Generation of monoclonal antibodies to cancer/testis (CT) antigen CT10/MAGE-C2

Ran Zhang1*, Yong Zhu1, Liang Fang1, Xue-Song Liu1, Ying Tian1, Li-Hua Chen1, Wei-Ming Ouyang1, Xiao-Guang Xu1, Jin-Long Jian1, Ali O. Güre2, Sheila Fortunato2, Gerd Ritter3, Lloyd J. Old2, Andrew J. G. Simpson2, Yao T. Chen1, Boquan Jin1 and Achim A. Jungbluth2

1Department of Immunology, Fourth Military Medical University, Xi’an 710032, P. R. China
2Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, Box 32, New York, NY 10021, USA
3Weill Medical College of Cornell University, 1300 York Avenue, New York, NY 10158, USA
*These authors contributed equally to this work

Contributed by: LJ Old

CT10/MAGE-C2 is a recently identified antigen that, typically of cancer/testis (CT) antigens, can be found in various malignant tumors and in normal adult testis. As with many other CT antigens, our knowledge is based mainly on mRNA expression data. In the present study, we describe the generation of mAbs to CT10/MAGE-C2 for the analysis of its protein expression. Newly generated clones were chosen based on their reactivity in ELISA, immunoblotting, and immunohistochemistry (IHC). Emphasis was put on the reactivity of newly generated reagents on formalin-fixed, paraffin-embedded tissue to ensure their applicability to archival material. Eventually we selected two clones, LX-CT10.5 and LX-CT10.9, that showed intense reactivity to CT10/MAGE-C2 protein and CT10/MAGE-C2 mRNA-positive cell lines, but no cross-reactivity with other CT antigens. Both mAbs show superior staining characteristics in IHC and are applicable to frozen and paraffin sections. In tests, CT10/MAGE-C2 displays the typical CT pattern with regard to staining of germ cells, which is intense during the early maturation stages. In tumors, we analyzed a limited number of cases displaying the typical heterogeneous CT expression pattern. Interestingly, immunoreactivity was seen solely in the nucleus: No staining was seen in the cytoplasm of tumor cells.

Keywords: cancer/testis, tumor antigens, CT10/MAGE-C2, monoclonal antibodies, ELISA, immunohistochemistry

Introduction

CT antigens are found in various types of malignant tumors, whereas in normal adult tissues, their expression is generally restricted to testicular germ cells. To date, over 40 CT genes or gene families have been identified, with MAGE-A1 being the prototype (1, 2, 3). CT antigens can elicit autologous immune responses in tumor patients, and immunological methods such as autologous T cell assays and SEREX (serological analysis of recombinant cDNA expression libraries) have been used to identify many CT antigens (4, 5, 6, 7). Other techniques that were used for CT gene identification include representational-difference analysis and database mining (8, 9).

CT10/MAGE-C2, a novel CT antigen, was recently identified in melanoma cell line SK-MEL-37 by representational-difference analysis, a PCR-based subtractive hybridization method that isolates differently expressed genes by comparing the expression profile of two cDNA libraries (8, 10). The CT10/MAGE-C2 gene shows significant homology to CT7/MAGE-C1 and exhibits a typical CT antigen mRNA expression pattern. CT10/MAGE-C2 maps to chromosome Xq27, in close proximity to the CT7/MAGE-C1 and MAGE-A genes (8). Moreover, CT10/MAGE-C2 has a 3-bp difference to MAGE-C2 and HCA587, which have identical cDNA sequences (11). HCA587 mRNA has been detected in 59/105 (56%) of hepatocellular carcinoma patients tested (12). T lymphocyte precursors recognizing antigens encoded by MAGE-C2 have been identified both before and after vaccination with MAGE-A antigens in the recent evaluation of several small-scale therapeutic MAGE-A protein or peptide vaccines. In one of the vaccinated patients, a majority of the antitumor CT1 clones present recognized MAGE-C2-encoded antigens (13, 14). However, to date no data are available concerning CT10/MAGE-C2 expression at the protein level in either normal or tumor tissues. Consequently, in the present study we address this lack of knowledge by describing the generation of two mAbs to CT10/MAGE-C2 (LX-CT10.5 and LX-CT10.9) and their application for protein expression analysis in a limited number of tissues.

Results

After BALB/c mice were immunized with CT10/MAGE-C2 protein, spleen cell fusions were undertaken and hybridoma supernatants were screened by indirect solid phase ELISA (Figure 1). A total of 11 clones (LX-CT10.1 to LX-CT10.12) were positive for CT10/MAGE-C2 and negative for NY-ESO-1, SSX2, MAGE-A3, CT7, and CT16 (Figure 1).

