

Recently, some research groups have reported that monoclonal antibodies for insulin-like growth factor-1 receptor (IGF-1R), δ-like 4 ligand (DLL4), and CD47 efficiently eliminate colon cancer and leukemia CSCs/TICs.[33–36] These approaches are also fascinating, and a reasonable option for elimination of CSCs/TICs. An antibody is a relatively stable protein, but the half-life in peripheral blood is approximately 2 to 3 weeks, and therefore serial administration is needed to maintain the effects of the antibody. On the other hand, antigenic peptide vaccination can induce specific CTLs as memory cells *in vivo*, such that the specific immunity will last for several years. Thus, peptide vaccination therapy may also be useful for prevention of post-treatment cancer recurrence.

In conclusion, we report here the novel finding that colon cancer CSCs/TICs are as sensitive to CTLs as are non-CSCs/TICs, and that CEP55, a tumor-associated antigen, is a suitable antigen for targeting colon cancer CSCs/TICs.

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