Clinical Cancer Research

Olfactory Receptor Family 7 Subfamily C Member 1 Is a Novel Marker of Colon Cancer–Initiating Cells and Is a Potent Target of Immunotherapy

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Abstract

Purpose: Cancer-initiating cells (CICs) are thought to be essential for tumor maintenance, recurrence, and distant metastasis, and they are therefore reasonable targets for cancer therapy. Cancer immunotherapy is a novel approach to target cancer. In this study, we aimed to establish novel CIC-targeting immunotherapy.

Experimental Design: Colorectal cancer (CRC) CICs were isolated as side population (SP) cells. The gene expression profile of CRC CICs was analyzed by cDNA microarray and RT-PCR. Protein expression of olfactory receptor family 7 subfamily C member 1 (OR7C1) were analyzed by Western blot and immunohistochemical staining. The functions of OR7C1 were analyzed by gene overexpression and gene knockdown using siRNAs. OR7C1-positive cells were isolated by a flow cytometer and analyzed. CTLs specific for OR7C1 peptide were generated, and the antitumor effect was addressed by mice adoptive transfer model.

Results: OR7C1 has essential roles in the maintenance of colon CICs, and the OR7C1-positive population showed higher tumorigenicity than that of the OR7C1-negative population, indicating that OR7C1 is a novel functional marker for colon CIC. Immunohistochemical staining revealed that OR7C1 high expression was correlated with poorer prognosis in CRC patients. OR7C1-derived antigenic peptide-specific CTLs showed specific cytotoxicity for CICs, and an OR7C1-specific CTL clone showed a greater antitumor effect than did a CTL clone targeting all cancer cells in a CTL adoptive transfer mouse model.

Conclusions: OR7C1 is a novel marker for colon CICs and can be a target of potent CIC-targeting immunotherapy. *Clin Cancer Res; 22(13); 3298–309. ©2016 AACR.*

Introduction

Colorectal cancer (CRC) is one of the most common malignancies throughout the world, with more than 140,000 new cases every year in United States (1). Recent progress in CRC treatments has improved the prognosis, but advanced disease with recurrence or distant metastasis is usually incurable and has an unfavorable prognosis (2, 3). Cancer stem–like cells/cancer-initiating cells (CSC/CIC) are defined by their ability of tumor initiation, self-renewal, and differentiation (4). CICs are resistant to standard cancer therapies including chemotherapy and radiotherapy, and they are therefore thought to be responsible for tumor recurrence and metastasis (5,6). CICs are a reasonable target for cancer treatment, and elimination of CICs is essential to cure cancer.

Cancer immunotherapy is a novel approach to target cancer and is expected to become the fourth most widely used cancer therapy after surgery, chemotherapy, and radiotherapy. Identification of the first human tumor-associated antigens (TAAs) has enabled cancer immunotherapy to be performed using antigenic peptides derived from TAAs (7–10). Clinical trials using antigenic peptides derived from TAAs are now being conducted in several facilities, and some trials have shown fascinating and promising results; however, the results of most studies using antigenic peptide vaccination have been disappointing (11). Recently, melanoma-initiating cells (melanoma stem cells) have been isolated as $CD271^+$ cells, and $CD271^+$ melanoma cells lack the expression of melanoma antigens including TYR, MART1, and MAGEs. Low expression levels of melanoma antigens in melanoma-initiating cells are thought to be the reason for resistance to

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Translational Relevance

Cancer stem–like cells (CSC)/cancer-initiating cells (CIC) are a small subpopulation of cancer cells endowed with higher tumorigenicity and are resistant to standard therapy. Cancer immunotherapy is a novel approach to target cancer, and we aimed to establish CIC-targeting immunotherapy. OR7C1 is a novel marker of CRC CICs and has an essential role in the maintenance of CICs. OR7C1 is a novel cancer testis antigen, and can be a target of CTLs. Targeting CICs by OR7C1-specific CTLs clones showed potent antitumor effects. These observations indicate that treatment-resistant CICs can be targeted by CTLs and antigenic peptides derived from OR7C1 are promising candidates for CIC-targeting immunotherapy.

peptide vaccination therapies using melanoma antigens (12). And recent studies indicate that CICs may contribute to make immunosuppressive microenvironment (13). We therefore hypothesized that CIC-targeting immunotherapy might improve the efficiency of cancer immunotherapy. To address the feasibility of CIC-targeting immunotherapy, we isolated CRC CICs as side population (SP) cells by Hoechst 33342 staining and found that SP cells are susceptible to CTLs as are non-CIC main population (MP) cells (14). We also reported that CICs of malignant fibrous histiocytoma could be recognized by autologous CTLs derived from tumor-infiltrating lymphocytes (15). Thus, CTLs can recognize treatment-resistant CICs.

In this study, we identified a novel CIC antigen in CRC, olfactory receptor family 7 subfamily C member 1(OR7C1). A gene knockdown study using siRNAs and an OR7C1 overexpression model showed that OR7C1 has a role in the maintenance of CICs of CRC. A high expression level of OR7C1 was shown to be a poor prognostic factor in the immunohistochemical staining of OR7C1. An OR7C1-specific CTL clone showed significantly stronger antitumor effect than that of a shared antigen-specific CTL clone in an *in vivo* CTL adoptive transfer model. These results indicate that the CIC antigen OR7C1 is a novel marker for CRC CICs and is a promising target for CIC-targeting immunotherapy.

Materials and Methods

Cell lines, tissue samples, and blood samples

Colon adenocarcinoma cell lines SW480 (HLA-A*0201/2402, B*0702/1508, Cw*0702/0704), HT29 (HLA-A1/24), HCT15 (HLA-A*0201/2402, B*08/3501, Cw*07/04) were kind gifts from Dr. K. Imai (Sapporo Medical University, Sapporo, Japan) at 1999. All cells was cultured in DMEM (Sigma Chemical Co.) supplemented with 10% FBS (Life Technologies, Inc.). HCT15-B2M, a stable transfectant of HCT15 cells with *β2 microglobulin* cDNA, was cultured in DMEM supplemented with 10% FBS and 10 μg/mL puromycin (Sigma). SW480, HT29, and HCT15 cells were authenticated by us using the PCR-SSCP (Single Strand Conformation Polymorphism) method as described previously (16). Erythroleukemia cell line K562 was purchased from ATCC and was cultured in RPMI1640 (Sigma) supplemented with 10% FBS. HLA-A*2402 stably transfected TAP-deficient cell line T2 A-A*2402 (T2-A24) cell was a kind gift from Dr. K.Kuzushima (Aichi Cancer Center Research Institute, Nagoya, Japan) and was cultured in RPMI1640 supplemented with 10% FBS and 0.8 mg/mL G418 (Life Technologies, Inc.). No specific authentication of K562 cells and T2-A24 cells were performed.

Three pairs of frozen colorectal adenocarcinoma and corresponding non-neoplastic colorectal tissues, which were used for RT-PCR, were obtained from surgically resected tissues removed at Kushiro City General Hospital. Sixty-seven specimens, used for immunohistochemical staining of OR7C1, were obtained from surgical resection tissues at Department of Gastroenterological Surgery I, Hokkaido University Graduate School of Medicine (Sapporo, Japan). Normal tissues used for immunohistochemical staining of OR7C1 were obtained from pathologic dissection at the Department of Pathology, Sapporo Medical University School of Medicine (Sapporo, Japan). To obtain CTL clones, we used blood from three healthy volunteer donors and three colon cancer patients at Higashi Sapporo Hospital (Sapporo, Japan). We obtained informed consent from all patients and volunteer donors according to the guidelines of the Declaration of Helsinki.

SP analysis and flow cytometry analysis of OR7C1

SP analysis and flow cytometry analysis of OR7C1 were performed as described previously and as described in the Supplementary Materials and Methods (14,17).