Western blot analysis was used to assess antibodies for recognition of CT10/MAGE-C2 antigen that is naturally expressed in human tumor cell lines. The cell lines used in this analysis were pretyped for CT10/MAGE-C2 RNA expression by RT-PCR. Monoclonal Abs LX-CT10.5 and LX-CT10.9 recognized a protein with an apparent molecular weight of 49 kDa in NP-40 detergent lysates of CT10/MAGE-C2 expressing human melanoma cell lines (SK-MEL-37 and SK-MEL-55). This reactivity was not observed with a detergent lysate of a human melanoma cell line (SK-MEL-24) which does not express CT10/MAGE-C2. The 49 kDa protein co-migrated with the E. coli-derived recombinant CT10/MAGE-C2 protein recognized by those antibodies (Figure 2).
Figure 1

Solid phase ELISA of ascites fluid from 11 newly generated anti-CT10/MAGE-C2 clones (LX-CT10.1 to LX-CT10.12). (A) Titration assay using ascites from clones LX-CT10.1 to LX-CT10.12 and full-length recombinant CT10/MAGE-C2 protein. (B) Specificity analysis showing reactivity with CT10/MAGE-C2 and no reactivity with CT antigens NY-ESO-1, SSX2, MAGE-A3, CT7, and CT16.

Figure 2

Immunoblotting of human cancer cell lines pretyped by RT-PCR for CT10/MAGE-C2 mRNA expression with mAb LX-CT10.5 (NP-40 detergent lysates, chemiluminescence detection). Positive reaction of mAb LX-CT10.5 with a protein of an apparent molecular weight of 49 kDa (lane 1) in lysates of CT10/MAGE-C2 mRNA-positive melanoma cell lines SK-MEL-37 (lane 2) and SK-MEL-55 (lane 3); no reactivity was seen with the CT10/MAGE-C2 mRNA-negative melanoma cell line SK-MEL-24 (lane 4); the proteins detected by mAb LX-CT10.5 co-migrate with the 49 kDa E. coli-derived recombinant CT10/MAGE-C2 protein (arrow, lane 5).

The positive clones were then tested for reactivity by IHC employing various antigen-retrieval techniques. Testis with preserved spermatogenesis served as an initial test tissue. Clones LX-CT10.4, LX-CT10.5, LX-CT10.9, and LX-CT10.10 showed superior staining of testicular germ cells in the typical CT antigen pattern (Figure 3). Subsequent IHC analysis of snap-frozen testicular tissue showed immunoreactivity of clones LX-CT10.5 and LX-CT10.9 only; LX-CT10.4 and LX-CT10.10 were negative. Consequently, clones LX-CT10.5 and LX-CT10.9 were used for further analysis (Figure 3). Both clones showed a similar staining pattern that was optimal using DAKO hipH antigen retrieval solution. In testicular tissues, both clones had intense nuclear staining of the spermatogonia and less intense staining of spermatocytes. No staining was seen in Sertoli cells or interstitial tissue. In an RT-PCR/IHC parallel analysis, 28 lung tumor specimens were studied side by side for mRNA and protein expression. Twenty tissues were CT10/MAGE-C2 mRNA-positive, and eight tissues were CT10/MAGE-C2 mRNA-negative. In 20 cases, RT-PCR and immunostaining were congruent (RT-PCR/IHC: 13x +/+, 7x --); 8 cases showed discrepant results (RT-PCR/IHC: 7x +/-; 1x +/). In all analyzed tissues, immunostaining was exclusively nuclear (Figure 3). The staining was mostly heterogeneous, involving only portions of the tumor. No significant staining was seen in the cytoplasm or in any normal tissue adjacent to the tumor. In the tissue microarray of normal tissues, staining was seen in testicular tissue, but not in any other tissue (esophagus, stomach, duodenum, ileum, colon, liver, gall bladder, pancreas, mesentery, thyroid gland, salivary gland, adipose tissue, skeletal muscle, adrenal gland, lymph node, peripheral nerve, thymus, spleen, tonsil, lung, prostate, kidney, urinary bladder, mammary gland, skin).
Discussion

Although they were originally identified in melanoma, CT antigens have since been found in a wide array of tumors, but not in normal tissues except for germ cells and, occasionally, placenta (15). Due to this expression pattern, and due to their ability to elicit either cytotoxic T cell or serological immune responses in the autologous host, CT antigens have generated considerable interest as targets for cancer immunotherapy (2, 16, 17). Several early-phase clinical trials employing CT antigens have been initiated or completed. Immune responses were clearly demonstrated and were indicative of clinical efficacy (18, 19, 20). For an accurate assessment of the suitability of individual CT antigens as potential vaccines, and subsequently as an eligibility criterion for patient entry into clinical trials, it is essential that the antigen distribution within, and between, tumors be determined using specific and carefully characterized mAbs. Due to the techniques currently employed to isolate CT antigens, information about their mRNA expression is readily available, but protein expression analyses require the laborious and time-consuming generation of serological reagents and are hence scarce. Consequently, much less is known about the actual expression of these antigens at the protein level. Monoclonal Abs have been developed for only some of the more than 40 CT antigens, such as MAGE-A1, MAGE-A4, MAGE-A11, CT7/ MAGE-C1, and NY-ESO-1 (18, 19, 20, 21, 22, 23, 24, 25, 26). In the present study, we report the generation of a mAb to CT10/ MAGE-C2, a recently identified CT antigen.

