

Tumor-specific Immunological Recognition of Frameshift-mutated Peptides in Colon Cancer with Microsatellite Instability¹

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ABSTRACT

Colorectal cancers with microsatellite instability (MSI+ CRCs) caused by dysfunction of DNA mismatch repair have unique clinicopathological characteristics including good prognosis with T-cell infiltration in tumor. To identify tumor antigens that induce immune response against MSI+ CRC, SEREX (serological analysis of recombinant cDNA expression cloning) was applied. By screening a λ phage cDNA library constructed from three MSI+ CRC cell lines with serum from a patient with MSI+ CRC with abundant T-cell infiltrates in tumor, 64 antigens were isolated. Immunogenicity of each antigen was evaluated by screening sera from patients with various cancers and from healthy individuals, and specific IgG antibodies (Abs) for 49 antigens were detected only in MSI+ CRC patients. A frameshift mutation in the repetitive G sequences (microsatellite) in the coding region of CDX2, one of the identified antigens, was found in the tumor tissue of the patient who had anti-CDX2 serum Ab. The Ab recognized both the COOH-terminal tumor-specific peptides created by the frameshift mutation and the NH₂-terminal normal peptides of CDX2 when Western blot analysis was performed using various bacterial recombinant CDX2 proteins including the normal and altered peptides, which indicated that immune response could be raised against tumor-specific peptides generated through MSI. The anti-CDX2 Ab was detected only in the patient with the CDX2 frameshift mutation in tumor and disappeared 7 years after the curative resection, suggesting that this immune response may also be useful as a tumor marker. No altered subcellular localization and transcription ability was demonstrated in the mutated CDX2, although decreased expression was suggested in immunohistochemical analysis. Therefore, tumor-specific peptides generated by MSI may be involved in antitumor immune responses and may be useful for the development of diagnostic and therapeutic methods for patients with MSI+ CRC.

INTRODUCTION

Approximately 15% of CRCs show MSI,³ a genetic defect resulting in the progressive accumulation of insertion/deletion mutations at repeated nucleotide sequences (1, 2) caused by inactivation of DNA

MMR genes, including *hMSH2*, *hMLH1*, *PMS1*, *PMS2*, *hMSH6/GTBP*, and *hMSH3* (3). HNPCCs show MSI because of germ-line mutations of the MMR genes (4), and sporadic MSI+ CRCs usually occur because of transcriptional silencing of *hMLH1* by methylation of the promoter (5, 6). These MSI+ CRCs are characterized by preferential location in proximal colon, high prevalence of mucinous and medullary histotypes with poor differentiation (1, 7–9). Despite the malignant histopathological features, it has been reported that patients with MSI+ CRC show better survival rates than those with MSI– CRC (9–13). MSI+ CRCs have a pronounced infiltration of activated cytotoxic CD8+ T cells in tumors (14–18). A higher content of activated cytotoxic intraepithelial lymphocytes was associated with improved survival in MSI+ CRC patients (18). Tumor cells near activated CTLs underwent apoptotic cell death (14). These observations indicate that T-cell responses may be involved in the good prognosis of MSI+ CRC patients.

MSI may affect repetitive nucleotide sequences (microsatellite) in the coding region of expressed genes, resulting in frameshift mutations that cause unique peptide sequences at their COOH terminus. These tumor-specific peptides may induce immune responses and may be involved in antitumor effects in MSI+ CRCs. Several proteins that contain microsatellites at the coding region, including TGFBR2, BAX, IGFIIR, hMSH3, hMSH6, and caspase-5, have previously reported to have frameshift mutations in MSI+ CRCs (19). These “target genes” sometimes act as tumor suppressor genes. In addition, these may be useful as targets for immunotherapy.

Various immunotherapies have recently been developed with the identification of tumor antigens recognized by T cells (20, 21). These tumor antigens were identified by various methods, including cDNA expression cloning with tumor-reactive T cells (22–25). However, it is not easy to generate tumor-reactive T cells in many cancers, including CRC, and only limited antigens have been identified in CRC. SEREX is a cDNA expression cloning of tumor antigens using patient’s serum IgG Ab for screening (26). Recognition by IgG Ab indicates that CD4+ T cells are activated to the same antigens. In addition, tumor antigens recognized by CD8+ T cells, including MAGE-A1, NY-ESO-1, and tyrosinase, could also be isolated by SEREX (26, 27).

In this study, we have applied SEREX to identify tumor antigens that induced immune response against MSI+ CRC in a patient with a good prognosis and T-cell infiltration in tumor tissues. In this patient, IgG Abs specific for both the COOH-terminal-unique peptides, generated by frameshift mutation that is possibly caused by MSI, and the NH₂-terminal normal peptides of CDX2 were identified. Therefore, unique peptides generated by MSI could be recognized by the patient’s immune response and may be involved in antitumor effects. The IgG Ab for the mutated CDX2 disappeared 7 years after curative resection. Therefore, tumor-specific peptides caused by MSI may be useful for the development of diagnostic or therapeutic methods for patients with MSI+ CRC.

MATERIALS AND METHODS

Cell Lines and Cancer Tissues. Human MSI+ CRC cell lines DLD-1, Lovo (28, 29) and Nota were cultured in RPMI 1640. COS7 and SW480 were

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³ The abbreviations used are: MSI, microsatellite instability; poly(A), polyadenylic acid; SEREX, serological identification of tumor antigens by cDNA expression cloning; CRC, colorectal cancer; MMR, mismatch repair; HNPCC, hereditary nonpolyposis CRC; TIL, tumor-infiltrating lymphocyte; Ab, antibody; CDX2, caudal-type homeobox transcription factor 2; TGFBR2, transforming growth factor- β type 2 receptor; BAX, BCL-2-associated protein X; IGFIIR, insulin-like growth factor 2 receptor; HNP-8, HNPCC patient no. 8; MSI-H, high level of MSI; MBP, maltose-binding protein; HA, hemagglutinin; SI, sucrase-isomaltase; PBMC, peripheral blood mononuclear cell.

cultured in DMEM. Media were supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 µg/ml streptomycin. Nota was established from MSI+ CRC of an HNPCC patient. Other cell lines were purchased from American Type Culture Collection (Manassas, VA). Tumor tissues and sera were obtained from patients and healthy individuals. HNPCC patients fulfilled the HNPCC criteria (30). All of the samples were obtained with informed consent.