Xenograft model

Xenograft transplantation model was performed as described previously (18). Cancer stem cell frequency was calculated by web program Extreme Limiting Dilution Analysis (ELDA; http://bioinf.wehi.edu.au/software/elda/) software (19).

cDNA microarray analysis

Total RNAs (3 μg) derived from SW480 SP cells and SW480 MP cells were reverse transcribed to generate double-stranded cDNA and labeled using Cy3-cytidine or Cy5-cytidine. Then the labeled cRNAs were hybridized to a oligonucleotide microarray (Panorama Human Micro Array, Sigma) and scanned by Genepix 4000B Microarray Scanner. Microarray raw data and processed data have been deposited in the Array Express database (E-MTAB-2605).

RT-PCR analysis and quantitative RT-PCR analysis

RT-PCR analysis was performed as described previously and as described in Supplementary Materials and Methods (14,20).

Sphere formation assay

To assay sphere formation efficiency, 10^3 cells were plated in ultra-low attachment 6-well plates (Corning Incorporated Life Sciences) and cultured in DMEM/F12 (Life Technologies, Inc.,) supplemented with 20 ng/mL EGF and 20 ng/mL basic fibroblast growth factor (R&D Systems). Cells were incubated in a 5% CO_2 incubator for 2 weeks, and the number of spheres was counted. The data was shown in (number of spheres) × 100/(numbers of seeded cells)%.

siRNA transfection

An *OR7C1* gene knockdown experiment was performed using siRNA. OR7C1 siRNA duplexes were designed and synthesized using the BLOCK-it RNAi designer system (Life Technologies, Inc.). The oligonucleotide encoding OR7C1 siRNA1 was 5′-TATACTTTGCAACTGGCGTCCTGGG-3′, siRNA2 was 5′-CCCAAGAATTTCTCCTCCTGGGATT-3′, siRNA3 was 5′-CAGAGATTCAGTTCATTCTCTTTGG-3′. Cells were seeded at

50% confluence and transfections were carried out using Lipofectamine RNAi max (Life Technologies, Inc.) in Opti-MEM according to the manufacturer's instructions.

Synthetic peptides and peptide-binding assay

Five peptides, OR7C1_34(10) (MYLVTFTGNL), OR7C1_93 (10) (TYAGCLSQIF), OR7C1_277(9) (MYTMVTPML), OR7C1_251(10) (FYGTGFGVYL) and OR7C1_131(9) (HYTVIMNPQL), were designed from the amino acid sequence of OR7C1 based on the HLA-A24–binding motifs. All of the peptides were synthesized at and purchased from Life Technologies, Inc. Peptides were dissolved in DMSO and stored at −80°C before use.

Peptide binding affinity to an HLA-A24 was assessed by HLA-A24 stabilization assay as described previously (21).

Preparation of APCs and CD8-positive T cells from PBMCs

PBMCs were isolated from three healthy volunteers and three colon cancer patients as described previously and as described in Supplementary Materials and Methods (21, 22).

CTL induction and establishment of CTL clones

CTL induction was performed as described previously (23). To obtain CTL clones, standard limiting dilution was performed as described previously (22). The lytic activity of CTLs was tested by a ^{51}Cr release assay and IFN-γ ELISpot assay as described previously (21, 22).

CTL adoptive transfer

NOD/SCID mice were inoculated subcutaneously in the back with 1×10^3 SW480 SP cells. Three weeks later, when the tumor had started to become palpable, 5×10^4 OR7C1_ 93(10)-specific CTL clone cells or 5×10^4 Cep55/c10orf3_ 193(10)-specific CTL clone cells or PBS were injected intravenously. The same adoptive transfer procedure was performed 4 weeks after inoculation with SP cells. Tumor size was assessed weekly.

Statistical analysis

Data for samples in the xenograft model, cytotoxicity assay and CTL adoptive transfer model were analyzed by Student *t* test, with $P < 0.05$ conferring statistical significance. Statistical analyses were performed with SPSS (version 11 for Windows; SPSS Inc), and GraphPad Prism (version 4.0 for Windows; GraphPad Software Inc) was used for plotting Kaplan–Meier curves. Pearson χ^2 square tests were used to determine the significance of associations between characteristic variables. Survival rates were calculated using Kaplan–Meier method, and differences between groups were tested using the log-rank test.

Results

Identification of a novel CIC antigen, OR7C1

We previously reported that side population (SP) cells derived from SW480, HT29. and HCT15 CRC cells showed high tumor-initiating ability, high expression levels of stem cell markers including *SOX2*, *POU5F1*, and *Nanog*, and a high level of resistance against chemotherapeutic agents compared with those of MP cells (14). In this study, we isolated SP cells from SW480, HT29, and HCT15 cells as sources of CRC CICs to screen for novel CIC-related antigens. SP cells derived from SW480, HT29, and HCT15 cells showed higher expression levels of stem cell markers including *SOX2*, *POU5F1*, and *LGR5*, higher tumor-initiating ability higher levels of tumor-initiating cells and higher sphere formation ability than those of MP cells (Supplementary Fig. S1A–S1D; Table 1). CRC CIC–related genes were screened by a cDNA microarray using mRNAs of SP cells and MP cells derived from SW480 cells. Approximately 600 genes showed more than 2-fold higher expression levels than those in MP cells (Supplementary Table S1). We further investigated the expression profiles of candidate genes by RT-PCR. Most of the genes showed ubiquitous expression in normal organs; however, one SP-specific gene, olfactory receptor, family 7, subfamily C, member 1 (*OR7C1*), showed expression only in the testis among adult normal tissues and fetal tissues (Fig. 1A). *OR7C1* mRNA was preferentially expressed in SP cells derived from SW480, HT29, and HCT15 cells rather than in MP cells (Fig. 1B). Therefore, OR7C1 is a novel cancer/testis (CT) antigen and is also a novel CIC antigen. The expression of *OR7C1* mRNA in human primary carcinoma tissues was examined by RT-PCR. *OR7C1* mRNA was expressed in all primary CRC tissues, but its expression was not detected in adjacent normal colon mucosa tissues. Stem cell markers (*SOX2*, *POU5F1*, *LGR5*) were also detectable in only CRC tissues (Fig. 1C). We then evaluated the expression level of OR7C1 protein using a polyclonal anti-OR7C1. The specificity of the OR7C1 antibody was confirmed by Western blotting using 293T cells transiently transfected with OR7C1 DNA (Supplementary Fig. S1E). OR7C1 protein was preferentially expressed in SP cells derived from SW480, HT29, and HCT15 cells rather than in MP cells as shown by Western blot analysis (Fig. 1D). Immunohistochemical staining revealed that OR7C1-positive cells were present in primary spermatocytes, secondary spermatocytes, spermatid and sperm of the testis. OR7C1 was not detected in normal colon tissues (Fig. 1E).

To investigate the expression of OR7C1 protein in human CRC tissues, we stained 67 human CRC tissues using anti-OR7C1 antibody and 56 cases showed OR7C1-positive staining (positivity: 83.5%). The clinicopathologic status of each of the patients is summarized in Supplementary Table S2. No significant correlations with OR7C1 staining were found for median age of the patients, gender, clinical stage, site of the lesion and pathologic grade (Table S2). We classified the OR7C1 staining into four groups according to the staining intensity of cancer cells (intensity score (IS): IS 0: negative, IS 1+: weakly positive, IS 2+: moderately positive, IS 3+: strongly positive), and IS 0 and 1+ were classified as low OR7C1 IS and IS 2+ and 3+ were classified as high OR7C1 IS (Fig. 1F). The 10-year survival rates of low OR7C1 IS and high OR7C1 IS were 100% and 63.1%, respectively. The high OR7C1 IS group showed remarkably poorer prognosis than that of the low OR7C1 IS group (Fig. 1G, $P<0.001$). Strikingly, all low OR7C1 IS patients were alive at the time of investigation. We wanted to determine whether OR7C1-intensity would be a prognosis factor, but we were not able to perform the univariate analysis because all of the low IS group patients were alive.