Analysis of MSI. Genomic DNAs were extracted from specimens of CRCs and corresponding normal mucosas using SDS-proteinase K and phenol. MSI status was evaluated by PCR with seven microsatellite markers (BAT25, BAT26, D2S123, D5S346, D17S250, TGFBR1, and D18S58) selected from the international guidelines suggested by the National Cancer Institute collaborative meeting on MSI+ CRC (31). PCR was performed using the Ex Taq kit (Takara shuzou, Kyoto, Japan) and fluorescence-labeled primers, as described previously (32). Fluorescence-labeled PCR products were analyzed on ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA) using GeneScan 2.1 software (Perkin-Elmer), as described previously (33).

Profile of the Patient Whose Serum Was Used for Screening of cDNA Library. A 45-year-old male, HNP-8, had two colon adenocarcinomas in the transverse and the descending colon. The clinical stages were Dukes' class B2 and B1, respectively. He received a diagnosis of HNPCC according to the criteria of HNPCC (30) and underwent total colectomy. Tumors showed MSI-H phenotype having mutations in six of seven MSI markers. Pronounced CD8+ T-cell infiltrates were observed in the tumor. He has been disease-free for 7 years after surgical resection of tumors.

Screening of cDNA Library for Isolation of Tumor Antigens and Screening of Sera for IgG Ab Specific for Each Identified Antigen. Total RNAs isolated from three MSI+ CRC cell lines, DLD-1, LoVo, and Nota, were mixed and poly(A)+ RNA was purified with latex beads coated with oligo-dT (Oligotex-dT30 super; Takara shuzou). A cDNA library was constructed into the λ-ZAP Express vector using λ-ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) and packaged into λ phage particles, resulting in 1.8×10^6 primary recombinants. Serum from HNP-8 was first treated with lysates of *Escherichia coli* XL1 blue MRF' and ZAP express phages expressed on nitrocellulose membranes (Hybond-C; Amersham Pharmacia, Buckinghamshire, England) to absorb antibacterial and antiphage Abs. cDNA library screening was performed as previously reported (34). Briefly, proteins were expressed in *E. coli* (XL1 blue MRF') by transfection of the phage cDNA library and transferred onto isopropyl-1-thio-β-D-galactopyranoside-treated membranes. The membranes were incubated with the 1:100 diluted serum for 4 h at room temperature. The reactive plaques were detected by alkaline phosphatase-conjugated goat antihuman IgG (Fc) Abs (ICN Pharmaceuticals, Aurora, OH) and visualized with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate color development. The insert cDNA of the positive clones were amplified by PCR using the Ex Taq kit (Takara shuzou) and vector-specific primers (T3 primer and T7 primer), and their nucleotide sequences were determined using the Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer) and ABI PRISM 310 Genetic Analyzer (Perkin-Elmer). The sequences of cDNA were analyzed using the BLAST search of genetic databases provided by the National Center of Biotechnology Information (NCBI, <http://www.ncbi.nlm.nih.gov>). The presence of IgG Ab specific for each isolated antigen was screened in sera from various cancer patients and healthy individuals with nitrocellulose membranes in which phages encoding antigen and phage with no insert were mixed at a ratio of 2:3.

Analysis of Mutation in Coding Repetitive Sequences of the Identified Antigens. Mononucleotide repeats more than six nucleotides and dinucleotide repeats more than three dinucleotide units in the coding region of the identified antigen genes were searched in gene databases of the NCBI GenBank database. Frameshift mutations in coding repetitive sequences were analyzed using genomic DNA extracted from tumor tissues of MSI+ CRC patients whose sera reacted with antigens. PCR was performed with primers corresponding to selected coding regions (primer sequences and annealing temperatures available on request) using Takara Ex Taq kit (Takara shuzou). After electrophoresis, PCR products were purified using the QIAquick Gel Extraction kit (Qiagen, Valencia, CA) and sequenced as described above.

Production of Recombinant CDX2 Proteins. To prepare the full-length wild-type CDX2 (wtCDX2-pMAL), the coding region was amplified by PCR and subcloned into pMALc2x expression vector (New England Biolabs, Beverly, MA). The primer sets were as follows: 5'-ATTTTCAGAATTCATG-

TACGTGAGCTACCTCCTG-3' (CDX2 sense) and 5'-AGTGCCAAGTTGGGAGGGGAGGGGTCTCTC-3' (CDX2 antisense). To generate mutated CDX2 (mutCDX2-pMAL), one G insertion at seven G repeats in exon 3 was introduced by site-directed mutagenesis using PCR, as described previously (35). The primers were as follows: pMAL sense primer (5'-TAACAACAACCTCGGGATCG-3') and pMAL antisense primer (5'-TTTCCCAGTCACGACGTTGT-3'), complementary to the neighboring sequences of the polylinker site; mEcoRI primer (5'-AGGATTTACAGAGTCTGCTCC-3'), corresponded to the polylinker sequence located between pMAL sense primer and the inserted DNA with one mismatched nucleotide (underlined) that destroys the EcoRI site; CDX2 mut primer (5'-TTTAGCACCCCCCAGTTGGC-3'), complementary to the region to be mutagenized. First, PCR were done with pMAL sense primer and CDX2 mutant primer, and with mEcoRI primer and pMAL antisense primer using wtCDX2-pMAL as a template. Amplified DNA fragments were mixed and further amplified by PCR, using pMAL sense primer and pMAL antisense primer. The PCR product was digested with EcoRI and HindIII and inserted into pMALc2x (New England Biolabs). DNA fragments encoding the COOH-terminal 24 amino acids of wild-type CDX2 (wtCDX2₂₉₀), the COOH-terminal 46 amino acids of mutated CDX2 (mutCDX2₂₉₀), and the COOH-terminal 30 amino acids of mutated CDX2 (mutCDX2₃₀₆) were prepared by PCR from wtCDX2-pMAL or mutCDX2-pMAL and were subcloned into pMALc2x (New England Biolabs). The primers were as follows: 5'-ATTTTCAGAATTCGCCTCAGTGTCTGCTCTGTC-3' (CDX2₂₉₀ sense) and CDX2 antisense primer for wtCDX2₂₉₀, CDX2₂₉₀ sense primer and CDX2 antisense primer for mutCDX2₂₉₀, and 5'-ATTTTCAGAATTCGGTGCTAAACCCACCGT-3' (CDX2₃₀₆ sense) and CDX2 antisense primer for mutCDX2₃₀₆. Each subcloned DNA construct was transformed into competent *E. coli* strain DH5α (Toyobo, Osaka, Japan), and the proteins expressed as maltose binding protein (MBP) fusion proteins were purified with Amylose resin column (New England Biolabs) according to the manufacturer's instructions.

Western Blot Analysis with Sera. Purified recombinant proteins were separated by 10% SDS-PAGE and transferred to nitrocellulose membrane (Hybond-C; Amersham Pharmacia). After overnight incubation with 1:100 diluted sera at 4°C, the membranes were incubated with alkaline phosphatase conjugated goat antihuman IgG (Fc) Ab (ICN Pharmaceuticals) for 1 h at room temperature. Positive proteins were detected by incubation with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Competitive ELISA with Synthesized Peptides. ELISA was performed as following. Three 20-mer peptides from the COOH-terminal 30 amino acids of mutated CDX2 were synthesized: GAKPHRHPVTHRGLQRQNS, RHPVTHRGLQRQNSRLSHE, and HRGLQRQNSRLSHEERGLC. Peptides were synthesized with a multiple peptide synthesizer (ACT 396; Advanced ChemTech, Louisville, KY) using a solid-phase method based on F-moc [N-(9-fluorenyl)methoxycarbonyl] chemistry. Microtiter 96-well plates were coated with 100 µl of 10 µl/ml-purified recombinant MBP-COOH-terminal mutated CDX2 (mutCDX2₃₀₆) or MBP as a negative control at 4°C overnight. One hundred µl of 1:400 diluted serum from HNP-8 and 100 ng of each synthesized peptides were added to the mutCDX2₃₀₆-coated wells of ELISA plates and incubated at 4°C overnight. After washing, the plates were incubated with 1:3000 diluted horseradish peroxidase-conjugated antihuman IgG Ab (ICN Pharmaceuticals) for 1 h at room temperature. The color development was achieved by a solution containing tetramethylbenzidine and hydrogen peroxide (Sigma Chemical Co., St. Louis, MO) and stopped by adding 1N H₂SO₄. The absorbance was measured at 450 nm by microplate reader (Model550, Bio-Rad Laboratories, Hercules, CA).

Immunohistochemical Analysis. Immunohistochemical analyses for T cell subsets and expression of CDX2 were performed following a standard protocol (36). The primary Abs used were: anti-CD4 (mouse monoclonal 1F6; 1:20; Nichirei, Tokyo, Japan) and anti-CD8 (mouse monoclonal C8/144B; 1:25; Nichirei) and anti-CDX2 rabbit polyclonal Ab (1:500) that was generated to recognize the 149 amino acids within exon 1 of CDX2, as described previously (37). Sections of formalin-fixed, paraffin-embedded specimens were deparaffinized. Heat-induced epitope retrieval in 10 mM citric acid buffer was performed by autoclaving (CD4) or microwaving (CD8 and CDX2). Endogenous peroxidase activity was removed by incubation with 0.3% H₂O₂. For CDX2 staining, the section was incubated with anti-CDX2 Ab overnight at 4°C followed by incubation with peroxidase-conjugated goat antirabbit Ab (DAKO, Glostrup, Denmark), and bound peroxidase was detected using dia-

minobenzidine tetrachloride. For the CD4 and CD8 staining, the sections were incubated with anti-CD4 Ab or anti-CD8 Ab for 1 h at room temperature followed by incubation with biotinylated goat antimouse Ab (DAKO). The bound Ab was visualized using the streptABComplex/horseradish peroxidase (HRP) reagent (DAKO) and diaminobenzidine tetrachloride. CD8+ T-cell infiltration in tumor tissue was evaluated by counting the number of immunoreactive cells within tumor cell nests, as described previously (38). Five areas with abundant distribution were selected and evaluated with a microscopic field of $\times 200$ (0.933 mm^2).

Subcellular localization of the mutated CDX2 was evaluated by immunocytochemistry of COS7 cells transiently transfected with influenza virus HA-tagged mammalian expression vector pHM6 (Roche Diagnostics Corp.) containing CDX2 using anti-HA Tag rabbit immunoaffinity purified Ab (Upstate, Lake Placid, NY). COS7 cells were fixed with 2% paraformaldehyde 24 h after transfection using LipofectAMINE PLUS Reagent (Invitrogen, Carlsbad, CA) and were incubated overnight at 4°C with 1:50 diluted anti-HA rabbit polyclonal Ab, and CDX2 was detected with a biotin-labeled antirabbit IgG Ab (ICN Pharmaceuticals) and streptavidin-biotin-peroxidase procedure using VECTASTAIN Elite ABC standard kit (Vector Laboratories, Burlingame, CA) followed by diaminobenzidine color development.

Evaluation of Transcription Activity of the Mutated CDX2. To examine the transcription activities of wild-type and mutant CDX2, we performed a luciferase assay using 293T cells and SW480 cells, as described previously (39). The full-length coding regions of wild-type and mutant *CDX2* were subcloned into pcDNA3.1- (Invitrogen), resulting in pcDNA3.1-wt CDX2 and pcDNA3.1-mut CDX2, respectively. The reporter vector containing Luciferase under control of the promoter of *SI*, one of the intestine-specific genes for which transcription is regulated by CDX2 (40), was constructed as described previously (41). Briefly, the promoter region (-183 to $+54$) of *SI* gene was amplified by PCR using the forward primer $5'$ -GTGGTGGGTACCTGACAGTACAATTACTAA- $3'$ and the reverse primer GTGGTGAAGCTTAGCCTGTTCTCTTTGCTA- $3'$ and subcloned into the *KpnI* and *HindIII* site in the luciferase reporter plasmid, pGL3 Basic vector (Promega). Preconfluent cells were cotransfected with pGL3 Basic-SI promoter and pcDNA3.1-wt CDX2 or

pcDNA3.1-mut CDX2 using LipofectAMINE PLUS Reagent (Invitrogen). The *Renilla* luciferase control reporter, pRL-SV40 vector (Promega) was also cotransfected as a control for transfection efficiency. Forty-eight h after transfection, assay was performed using Dual Glo Luciferase Assay System (Promega) and Wallac 1420 ARVOSx multilabel counter (Perkin-Elmer). Firefly luciferase expression was normalized to *Renilla* luciferase expression. Results are reported as luciferase activity relative to the pGL3 Basic promoterless construct. Transfection experiments were performed five times in triplicate. The results were expressed as the means \pm SE.

RESULTS

Isolation of Tumor Antigens Recognized by IgG Abs in the Serum from a Patient with MSI+ CRC. To identify tumor antigens possibly involved in antitumor immune responses against MSI+ CRC, we have used samples from a patient HNP-8 with HNPCC. The tumor showed MSI-H phenotype having mutations in six of seven MSI markers. The patient is disease free for 7 years after the surgical resection of two colorectal adenocarcinomas in the transverse and descending colon. Immunohistochemical analysis revealed that the tumor tissues contained abundant CD8+ T-cell infiltrates (median $189/0.94 \text{ mm}^2$; Fig. 1, A, C, and D), but not many CD4+ T cells (Fig. 1B). These results suggest that CD8+ T-cell response against tumor cells might be associated with the good prognosis of HNP-8. Because living TILs and tumor cells were not available, we have applied SEREX using serum from the patient HNP-8.