Next, we investigated overall survival according to OR7C1-positive levels. OR7C1-positive rate showed a moderate positive correlation with OR7C1 staining intensity (Supplementary Fig. S1F, $R = 0.576$, $P = 0.0002$). The cases were classified into four groups according to the OR7C1-positive proportion [proportion score (PS): PS: negative, PS 1: <10%, PS 2: 10%–50%, PS3: >50%). The 10-year survival rates of patients in the OR7C1 PS1, PS2, and PS3 groups were 86.5%, 89.0%, and 60.0%, respectively, and the OR7C1PS 3 group showed poorer prognosis than that in the OR7C1 PS1 or PS2 group (Supplementary Fig. S1G). Univariate

Table 1. Tumor-initiating ability of colon cancer cell line

Cells			Injected cell number 10^4	10^3	10^2	10^1	CIC Frequency	95% CI	*P*
SW480	SP cells		7/7	6/8	5/5	1/2	1/327	137–781	0.00000129[a]
	MP cells		6/8	5/7	1/5	0/2	1/3,522	1,568–7,909	
	Mock		0/5	0/5	0/5	0/5	–	18,544-	0.000123[a]
	OR7C1-Overexpression		5/5	1/5	0/5	0/5	1/3,055	1,099–8,498	
	SP cells	Control si	5/6	11/11	2/6	0/6	1/1,176	547–2,526	
		si1	3/6	1/11	0/6	0/6	1/13,583	4,982–37,035	0.00000206[a]
		si2	n.d.	1/5	n.d.	n.d.	1/4,482	629–31,944	0.12
		si3	n.d.	1/5	n.d.	n.d.	1/4,482	629–31,944	0.12
		$OR7C1^+$	n.d.	5/5	2/5	1/5	1/143	46.4–446	0.0000026[a]
		$OR7C1^-$	n.d.	0/5	0/5	0/5	–	1,853.1-	
HCT15	Mock		5/5	4/5	2/5	0/5	1/463.3	176.6–1,217	0.00134[a]
	OR7C1-Overexpression		5/5	5/5	5/5	0/5	1/42.2	16–113	
	SP cells	control si	n.d.	5/5	n.d.	n.d.			n.d.
		si1	n.d.	5/5	n.d.	n.d.			
		si2	n.d.	5/5	n.d.	n.d.			
		si3	n.d.	4/5	n.d.	n.d.			
		$OR7C1^+$	n.d.	5/5	3/5	1/5	1/92.5	32.9–262	0.000153[a]
		$OR7C1^-$	n.d.	1/5	2/5	0/5	1/1,633.3	478.7–5,576	
HT29	Mock		5/5	4/5	3/5	2/5			n.d.
	OR7C1-Overexpression		5/5	5/5	5/5	5/5			
	SP cells	Control si	n.d.	5/5	n.d.	n.d.			n.d.
		si1	n.d.	2/5	n.d.	n.d.			
		si2	n.d.	4/5	n.d.	n.d.			
		si3	n.d.	1/5	n.d.	n.d.			
		$OR7C1^+$	n.d.	5/5	5/5	3/5	1/11.4	3.93–35.3	0.544
		$OR7C1^-$	n.d.	5/5	5/5	2/5	1/18.9	6.1–61.4	

NOTE: The analysis was completed 10 weeks following injection. Data are numbers of tumor-initiation/numbers of injections. Differences of estimated frequencies of CICs were analyzed by χ^2 test.
Abbreviation: CI, confidence interval.
[a]$P < 0.05$.

analysis showed that OR7C1 PS3 colorectal carcinoma is a risk factor for poor prognosis after surgical resection. (Table 2; relative risk: 5.08, $P = 0.013$).

A recent study showed that increased expression of ALDH1 was associated with poor clinical outcome in colorectal cancer cases (24). We thus investigated the expression of ALDH1 and classified the expression into IS0-IS3. The positive frequencies of OR7C1 and ALDH1 showed a statistically significant correlation (Supplementary Fig. S1H, $R = 0.426$, $P < 0.01$). We found that the prognosis of ALDH1 strong intensity (IS2 and IS3) showed a tendency to be related to poorer prognosis but did not reach statistical significance (Fig. 1H). Univariate analysis showed that ALDH1 highly positive (PS3) colorectal carcinoma is a risk factor for poor prognosis after surgical resection (Table 2; relative risk: 4.07, $P = 0.029$). Finally, we performed the multivariate analysis, and only OR7C1 high positivity (PS3) was an independent risk factor for poor prognosis (Table 2).

OR7C1 has a role in the maintenance of CRC CICs

To evaluate the functions of OR7C1 in colon cancer cells, we generated OR7C1-overexpressed SW480 cells (SW480-OR7C1 cells). The expression of *OR7C1* mRNA was confirmed by quantitative reverse transcription PCR (qRT-PCR; Fig. 2A). The expression levels of stem cell markers including *SOX2*, *LGR5*, and *POU1F5* were higher in SW480-OR7C1 cells than in SW480 cells (Fig. 2A). The ratio of SP cells in SW480-OR7C1 cells was higher than that in SW480 cells (Fig. 2B). SW480-OR7C1 cells showed higher sphere-forming ability than that of SW480 cells (Fig. 2C). The tumorigenicity of SW480-OR7C1 cells *in vivo* was examined by injecting cells into NOD/SCID mice. Tumor formation was initiated with as few as 10^3 SW480-OR7C1 cells. On the other hand, SW480 cells did not initiate the formation of a tumor even with injection of 10^4 cells (Fig. 2D; Table 1). To generalize these observations, the same experimental procedure was performed by using two additional CRC cell lines (HCT15 cells and HT29 cells), and similar results were obtained (Table 1; Supplementary Fig. S2). The frequency of CICs was significantly higher in SW480-OR7C1 cells than in SW480 cells (Table 1). These results indicate that CRC cells with overexpression of the *OR7C1* gene have properties of CIC.

To confirm the functions of OR7C1 in CRC cells, we performed *OR7C1* gene knockdown experiments using SW480 SP cells. We designed three different siRNAs and confirmed the suppression of *OR7C1* mRNA by siRNAs (Fig. 2E). Gene knockdown of *OR7C1* decreased the expression levels of stem cell markers including *SOX2*, *LGR5*, and *POU1F5* (Fig. 2E) and the percentage of SP cells in SW480 cells (Fig. 2F). Gene knockdown of *OR7C1* significantly decreased sphere-forming ability of SW480 SP cells (Fig. 2G). We evaluated the tumorigenicity of SW480 SP cells in which OR7C1 mRNA was knocked down by siRNAs. Control siRNA-transfected SW480 cells initiated tumor formation with as few as 10^2 cells, whereas OR7C1 siRNA-transfected SW480 cells needed 10^3 cells to initiate tumor formation (Table 1). The tumor was significantly smaller with OR7C1 siRNA-transfected SW480 SP cells than with control siRNA-transfected cells (Fig. 2H). Similar results were obtained by SP cells derived from two additional CRC cell lines, HCT15 and HT29 (Table 1 and Fig. S3). These results indicate that *OR7C1* has an essential role in the maintenance of CRC CICs.