A total of 1.0×10^6 recombinant clones of a λ phage cDNA library constructed from the mixture of mRNA of three MSI+ CRC cell lines, DLD-1, LoVo, and Nota, that have frameshift mutations in *TGFBR2*, *BAX*, *IGF1R*, or *caspase-5*, were screened with serum from HNP-8, and 486 positive clones representing 64 distinct genes were isolated. Twenty antigens were isolated more

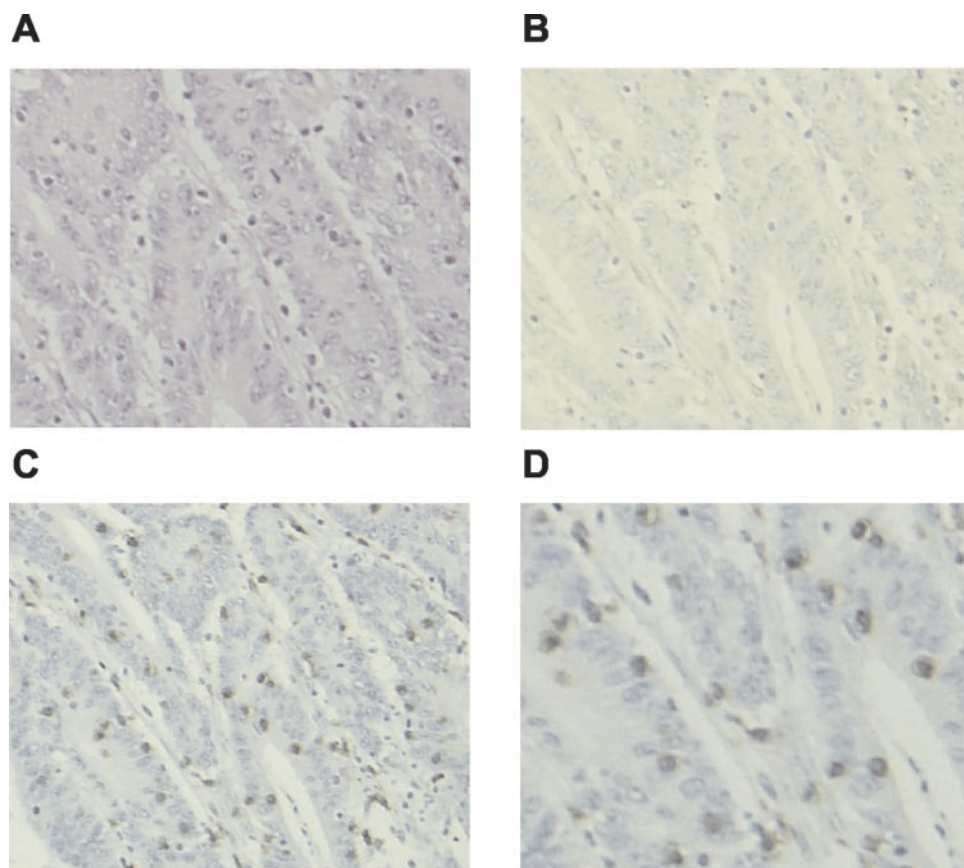


Fig. 1. Infiltration of T cells in the MSI+ CRC tumor tissue of HNP-8. A, H&E staining shows abundant infiltration of mononuclear cells within cancer cell nests. B, infiltrated T cells are not stained with anti-CD4 Ab. C, CD8 staining shows that infiltrated cells are CD8+ T cells. D, a high magnification view of C showing abundant infiltration of CD8+ T cells within cancer cell nests. A, B, and C, $\times 200$; D, $\times 400$.

Table 1 cDNAs isolated by SEREX and the presence of specific IgG antibodies in sera from patients with various cancers and healthy individuals^a

Antigen (UniGene cluster or Accession number)	No. of isolated clones	No. of sera reacted with the antigens ^b					
		MSI(+) CRCs n = 13	MSI(-) CRCs n = 14	Renal cell cancers n = 10	Esophageal cancers n = 10	Pancreatic cancers n = 14	Healthy individuals n = 38
1 Poly(A)-binding protein, cytoplasmic1	268	1	0	2	1	0	0
2 Poly(A)-binding protein, cytoplasmic4	45	1	0	0	0	0	0
3 Galectin-4	42	6	7	10	8	4	8
4 Homo sapiens DNA from chromosome 19 (Hs.71779)	19	1	0	0	0	0	0
5 Cisplatin resistance-associated overexpressed protein	12	1	1	0	1	0	0
6 Protein phosphatase 1B, β isoform	9	1	0	0	0	0	0
7 Caudal-type homeobox transcription factor 2	8	1	0	0	0	0	0
8 Eukaryotic translation initiation factor 4 γ , 1	7	2	2	0	1	0	0
9 Hypothetical protein MGC11256 (Hs.28029)	6	1	0	0	0	0	0
10 DKFZP434M154 protein (Hs.154680)	4	1	0	0	0	0	0
11 Ubiquitin-like, containing PHD and RING finger domains, 1	3	1	0	3	0	0	0
12 Human DNA sequence from 1q42 (AL160408)	3	1	0	0	0	0	0
13 PDZ domain protein (Hs.321197)	2	10	7	10	5	4	20
14 Human clone 295, region surrounding MODY3 mRNA (Hs.204166)	2	3	0	0	0	0	2
15 Retinoblastoma-binding protein 6	2	1	0	0	0	0	2
16 RAB1A, member RAS oncogene family	2	1	0	0	0	0	0
17 Eukaryotic translation elongation factor 1 α 1	2	1	0	0	0	0	0
18 Caudal-type homeobox transcription factor 1	2	1	0	0	0	0	0
19 ADP-ribosylation factor GTPase-activating protein 3	2	1	0	0	0	0	0
20 SH3 protein interacting with Nck, M _r 90,000 (Hs.102929)	2	1	0	0	0	0	0

^a List of cDNAs isolated more than two clones by immunoscreening with serum from HNP-8.

^b Reactivity of serum against each isolated antigen on nitrocellulose membrane was evaluated at 1:100 dilution.

than once (Table 1). Poly(A)-binding protein, cytoplasmic 1 (PABPC1) was the most frequently isolated (268 clones). Poly(A)-binding protein, cytoplasmic 4 (PABPC4), galectin-4, cisplatin resistance-associated overexpressed protein, and Homo sapiens DNA from chromosome 19 were also isolated frequently. Among the identified antigens, galectin-4, CDX1 and CDX2 are intestine-specific genes (42, 43) and down-regulation of CDX1 and CDX2 was previously reported to be associated with development of CRCs (44). Overexpression in tumor cells of Eukaryotic translation initiation factor 4 γ 1 (EIF4G1) and Eukaryotic translation elongation factor 1 α 1 (EEF1A1) was shown (45, 46). Galectin-4 and EIF4G1 were previously isolated as tumor antigens by SEREX from colon cancer and small cell lung carcinoma, respectively (46, 47). Eight positive clones corresponding to CDX2, one of the MSI target genes, were isolated, but no mutation was found among the isolated CDX2 clones. Possible mutations at the coding repetitive sequence were also analyzed among the other isolated clones encoding known genes, but no mutation was found. The other MSI target genes, which have frequently mutations in MSI+ CRC, such as *TGFBRII*, *BAX*, *IGFIIR*, *hMSH3*, *hMSH6*, and *caspase-5*, were not isolated in this screening.

Recognition of the Isolated Antigens by Patients with Various Cancers and Healthy Individuals. To identify tumor-specific antigens that might induce immune responses only in patients with cancers, particularly MSI+ CRC, immunogenicity of each identified tumor antigen was evaluated by screening specific IgG Abs among sera from various cancer patients and healthy individuals. Of the 64 antigens screened, 10 antigens reacted with sera from healthy individuals, including galectin-4. Although galectin-4 has been identified as a colon cancer antigen (47), 8 of 38 sera from healthy individuals reacted with galectin-4 (Table 1), indicating immune response against galectin-4 is not specific for patients with CRCs. Among the remaining 54 tumor antigens, 21 antigens reacted with sera from several cancer patients. These were expressed ubiquitously in various tissues when analyzed by reverse transcription-PCR (RT-PCR; data not shown). Forty-nine antigens reacted with only sera from MSI+ CRC patients, suggesting that these antigens might be specific for MSI+ CRC.

Recognition of Tumor-specific COOH-terminal CDX2 Peptides

Generated by the Frameshift Mutation. To examine whether tumor-specific peptides generated by frameshift mutation at coding repetitive sequences were recognized by the sera, the presence of the coding repeats was first searched for using gene databases in the 49 antigens that reacted exclusively with sera from MSI+ CRC patients, because there may be mutations in autologous tumor cells; even when the isolated clones do not contain any mutation. Nine mononucleotide repeats and 1 dinucleotide repeat were identified in eight antigens as shown in Table 2. DNA sequences of the identified repetitive regions were determined in the genomic DNAs of the corresponding tumor tissues from MSI+ CRC patients whose sera reacted with the antigens. One G insertion in the 7G repeats of the *CDX2* coding region was found in one allele of genomic DNA tumor cells from HNP-8. No mutation was found in the other nine repetitive regions. This G insertion in *CDX2* was not found in the genome of PBMCs from the same patient, indicating that it was a mutation that occurred in tumor cells. This mutation resulted in a frameshift change with a new stop codon at 22 amino acids downstream and the substitution of the normal COOH-terminal 8 amino acids with the tumor-specific 30 amino acid peptides (Fig. 2A).

To test whether IgG Ab recognizes the tumor-specific COOH-terminal peptide generated by the frameshift mutation, recognition by the HNP-8 serum of various bacterial recombinant CDX2 proteins containing mutant or normal peptides was examined by Western blot analysis (Fig. 3). The serum IgG Ab recognized both the full-length wild-type and mutated CDX2 proteins (Fig. 3A). The recognition of wild-type CDX2 explained the reason why the isolated CDX2 clones did not contain mutation in the cDNA expression cloning. The serum also recognized both recombinant proteins containing the COOH-terminal mutated peptide, mutCDX2₂₉₀ and mutCDX2₃₀₆, but not the wild-type COOH-terminal protein wCDX2₂₉₀ (Fig. 3B). The frameshift mutation at the coding 7G repeat of *CDX2* was found only in the tumor cells of HNP-8 among 27 patients with CRC analyzed, and anti-CDX2 Ab was only detected in the HNP-8 serum (Fig. 3C). To confirm the serum recognition of the mutated CDX2 peptide and identify the epitope, we have performed a competitive inhibition assay

Table 2 A mutation in the microsatellite in the coding region of CDX2 among the isolated antigens

	Antigen ^a	Type of coding repeats	Identified mutation in cancer cells ^b	No. of reactive sera from MSI+ CRC (n = 13)
1	<i>Poly(A)-binding protein, cytoplasmic4</i>	A7		1
2	<i>Ubiquitin C</i>	G7		1
3	<i>Caudal-type homeobox transcription factor 2</i>	G7	G8	1
4	<i>Tight junction protein 2</i>	T7, A7		1
5	<i>Coilin</i>	A8		1
6	<i>5-methyltetrahydrofolate-homocysteine methyltransferase</i>	A8		1
7	<i>Retinoblastoma 1</i>	(AG)5		1
8	<i>ADP-ribosylation factor GTPase activating protein1</i>	T7, A8		1

^a Genes with mononucleotide repeats more than six nucleotides and dinucleotide repeats more than three repeat units.

^b Mutations in the coding repeats were analyzed by sequencing genomic DNA from the corresponding cancer tissues.

A

Wild-type (G)7

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          306                               313
... CCA ACT GGG GGG GTG CTA AAC CCC ACC GTC ACC CAG TGA
   P   T   G   G   V   L   N   P   T   V   T   Q   stop