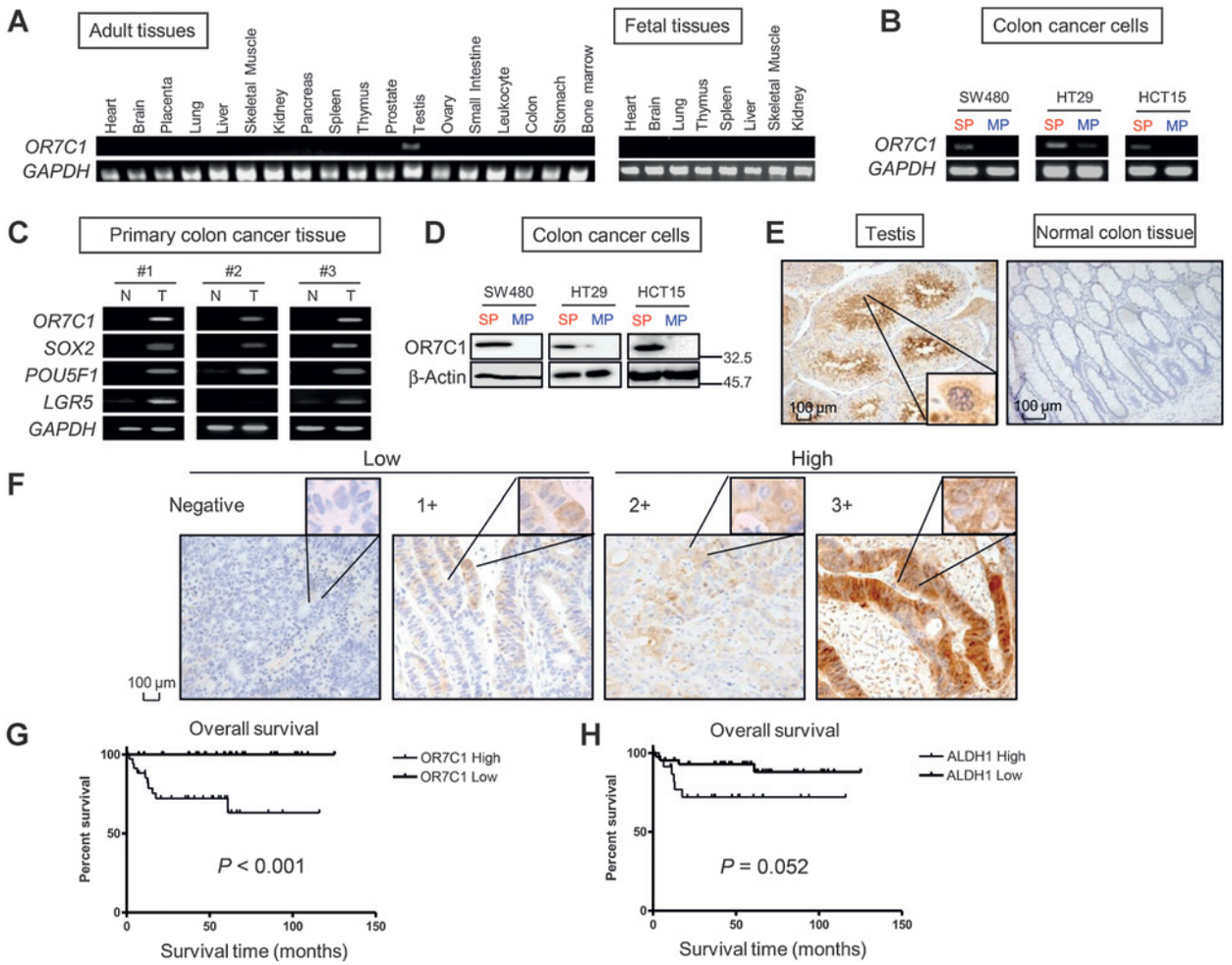

Figure 1.
Identification of a novel cancer/testis antigen, OR7C1. A, RT-PCR in normal adult and fetal human tissues. The expression of *OR7C1* mRNA was evaluated by RT-PCR using cDNAs of adult organs (heart, brain, placenta, lung, liver, skeltal muscle, kidney, pancreas, spleen, thymus, prostate, testis, ovary, small intestine, leukocyte, colon, stomach, and bone marrow) and fetal organs (heart, brain, lung, thymus, spleen, liver, skeletal muscle, and kidney). *GAPDH* was used as a positive control. B, RT-PCR in human colon cancer cell lines. *GAPDH* was used as a positive control. C, RT-PCR of OR7C1 and CSCs/CICs markers in primary colon cancer tissues. T, colorectal adenocarcinoma tissues; N, non-neoplastic colorectal tissues. *GAPDH* was used as a positive control. D, Western blot analysis of OR7C1 in human colon cancer cell lines. β-Actin was used as a positive control. E, immunohistochemical staining of OR7C1. Human testis and normal colon tissues were immunohistochemically stained using an anti-OR7C1 antibody. Brown color indicates positive staining of OR7C1. Magnification, ×100. F, immunohistochemical staining of OR7C1 in CRC tissues. The tissues were classified into four groups according to the intensity of OR7C1 staining. Intensity score 0, negative; intensity score 1+, weak positive; intensity score 2+, moderately positive; intensity score 3+, strong positive. G, Kaplan–Meier curves for overall survival depending on OR7C1 intensity. TheOR7C1 high IS group included IS2 and IS3, and the OR7C1 low IS group include IS0 and IS1. H, Kaplan–Meier curves for overall survival depending on ALDH1 intensity.

OR7C1 is a novel marker of CRC CICs

OR7C1 overexpression and knockdown experiments indicated that OR7C1 has a role in CRC CICs, and we therefore characterized OR7C1-positive ($OR7C1^+$) cells derived from CRC cells. The polyclonal antibody we used in this study is specific for the putative extracellular domain of OR7C1, and we evaluated staining of CRC cells by flow cytometry. OR7C1-overexpressed SW480 cells were stained as a positive control and analyzed by flow cytometry. The specificity of OR7C1 staining was also confirmed by flow cytometry in 293T cells transiently transfected with OR7C1 (data not shown), indicating that the polyclonal antibody could detect OR7C1 protein by flow cytometry analysis. A cell population with positive OR7C1 staining was detected in OR7C1-overexpressed SW480 cells, and the percentage of $OR7C1^+$ cells was 95.0% (Fig. 3A). We further analyzed CRC cells and found that approximately 2.0% $OR7C1^+$ cells were present in SW480, HCT15, and HT29 cells (Fig. 3B). A higher level of *OR7C1* gene expression in $OR7C1^+$ cells was confirmed by qRT-PCR (Fig. 3C). The expression of stem cell markers in $OR7C1^+$ cells was analyzed by qRT-PCR, and $OR7C1^+$ cells showed higher expression levels of stem cell markers including *SOX2*, *LGR5*, and *POU1F5* than the levels in OR7C1-negative ($OR7C1^-$) cells (Fig. 3C). We then investigated whether the $OR7C1^+$ cells have properties of CICs. Xenograft transplantation of 10^3 $OR7C1^+$ cells and 10^3 $OR7C1^-$ cells derived from SW480, HCT15, and HT29 cells revealed that the tumor derived from $OR7C1^+$ cells was significantly larger than

Table 2. Multivariate analysis with Cox proportional hazards model for overall survival

	Total cases ($n = 67$)			
	Univariate analysis Risk ratio label		Multivariate analysis Risk ratio label	
Factor	**(95% CI)**	***P***	**(95% CI)**	***P***
OR7C1 Stain				
High intensity (+2/+3)†1	N.C.	–		
Wide area (≧50%)†2	5.08 (1.41–18.34)	0.013[a]	5.08 (1.41–18.34)†2	0.013[a]
ALDH1 Stain				
High intensity (+2/+3)†1	3.29 (0.92–11.75)	0.066	3.29 (0.92–11.75)†1	0.066
Wide area (≧50%)†2	4.07 (1.16–14.31)	0.029[a]		
Age		1.01 (0.95–1.06)	0.88	
Sex				
Female	–	–		
Male	1.08 (0.31–3.76)	0.89		
Advanced stage (stage III/IV)		2.71 (0.70–10.51)	0.15	
Location				
Cecum	1.49 (0.19–11.80)	0.70		
Ascending	0.46 (0.04–5.07)	0.53		
Transverse	1.75 (0.18–16.90)	0.63		
Descending	0.69 (0.04–10.98)	0.79		
Sigmoid/Rectum	0.51 (0.05–4.90)	0.56		
Differentiation				
High	–	0.46		
Medium	2.32 (0.62–8.68)	0.21		
Low	1.65 (0.18–14.87)	0.66		

NOTE: Multivariate analysis was done using stepwise method, and right lane shows only results of the final model. Univariate analysis of OR7C1-intensity cannot be calculated, as all of the low intensity cases are alive. ([a]$P < 0.05$). Each of stained intensity and stained area of OR7C1/ALDH1 were calculated as independent model, showed in Table as †1 or †2.