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Mutated (G)8

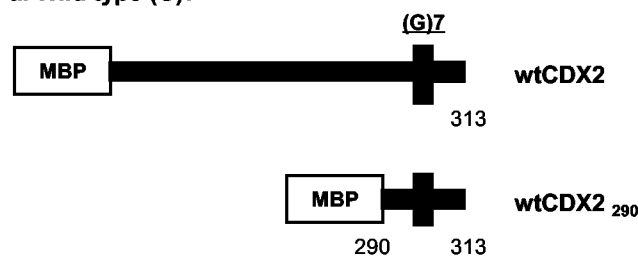
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          insertion
          ▼
... CCA ACT GGG GGG GGT GCT AAA CCC CAC CGT CAC CCA
   P   T   G   G   G   A   K   P   H   R   H   P
          GTG ACC CAC CGG GGT CTG CAG CGG CAG AGC AAT
          V   T   H   R   G   L   Q   R   Q   S   N
          TCC AGG CTG AGC CAT GAG GAG CGT GGA CTC TGC TAG
          S   R   L   S   H   E   E   R   G   L   C   stop

```

B

a. Wild type (G)7



b. Mutated (G)8

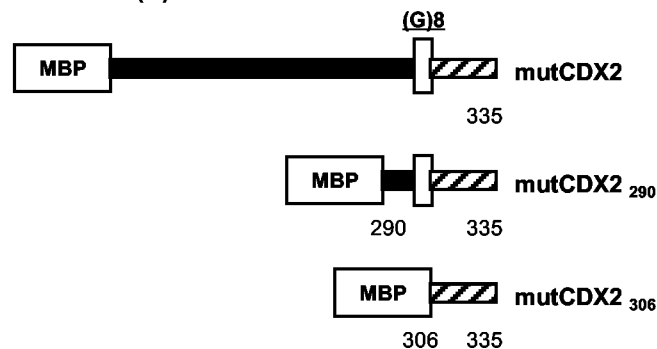


Fig. 2. Structures of wild-type and mutated recombinant CDX2. A, the nucleotide and amino acid sequences of wild-type and frameshift-mutated CDX2. The frameshift mutation caused by one G insertion in the coding 7 G microsatellite of HNP-8 tumor resulted in substitution with tumor-specific peptide consisting of 30 amino acids. B, the structures of recombinant wild-type and mutated CDX2 fusion proteins used in this study. MBP was fused at the NH₂ terminus of the recombinant proteins. a, recombinant wild-type CDX2 and the fragment; b, recombinant mutated CDX2 and 2 fragments. **Bold lines**, normal amino acid sequences shared with wild-type CDX2. **Open box**, eight guanidine repeats in the coding region. **Hatched domain**, tumor-specific COOH-terminal 30 amino acids caused by the frameshift mutation.

using ELISA with three overlapping 20-mer synthetic peptides that covered the 30-amino-acid mutated COOH-terminal CDX2 peptide. The absorbance of wells coated with the recombinant MBP-COOH-terminal mutated CDX2, incubated with serum alone, was 0.211. The absorbance of the CDX2-coated wells incubated with the serum-plus-each-20-mer peptide, GAKPHRHPVTHRGLQ-RQSNS, RHPVTHRGLQRQSNRSLSHE, or HRGLQRQSNRSLSHEERGLC, was 0.068, 0.069, or 0.150, respectively. Thus, the two NH₂-terminal overlapping peptides, but not the COOH-terminal peptide, blocked the serum binding to the recombinant MBP-COOH-terminal mutated CDX2, indicating that the serum recognized the epitope located around the common sequence, RHPVT, of the two NH₂-terminal overlapping peptides. Although the same mutations at coding-repeat sequences of *TGFBR2*, *BAX*, *IGF1R*, and *caspase-5*, were also found in the tumor cells of HNP-8 as the cDNA library, these proteins were not isolated by the cDNA cloning. This result may be attributable to the limitation of the SEREX method, which cannot isolate all of the antigens recognized by the contained Abs. Additional investigations of the immunogenicity of these mutated proteins are required by the screening of Abs against the recombinant proteins or synthesized peptides of these mutated target genes. These results indicated that the tumor-specific-CDX2-mutated peptides induced specific IgG responses to the mutated peptide as well as to the normal NH₂-terminal side peptide in HNP-8 whose tumor contained the CDX2 frameshift mutation. The IgG Ab specific for wild-type and mutated CDX2 had disappeared from the serum of HNP-8 7 years after surgical resection (Fig. 3D), suggesting the possibility of the diagnostic use of the Ab for the frameshift-mutated peptide in patients with MSI+ CRC.

Characterization of the Identified Mutated CDX2. We have attempted to evaluate the biological role of the identified CDX2 mutation in tumor development. We have first analyzed subcellular localization of the mutated CDX2 by immunocytochemical analysis of COS7 cells transfected with HA-tagged wild-type or mutated full-length CDX2 cDNA with anti-HA Ab. As shown in Fig. 4A, nuclear staining was observed in COS7 cells transfected with either wild-type or mutated CDX2, indicating that mutated CDX2 was also present in the nucleus, similar to wild-type CDX2, as described previously (48, 49). Staining of the colon cancer tissues of HNP-8 with anti-CDX2 polyclonal Ab that recognizes the peptide encoded by exon 1 on both wild-type and mutated CDX2, demonstrated positive staining in the nuclei of cancer cells as well as of normal intestinal epithelial cells, as shown previously (48, 49), although the staining of tumor cells appeared to be relatively reduced compared with the adjacent normal epithelial cells (Fig. 4B). The presence of IgG Ab specific for the mutated CDX2 in the HNP-8 serum and its disappearance after the resection suggested expression of mutated CDX2 protein in the tumor of HNP-8.

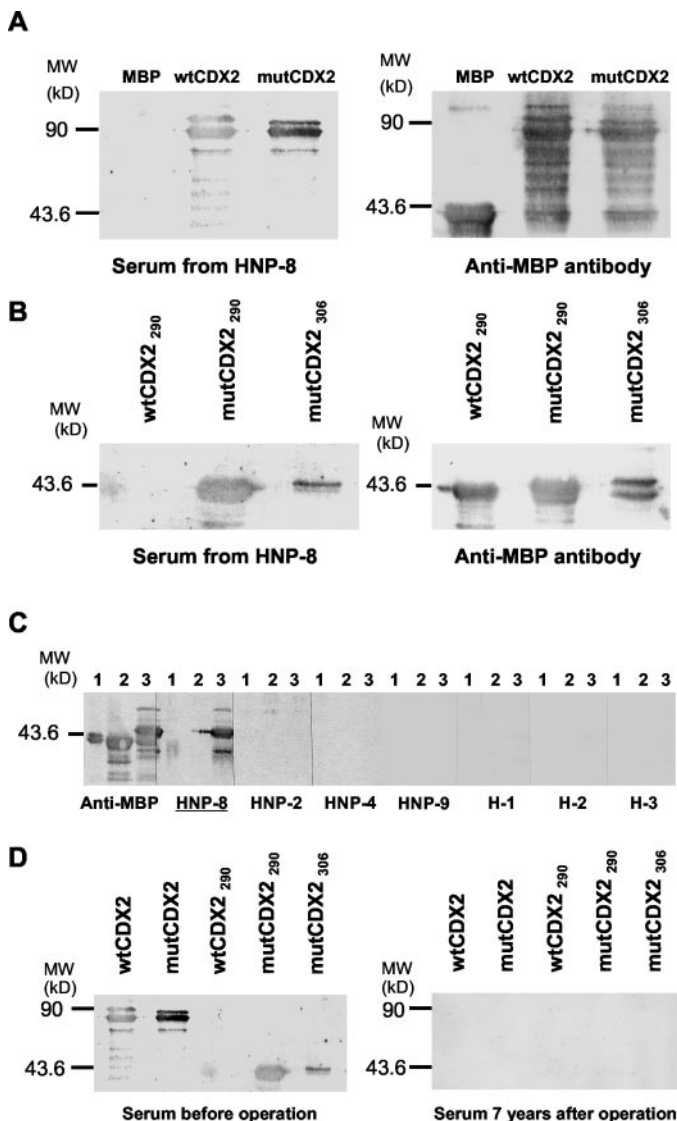