Abbreviation: CI, confidence interval.

that derived from OR7C1$^-$ cells (Fig. 3D). Xenograft transplantation of as few as 10^1 SW480 OR7C1$^+$ cells initiated tumor formation in 1 of 5 mice, whereas SW480 OR7C1$^-$ cells did not initiate tumor formation even with injection of 10^3 cells, and the percentages of CICs were higher in OR7C1$^+$ cells than that in OR7C1$^-$ cells (Table 1). HCT15 OR7C1$^+$ cells initiated tumor formation in 1 of 5 mice with injection of 10^1 cells, whereas HCT15 OR7C1$^-$ cells needed 10^2 cells to initiate tumor formation. On the other hand, no difference was observed in initiating tumor formation by HT29 OR7C1$^+$ cells and HT29 OR7C1$^-$ cells. OR7C1$^+$ cells showed higher proliferation ability *in vitro* (Fig. 3E) and the higher sphere-forming ability (Fig. 3F) than those of OR7C1$^-$ cells. These results indicate that OR7C1$^+$ colorectal cancer cells have characteristics of CICs and that OR7C1 is a novel marker for CRC CICs.

Establishment of CIC-targeting immunotherapy using antigenic peptide derived from OR7C1

OR7C1 is a novel CIC antigen and it has a role in the maintenance of CRC CICs. Furthermore, OR7C1 is a novel cancer/testis (CT) antigen, thus OR7C1 is a promising target of CIC-targeting cancer immunotherapy. We induced OR7C1-specific CTLs derived from HLA-A*2402–positive colon cancer patients and healthy volunteer donors to evaluate the immunogenicity of OR7C1. We designed and synthesized OR7C1 candidate peptides carrying HLA-A*2402, a frequent HLA allele in Asian and other areas, binding motifs, as described in a previous report (21). HLA-A24-binding ability was assessed by an HLA-A24 binding assay, and all of tested peptides showed higher binding affinity than that of the negative control peptide (Table S3).

PBMCs of 5 CRC patients and 3 healthy donors were stimulated with mixture of 5 peptides, and reactivity for each peptide was evaluated by an IFN-γ ELISpot assay and a ^{51}Cr release assay. The reactivity for each peptide is shown in Supplementary Fig. S4A and S4B, and OR7C1_277(9), OR7C1_34(10), OR7C1_251(10), and OR7C1_93(10) peptides were shown to be immunogenic epitopes. Among the tested peptides, OR7C1_93(10) showed highest CTL induction rates, inducing specific CTLs from 6 of 8 cases as detected by ELISpot (patients: 4, healthy donors: 2) and 5 of 8 cases by ^{51}Cr release assay (patients: 3, healthy donors: 2; Supplementary Fig. S4A and S4B). Therefore, OR7C1_93(10) peptide might be the most immunogenic epitope of OR7C1, and we thus generated an OR7C1_93(10)-specific CTL clone for further analysis (clone: #23). The OR7C1_93(10)-specific CTL clone exhibited cytotoxic activity for T2-A24 cells pulsed with OR7C1_93(10) peptide but not for T2-A24 cells without the peptide or K562 cells (Fig. 4A). To confirm that the OR7C1_93(10) peptide is endogenously expressed in CRC CICs, we performed a cytotoxicity assay using SP and MP cells derived from SW480, HCT15-B2M, and HT29 cells. The OR7C1_93(10)-specific CTL clone showed greater cytotoxicity for SP cells derived from SW480 cells, HCT15-B2M cells, and HT29 cells than for MP cells derived from SW480 cells, HCT15-B2M cells, and HT29 cells and for HLA-A*2402$^-$OR7C1$^-$ K562 cells (Fig. 4B). Moreover, the CTL clone showed significantly greater cytotoxicity for OR7C1$^+$ cells derived from SW480 cells, HCT15-B2M cells, and HT29 cells than for OR7C1$^-$ cells derived from SW480 cells, HCT15-B2M cells, and HT29 cells (Fig. S4C). The cytotoxicities for SP cells and OR7C1$^+$ cells derived from HCT15-B2M cells were blocked by an anti-HLA class I mAb (Fig. 4C and Supplementary Fig. S4D). These results indicate that OR7C1_34(10), OR7C1_93(10), OR7C1_251(10), and OR7C1_277(9) peptides are immunogenic and that OR7C1_93(10) peptide is endogenously processed and presented on the cell surface of colon CICs.

The OR7C1-specific CTL clone efficiently recognized isolated CICs. We then examined whether the CTL clone can recognize CICs specifically in CIC and non-CIC mixed conditions. OR7C1-specific CTL clone #23 was added to SW480 colon cancer cell

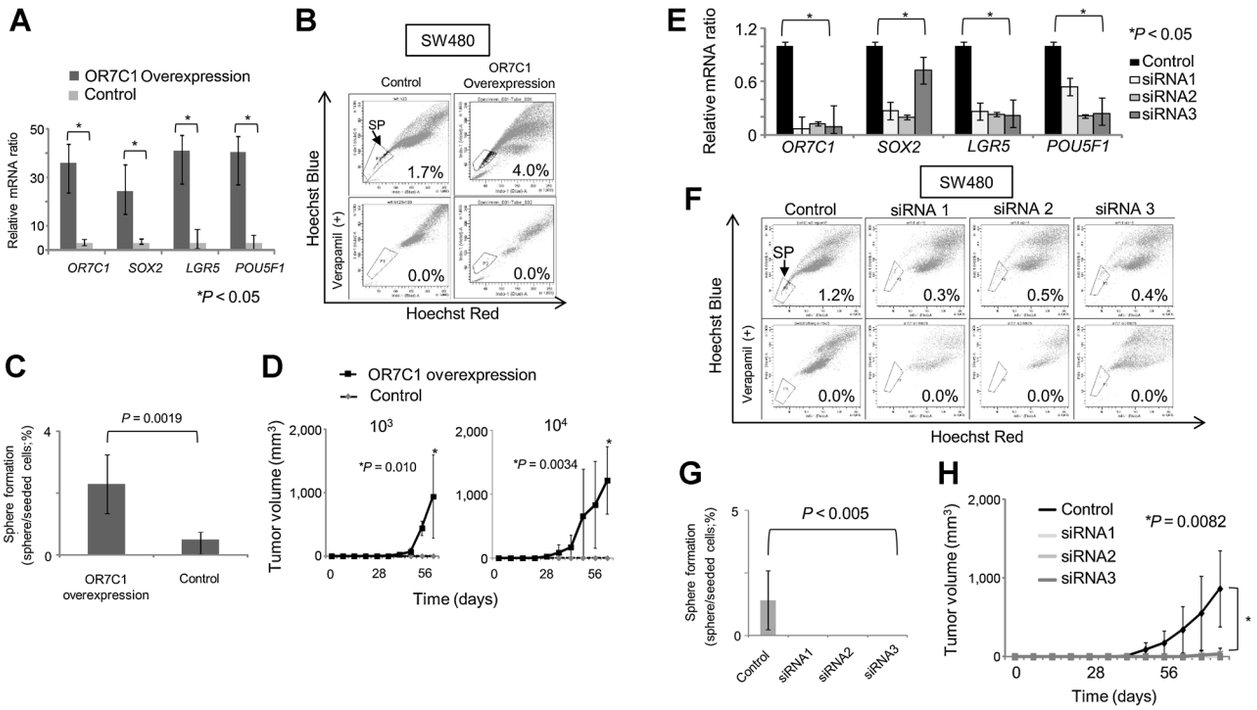

Figure 2.