Fig. 3. Recognition of wild-type and mutated CDX2 by IgG Ab in serum from HNP-8 whose tumor expresses CDX2 with a frameshift mutation. **A**, the serum of HNP-8 reacted with the recombinant full-length wild-type CDX2 (*wtCDX2*) and mutated CDX2 (*mutCDX2*) but not with MBP (*MBP*) in Western blot analysis (left panel). The expression of each recombinant protein is confirmed by anti-MBP Ab. (right panel). **B**, the serum of HNP-8 reacted with two different recombinant COOH-terminal mutated CDX2 proteins (*mutCDX2₂₉₀* and *mutCDX2₃₀₆*), but not with recombinant COOH-terminal wild-type CDX2 protein (*wtCDX2₂₉₀*). The expression of each recombinant protein is confirmed by anti-MBP Ab. (right panel) **C**, recognition of recombinant CDX2 by only serum from HNP-8. Recognition of recombinant proteins (Lane 1, MBP; Lane 2, *wtCDX2₂₉₀*; Lane 3, *mutCDX2₂₉₀*) was analyzed by Western blot analysis using sera from HNP-8 with the CDX2 mutation in tumor cells, three MSI+ CRC patients without the CDX2 mutation in tumor cells (*HNP-2*, *4*, and *9*), and three healthy individuals (*H-1*, *2*, and *3*). **D**, disappearance of serum IgG Ab specific for CDX2 7 years after surgical resection. Presence of IgG Ab specific for the mutated CDX2 proteins was analyzed in sera from HNP-8 before (left panel) and 7 years after surgical resection (right panel). MW (kD), *M_r* in thousands.

To examine whether the mutated CDX2 had altered transcription activity, a reporter assay was performed using luciferase reporter vector conjugated with the promoter of SI the transcription of which is regulated by CDX2. Transfection of either wild-type or mutated CDX2 into embryonal kidney cell line 293T cells, which do not express CDX2 (Fig. 4C,a), and colon cancer cell line SW480 cells, which do express CDX2 at a very low level (Fig. 4C,b), could activate the transcription of luciferase at a similar level. No dominant-negative effect of the mutated CDX2 was observed. These results suggest that mutated CDX2 has similar biological activity to wild type CDX2,

although it may be involved in colon cancer development through the down-regulation of its expression.

DISCUSSION

Although identification of tumor antigens has provided new opportunities for the development of immunotherapy, only limited antigens

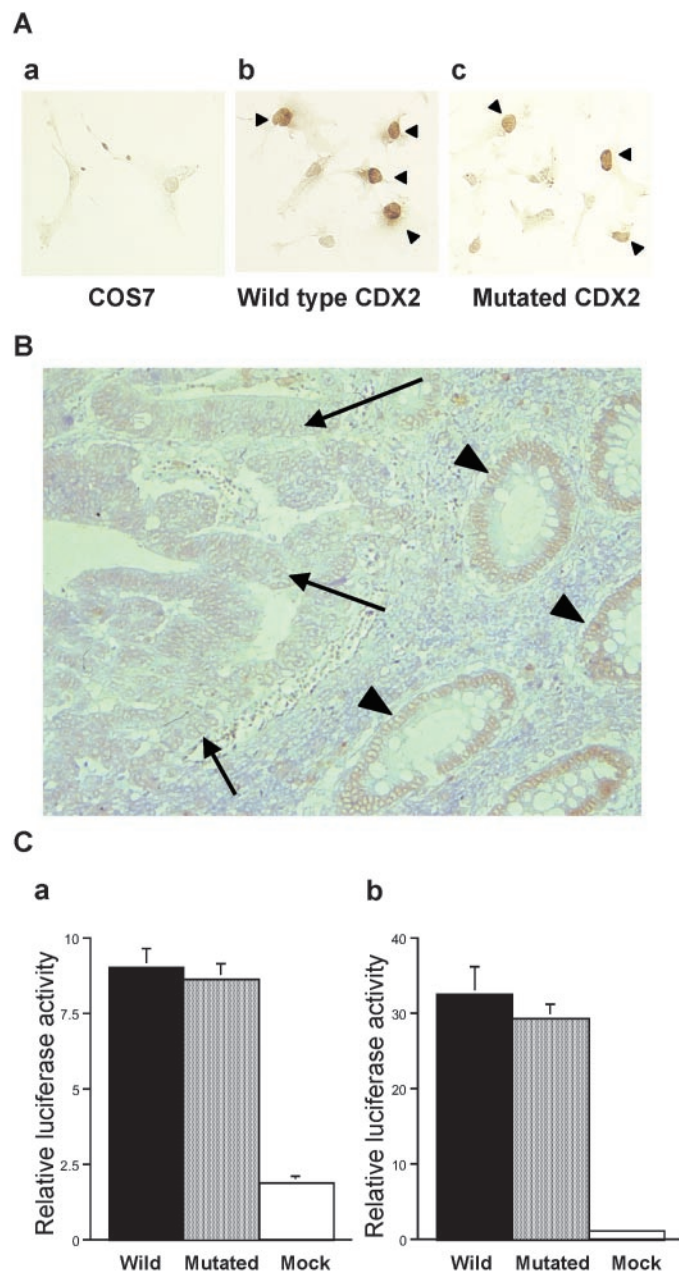


Fig. 4. Characterization of the mutated CDX2. **A**, nuclear localization of the mutated CDX2. Subcellular localization of wild-type and mutated CDX2 was evaluated by staining with anti-HA monoclonal Ab of COS7 cells transfected with HA-tagged wild-type CDX2 (**b**) and mutated CDX2 (**c**). Untransfected COS7 cell control (**a**). Arrowheads, positive staining of the nucleus of COS7 cells. **B**, expression of CDX2 in tumor tissue of HNP-8. Immunohistochemical analysis of HNP-8 tumor tissues with anti-CDX2 Ab that recognize shared portion of wild-type and mutated CDX2 demonstrated positive staining of nucleus in both normal mucosa (arrowhead) and tumor cells (arrow). Relatively decreased staining of tumor cells is shown. **C**, similar transcription activity of the mutated CDX2. 293T cells (**a**) and SW480 cells (**b**) were cotransfected with firefly luciferase reporter gene under control of SI promoter and pcDNA3.1-*wtCDX2* (*Wild*), pcDNA3.1-*mutCDX2* (*Mutated*), or empty pcDNA3.1- (*Mock*). pRL-SV40 containing *Renilla* luciferase was used to normalize transfection efficiency. Data are expressed as luciferase activity relative to the pGL3 basic promoterless construct. Values represent the means \pm SE for the five separate experiments performed in triplicate.

have thus far been identified in CRC. MSI+ CRCs caused by dysfunction of DNA MMR enzymes have unique characteristics, including a good prognosis with T-cell infiltration in tumor, suggesting that immune responses against altered peptides generated by frameshift mutations through MSI may contribute to the good prognosis. Thus, in this study, we have attempted to identify tumor antigens involved in antitumor immune responses in MSI+ CRC patient with abundant CD8+ T-cell infiltrates in tumor, and demonstrated for the first time that IgG Ab response to the tumor-specific COOH-terminal CDX2 peptide generated by the frameshift mutation through MSI was endogenously induced in the HNPCC patient. SEREX was found to be a useful method for the isolation of tumor-specific frameshift peptides.