Functional analysis of *OR7C1*. A, qRT-PCR of OR7C1 and CIC markers. The expression of *OR7C1, SOX2, LGR5*, and *POU5F1* mRNAs was examined by qRT-PCR. OR7C1-overexpressed SW480 cells (OR7C1 overexpression) and mock plasmid–transfected SW480 cells (control) were used. Relative expression in control cells is shown. Data are shown as means ± SD. B, SP ratios of OR7C1-overexpressed SW480 cells. C, sphere formation assay. Data are shown as ± SD. D, tumor growth of SW480 OR7C1-overexpressed cells and control cells. 10^1,10^2,10^3, or 10^4 of SW480 *OR7C1*-overexpressed cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data are shown as means ± SD. Engraftment rates are summarized in Table 1. Difference between SW480 *OR7C1*–overexpressed cells and control cells was examined for statistical significance by Student *t* test. E, qRT-PCR of OR7C1 and CICs markers. Total RNAs were isolated 48 hours after transfection of a negative control or *OR7C1*-specific siRNA 1, 2 and 3. Expression of *OR7C1, SOX2, LGR5*, and *POU5F1* mRNAs was examined by quantitative RT-PCR (qRT-PCR). Data are shown as means ± SD. F, SP ratios of SW480 cells in which OR7C1 mRNA was knocked down. G, sphere formation assay. Data are shown as means ± SD. H, tumor growth of SW480 OR7C1-knockdown cells and control cells. 10^1,10^2,10^3, or 10^4 SW480 *OR7C1*-knockdown cells and control cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. Data are shown as means ± SD. Engraftment rates are summarized in Table 1. Difference between SW480 *OR7C1*–knockdown cells and control cells was examined for statistical significance by Student *t* test.

culture twice (Fig. 4D), and the ratios of SP cells were determined 1 week after the second CTL mixture. SP cells were observed in control the $CD8^+$ T-cell mixture, but the frequency of SP cells was reduced in the OR7C1-specific CTL clone mixture (Fig. 4E), indicating that the OR7C1-specific CTL clone specifically recognizes SP cells.

We then investigated the potency of CIC-related antigens. We previously identified a novel TAA, CEP55 (22). CEP55 is expressed in both SP cells and MP cells derived from colon carcinoma cells, and a CTL clone specific for CEP55 peptide could recognize SP cells and MP cells at similar levels (14). In a previous review article, we proposed that TAAs that are recognized by CTLs can be divided into 3 categories according to the expression pattern in CICs and non-CICs: (i) CIC antigens, which are expressed preferentially in CICs, (ii) shared antigens, which are expressed in both CICs and non-CICs, and (iii) non-CIC antigens, which are expressed preferentially in non-CICs (25). In this study, we investigated the expression profiles of several known TAAs in SP cells and MP cells by RT-PCR. *OR7C1*, *MAGEA3*, and *MAGEA4* were preferentially expressed in SP cells (CIC antigens), *CEP55*, *BIRC5*, and *AURKA* were expressed in both SP cells and MP cells (shared antigens), and *EGLN3* was preferentially expressed in MP cells (non-CIC antigen; Fig. 4F). As both CIC antigens and shared antigens are expressed in CICs, we compared the potency of a CIC antigen and that of a shared antigen using CTL clones specific for OR7C1 and CEP55, respectively. The OR7C1-specific CTL clone showed greater cytotoxicity for SP cells than for MP cells, whereas the CEP55-specific CTL clone showed the same levels of cytotoxicity for both SP cells and MP cells (Fig. 4G). The antitumor effects *in vivo* of the OR7C1-specific CTL clone and the CEP55-specific CTL clone were compared by using a CTL therapeutic adoptive transfer model (Fig. 4H). Both OR7C1-specific and CEP55-specific CTL clones showed significant antitumor effects compared with the effects in the negative control group (Fig. 4I). Interestingly, the OR7C1-specific CTL clone showed a significantly greater antitumor effect than that of the CEP55-specific CTL clone (Fig. 4I). Similar results could be observed in two independent experiments. These results indicate that immunotherapy targeting only CICs using a CIC antigen-specific CTL is more potent than immunotherapy targeting all cancer cells using shared antigens.

Discussion

Several methods have been reported for isolation of CICs including the use of cell surface markers, use of SP cells,

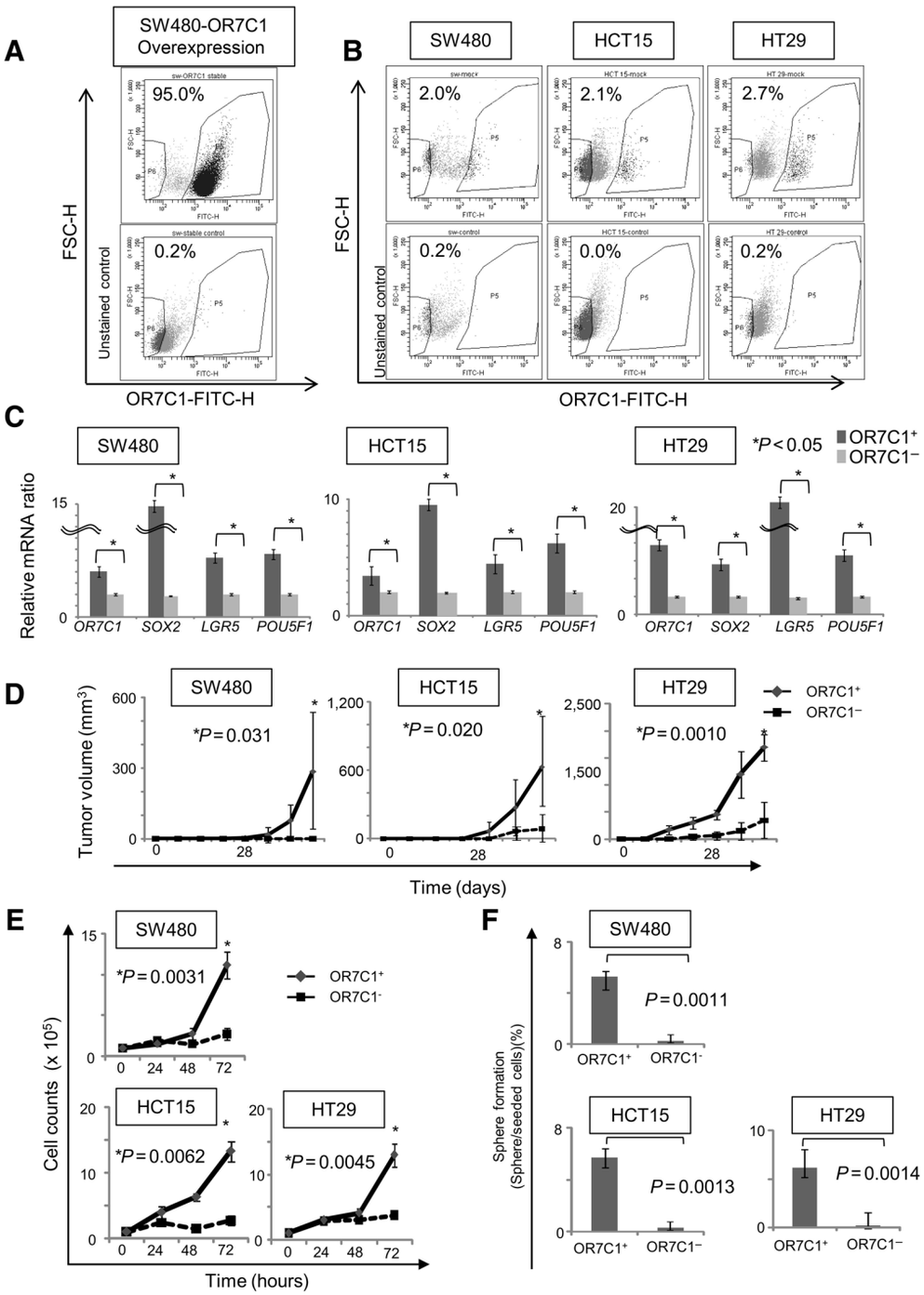

Figure 3.

OR7C1 is a novel colon CIC marker. A, OR7C1 expression in OR7C1-overexpressed SW480 cells determined by flow cytometry. Expression of OR7C1 was examined by flow cytometry using an anti-OR7C1 antibody. SW480-OR7C1 cells and SW480-mock cells were used. The gate for positive staining was defined by SW480-mock cells. The percentages indicate the positive frequencies of OR7C1. B, OR7C1 expression in SW480, HCT15, and HT29 cells. Expression of OR7C1 was examined by flow cytometry. The gate for positive staining was defined by samples without the anti-OR7C1 first antibody. C, Quantitative RT-PCR of *OR7C1* and CICs markers in OR7C1$^+$ cells and OR7C1$^-$ cells. OR7C1-positive (OR7C1$^+$) cells and OR7C1-negative (OR7C1$^-$) cells were isolated by flow cytometry from SW480, HCT15, and HT29 cells. Expressions of *OR7C1, SOX2, LGR5*, and *POU5F1* mRNAs in OR7C1$^+$ cells and OR7C1$^-$ cells were examined by qRT-PCR. D, tumor growth of OR7C1-positive cells and OR7C1-negative cells. 10^1,10^2, or 10^3 positive cells and negative cells were inoculated subcutaneously into the backs of NOD/SCID mice, and tumor growth was measured weekly. The graphs show the results for mice with injection of 10^3 cells. Data are shown as means ± SD. Engraftment rates are summarized in Table 1. Difference was examined for statistical significance by Student *t* test. E, proliferation ability *in vitro*. Growth of OR7C1$^+$ cells and OR7C1$^-$ cells *in vitro* was examined by cell counting. Data are shown as means ± SD. F, sphere formation assay. Data are shown as means ± SD.

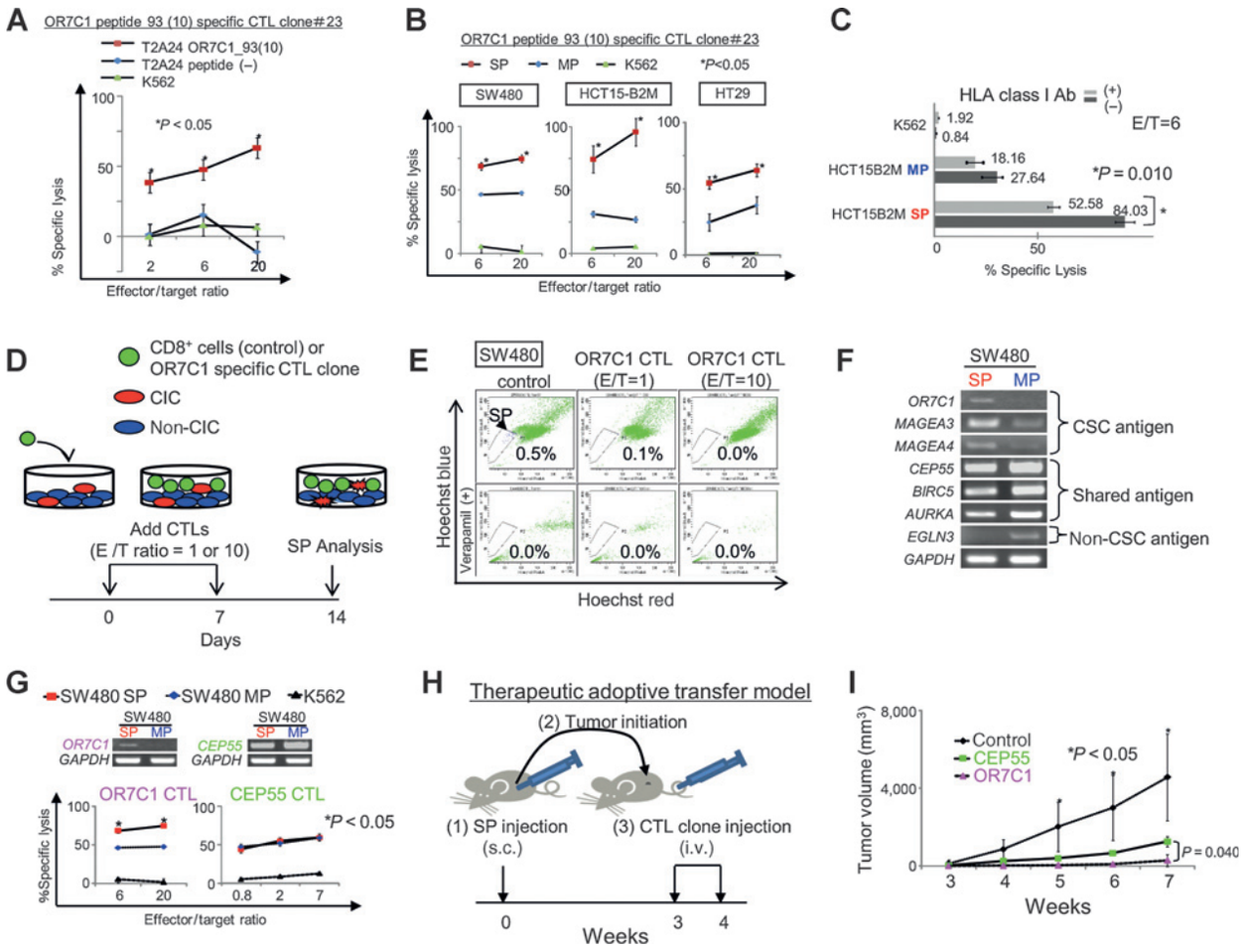

Figure 4.

Establishment of CIC-targeting immunotherapy using antigenic peptide derived from OR7C1. A, specificity of CTL clone #23 by ^{51}Cr release assay. CTL clone #23 was established from case G by the limiting dilution method. The cytotoxic activity of CTL clone #23 against OR7C1_93(10) peptide was examined by a ^{51}Cr release assay using OR7C1_93(10) peptide-pulsed T2-A24 cells as targets. K562 cells were used as a negative control. Data represent means ± SD. B, cytotoxicity against SP cells by CTL clone #23. Cytotoxic activities against SP cells and MP cells derived from SW480, HCT15-B2M, and HT29 cells were examined by a ^{51}Cr release assay using CTL clone #23. Data are shown as means ± SD. C, inhibition of cytotoxicity by anti-HLA class I antibody. The cytotoxicities of CTL clone #23 for SP cells were examined by a ^{51}Cr release assay using anti-HLA class I antibody (W6/32). SP cells and MP cells were isolated from HCT15-B2M cells. K562 cells were used as a negative control. The effector/target ratio (E/T) was 6. Data are shown as means ± SD. D, schema of *in vitro* CIC killing model. Cultured SW480 cells were mixed with CTL clone #23 at days 0 and 7, and SP ratios were analyzed at day 14. The ratio of CTL clone #23 and SW480 (E/T) was 1 or 10. Nonspecific CD8$^+$ T cells were used as a negative control. E, *in vitro* CIC killing reduced SP ratios. The SP ratios of SW480 cells co-cultured with OR7C1-specific CTL clone #23 or nonspecific CD8$^+$ T cells (control) were analyzed. F, classification of TAAs (CSC antigen, shared antigen, and non-CSC antigen). The expression of known TAAs was evaluated by RT-PCR using SP cells and MP cells derived from SW480 cells. OR7C1, MAGEA3, and MAGEA4 were preferentially expressed in SP cells (CSC antigen). CEP55, BIRC5, and AURKA were expressed in both SP cells and MP cells (shared antigen). EGLN3 was preferentially expressed in MP cells (non-CSC antigen). G, cytotoxicity against SP cells of CSC antigen–specific CTL clone and shared antigen-specific CTL clone. The cytotoxicities for SP cells using OR7C1-specific CTL clone (#23) as CSC antigen–specific CTLs and CEP55-specific CTL clone (#41) as shared antigen-specific CTLs were analyzed by a ^{51}Cr release assay. SP cells and MP cells were isolated from SW480 cells. K562 cells were used as a negative control. Data are shown as means ± SD. H, schema of adoptive transfer model. SP cells isolated from SW480 cells were injected into NOD/SCID mice subcutaneously at week 0. The SP cells initiated tumor formation about 2 weeks later. CTL clones (#23 and #41) were injected into NOD/SCID mice with tumors at weeks 3 and 4, and then tumor growth was observed. Nonspecific CD8$^+$ T cells were used as a negative control. I, potent antitumor effect of CSC antigen-specific CTL adoptive transfer model. SW480 SP cells were inoculated subcutaneously into the backs of NOD/SCID mice and a CTL clones specific for OR7C1 and CEP55 were injected. Tumor growth was measured weekly. Data are shown as means ± SD. Differences between groups were examined for statistical significance by Student *t* test.