Recognition of the mutated CDX2 peptides by tumor-infiltrating CD8+ T cells in patient HNP-8 could not be evaluated in this study because of unavailability of TILs and tumor cells from this patient. It has recently been reported that tumor-specific peptides generated by frameshift mutation in coding 10A repeat of TGFBR2 can be recognized by CTLs or helper T cells (50–52). In these studies, HLA-A2-binding peptides synthesized based on HLA-A2 peptide-binding motif, could induce CTLs that lysed CRC cell lines expressing mutated TGFBR2 from the PBMCs of healthy individuals. However, induction of CTLs from PBMCs or TILs of MSI+ CRC patients has not yet been evaluated. Another study (51) identified MHC class II-restricted epitopes by induction of CD4+ T cells from the PBMCs of healthy individuals and MSI+ CRC patients, and one of the peptides was shown to be recognized by TILs from one unusual MSI+ CRC patient who had dominant CD4+ T-cell infiltration in tumor, instead of CD8+ T cells. These studies suggested that tumor-specific peptides generated by MSI might be recognized by T cells, although endogenous induction of immune response in typical MSI+ CRC patients has not clearly been demonstrated. Although the IgG Ab response in the HNP-8 patient suggested activation of CD4+ T cells, most of the tumor-infiltrating T cells were CD8+ T cells with some CD4+ T cells in the cancer stroma. The CDX2-specific memory or activated CD4+ T cells primed by dendritic cells presenting CDX2 in lymph nodes, may present in peripheral blood, lymphoid organs, or peritumoral area, and may not infiltrate into tumor tissues, in contrast to CD8+ T cells that can recognize CDX2 peptides associated with HLA class I on tumor cell surface. We have attempted to induce the mutated CDX2-specific T cells from PBMCs of this patient by *in vitro* stimulation with the recombinant proteins; however, specific T cells could not be induced, probably because we used PBMCs obtained from the patient whose serum no longer contained anti-CDX2 Ab 7 years after the surgical resection of tumor (data not shown). However, previous isolation of tumor antigens recognized by CD8+ T cells as well as CD4+ T cells using SEREX may suggest possible recognition of the mutated CDX2 peptides by CD8+ TIL and involvement of the antitumor immune response in this patient. Further analysis of additional patients with MSI+ CRC to examine whether the tumor-specific peptides can be recognized by CD8+ TIL is important.

Frameshift peptides may be useful target antigens for the development of immunotherapy because they are tumor specific, possibly highly immunogenic, and less likely to develop antigen-loss variants if the mutations are involved in the proliferation and survival of tumor cells. Although the identified mutated CDX2 is not useful for a broad population of patients because of the low frequency of this CDX2 mutation (53–55), SEREX may identify more common peptides generated by MSI. Frameshift peptides may also be useful as tumor markers for the diagnosis of MSI+ tumor. In this study, IgG Ab specific for CDX2 had disappeared from the serum of the patient 7 years after the surgical resection, suggesting complete disappearance of tumor in the patient. These serum Ab or mutated antigens, if shed

in serum, may be useful for the diagnosis of disease, stage, and even MSI status.

CDX2, a member of the caudal-related homeobox family containing a homeodomain and conserved regions with *Drosophila melanogaster* protein *caudal* (42), is a transcription factor involved in the proliferation and differentiation of intestinal epithelial cells (56). In adult mice, CDX2 is preferentially expressed in intestinal epithelia, most abundantly in proximal colon (49), and regulates expression of genes important in differentiation and homeostasis of the intestine such as SI (40) and carbonic anhydrase I (57). CDX2 has been implicated in the development of intestinal tumors. Although homozygous null mice are embryonic lethal in the knockout mice, heterozygous null mice developed multiple intestinal polyps prominently in proximal colon (58–60). Decreased expression of CDX2 was reported in human CRC cell lines and CRC tissues (42, 48) and forced expression of CDX2 reduced the proliferation of colon cancer cell lines (44, 61). In this study, we have identified the mutation in CDX2 in MSI+ colon cancer and attempted to understand the role of the identified CDX2 mutation in tumor development because some of the MSI target proteins with frameshift mutations, such as TGFBR2 and BAX, were suspected to be involved in tumorigenesis (62, 63). Nuclear localization and similar transcription activity in the reporter assays in the CRC cell line indicate normal function of the mutated CDX2. However, relatively weak staining with anti-CDX2 Ab of tumor cells in the immunohistochemical analysis may suggest that the decreased expression was associated with the cancer development. Further investigation is required to clarify the role of the identified CDX2 mutation in the development of CRC.

In summary, we have demonstrated that immune responses to the tumor-specific peptides generated by frameshift mutation through MSI are endogenously induced in the MSI+ CRC patient with good prognosis and CD8+ T-cell infiltration in tumor, suggesting that these tumor-specific peptides may be useful as target antigens for immunotherapy and as tumor markers for the diagnosis of MSI+ tumors.

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