ALDEFLUOR assay and tumor sphere formation (6). In this study and our previous study, we confirmed that SP cells derived from CRC cells have higher tumor-initiating ability, higher sphere-forming ability, and higher expression levels of stem cell markers than those of MP cells derived from CRC cells. Thus, SP cells used in this study are enriched with CICs and are a reasonable source of CICs. In the microarray screening, we found an olfactory receptor family gene, OR7C1. OR7C1 is a member of the olfactory receptor family, characterized by G protein–coupled seven transmembrane receptors, and is expressed in the tongue (26,27). The olfactory receptor family includes about 350 genes and they are usually expressed in the olfactory epithelium. Several kinds of

olfactory receptors have been identified in tissues other than olfactory tissues, including the testis, tongue, and placenta; however, functions of most olfactory receptors expressed in nonolfactory tissues are still elusive (28). A previous study showed that an olfactory receptor, OR51E2, is expressed in prostate cancer cells and that activation of OR51E2 by its ligand, β-ionone and inhibited proliferation of prostate cancer cells (29). Another olfactory receptor family member, OR51E1, was reported to be a potential novel biomarker for small intestine neuroendocrine carcinoma (30). Expression of the olfactory receptor OR13C4 was shown to be significantly associated with risk of pancreatic cancer by pathway analysis in a genome-wide association study (31). These findings suggest that ectopically expressed olfactory receptors in cancer cells may have some roles; however, there has been no report in which the relationship between an olfactory receptor and CICs was described. In the current study, we analyzed the functions of OR7C1 by gene overexpression and gene knockdown by siRNAs, and we found that OR7C1 has roles in the expression of stem cell genes including *SOX2*, *POU5F1* and *LGR5*, in the frequency of SP cells, in sphere-forming ability in a floating condition and in tumor-initiating ability *in vivo*. These results indicate that OR7C1 has roles in the maintenance of CRC CICs, which might be controlled by the expression of stem cell molecules including SOX2, POU5F1, and LGR5. In the olfactory bulb, olfactory receptors receiving signals usually stimulate the G protein (Golf)-coupled cascade and then induce an increase of cyclic AMP (cAMP) synthesis by activation of adenylate cyclase. The increase in cAMP synthesis results in a change in the intracellular Ca^{2+} concentration by the opening of some channels and in depolarization of the cell membrane (28). It has been reported that the human sperm chemotaxis is mediated via this pathway (32). As OR7C1 is expressed in primary spermatocytes, secondary spermatocytes, spermatid, and sperm in the testis, it may have a role in sperm chemotaxis. Inactivation of OR51E2 could reduce growth of prostate cancer cells via this pathway and then phosphorylation of p38 and stress-activated protein kinase/c-Jun NH_2 -terminal kinase (SAPK/JNK) MAPKs (29). The signaling pathway that induces the expression of stem cell–related genes via OR7C1 is still elusive, but activation of adenylate cyclase by Golf might be a possible pathway, and our preliminary data suggested that activation of Akt might also be involved in OR7C1 signal transduction (data not shown).

Previous work by Ben-Porath and colleagues revealed that the embryonic stem cell–like gene expression signature in cancer is related to a higher grade in breast cancers, glioblastomas, and bladder cancers (33). They found that activation targets of NANOG, POU5F1, SOX2, and c-MYC are overexpressed in poorly differentiated tumors. These findings indicate that ES-like gene expression might play a role in the stem-like phenotypes of tumors that have a biologically high-grade malignant potential. ES genes are related to cancer stem cell/cancer-initiating cell phenotype. SOX2 has roles in the maintenance of CICs of breast cancer, glioma, and lung cancer (18,34,35), and the expression of SOX2 is related to poorer prognosis in several malignancies (36–39). Relationships of other ES genes including OCT4, NANOG, and LIN28 with CSCs/CICs were described in other reports (40–43). On the other hand, there are controversial reports regarding the intestinal stem cell marker LGR5. Ziskin and colleagues reported that LGR5 did not have prognostic significance in CRC patients (44). On the other hand, Saigusa and colleagues reported that LGR5 expression and CD44 expression in cancer stroma may be coordinately associated with tumor relapse in locally advanced rectal cancer after preoperative chemoradiotherapy and that they are related to poorer overall survival (45). Moreover, combined expression levels of Aldh1 which is one of the CIC markers, Survivin, and EpCAM are strong independent prognostic factors, with high HRs, for survival and tumor recurrence in colon cancer patients and therefore reflect tumor aggressiveness (24). In this study, we investigated whether OR7C1 influences clinical prognosis using immunohistochemical staining of CRC tissues. We confirmed that patients in the highly positive and intense OR7C1 group have a low rate of overall survival and that only OR7C1 high positivity was an independent risk factor of poor prognosis. The frequencies of OR7C1-positive cells, which are thought to have upregulation of the stem cell genes SOX2, POU5F1, and LGR5, might reflect the total number of high-grade CICs and might be related to poorer prognosis.

We previously reported that CRC CICs isolated as SP cells were sensitive to CEP55-specific CTLs as well as non-CICs, although CICs were resistant to chemotherapy agents (14). Our recent mouse DNA vaccination models indicated that CIC antigens are more potent than shared antigens and that shared antigens are more potent than non-CIC antigens (17,46). The potency of CIC-targeting immunotherapy was shown for mouse melanoma and squamous cell carcinoma (47). However, there is no evidence that CIC antigens are the most potent also in the human CTL system, and there are no such known human CIC antigens. In this study, we identified a novel CIC antigen, OR7C1, and we confirmed the potency of a CIC targeting immunotherapy by using a mouse CTL adoptive transfer model. Our data indicate that targeting only CICs with a CIC antigen is a better strategy than targeting all cancer cells with a shared antigen. As shared antigens are also expressed in CICs, the exact mechanism by which CIC antigen is more potent than a shared antigen is still elusive. One possible explanation is the limited number of CTLs *in vivo*. There are huge numbers of cancer cells in apparent clinical tumors (a clinically diagnosable tumor of only 1 cm in diameter contains approximately 10^9 cancer cells.). Therefore, targeting only CICs is more effective for eradicating CICs with limited numbers of CTLs and so might result in a significant antitumor effect. On the other hand, the effector/target ratio was relatively low with CTLs specific to a shared antigen as shared antigen-specific CTLs also recognize non-CICs, and CTLs may miss CICs. Thus, the antitumor effect of shared antigen-specific CTLs might be limited compared with that CIC antigen-specific CTLs. CIC-specific immunotherapy, however, misses non-CICs, which constitute the major population of cells in a tumor. As non-CICs have less tumor-initiating ability than do CICs, non-CICs may not be significant for tumor eradication.

In summary, we identified a novel CRC CIC marker, OR7C1. Expression of OR7C1 is a poor prognostic marker for CRCs. OR7C1_93(10) peptide is a promising candidate for CIC-targeting immunotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Writing, review, and/or revision of the manuscript: R. Morita, Y. Hirohashi, T. Torigoe
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R. Morita, T. Torigoe, A. Takahashi, H. Asanuma, Y. Tamura, T. Kubo, T. Terui, T. Kondo, A. Taketomi
Study supervision: T. Torigoe, Y. Tamura, T. Tsukahara, T. Kanaseki, T. Mizuguchi, K. Ishitani, S. Hashino, M. Asaka

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