As a result of our prior experience in generating mAbs against CT antigens, the present study uses several approaches to demonstrate mAb specificity, including ELISA, Western blot assays, and IHC analysis of RT-PCR pretyped tumor tissues.

In normal tissues, we found CT10/MAGE-C2 protein expression to be consistent with that generally exhibited by CT antigens, with antibody reactivity restricted to testis among the normal adult tissues assayed. As with other CT antigens encoded on the X chromosome, such as NY-ESO-1 and the MAGE-A antigens, CT10/MAGE-C2 protein is most abundant in spermatogonia and is present at lower amounts in later-stage germ cells such as spermatocytes (21, 22, 27).

In the context of cancer, we analyzed a limited number of RT-PCR pretyped lung tumor specimens. The majority of specimens show concordance between protein and mRNA expression. However, in a number of samples, the RT-PCR data did not agree with those generated by IHC. Similar discrepancies between mRNA and protein expression had previously been found for several other CT antigens (21, 22, 27, 28). This appears to be largely due to the heterogeneous expression of CT antigens in tumor tissue. In contrast, an almost...
complete concordance of protein and mRNA expression is generally seen for melanocyte differentiation antigens such as tyrosinase and Melan-A (MART-1), probably due to the much more continuous nature of their expression in tumors (29, 30, 31). Also, it is possible that the newly generated antibodies may be identifying an alternatively spliced protein or a truncated protein that the primers used in the present study were not able to detect. In this context, it should be kept in mind that the members of several CT antigen families show a high degree of homology, and cross-reactivities of the serological reagents cannot be fully excluded. In the limited number of tumor specimens we examined, CT10/MAGE-C2 protein was restricted to the nucleus of neoplastic cells. This contrasts with NY-ESO-1 and with most MAGE-A antigens, which are predominantly present in the cell cytoplasm. However, MAGE-A11 is also exclusively present in the nucleus in cancer cells (25).

Interestingly, at a molecular level CT10 shares significant similarity to CT7 which is typically expressed in the cytoplasm and nucleus of tumor cells (8, 24). Little is known about the biological function of CT antigens, although recent data indicate that CT antigens can act as co-repressors or co-activators of gene expression by interacting with DNA-binding proteins. In this context, the presence of CT10/MAGE-C2 in the nucleus is especially intriguing.

In the present study we report the generation of new mAbs to CT10/MAGE-C2. A preliminary IHC analysis confirms a typical CT expression pattern and suggests, on a cellular level, an exclusive nuclear localization of the protein. Further studies of the expression and localization of the CT10/MAGE-C2 protein are now possible and are necessary in order to analyze its presence in various tumors and help to clarify its biological function.

Abbreviations
CT, cancer/testis; IHC, immunohistochemistry

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References


### Materials and methods

#### Antigen and ELISA

Full-length recombinant human (rh) CT10/MAGE-C2 protein (372 aa) was generated in *E. coli* as previously described (32). Briefly, the complete CT10/MAGE-C2 coding region was cloned into pQE30 to generate an N-terminal His-tagged fusion protein.

For ELISA analysis of hybridoma supernatants, microtiter polystyrene plates (Nunc, AGA, Roskilde, Denmark) were coated with 5 µg/mL rhCT10/MAGE-C2 in coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6), and incubated overnight at 4°C. Various dilutions of hybridoma supernatant were added. Detection of the primary antibody was done with a peroxidase-labeled goat-antimouse IgG Ab (1:2000; DAKO, Glostrup, Denmark). TMB substrate (Sigma, St. Louis, MO, USA) was used as chromogen. Results were analyzed at 450 nm on a microplate reader (BIO-RAD, Hercules, CA, USA).

#### Production of hybridomas

Purified rhCT10/MAGE-C2 protein was used to immunize BALB/c mice, and hybridomas were generated and cloned as previously described (32). In brief, mice were immunized three times with 20 µg rhCT10/MAGE-C2 protein at 3-wk intervals, followed by a booster injection 10 d after the final immunization. Complete Freund's adjuvant was used for the first injection; IFA was used thereafter, except for the booster injection, which was administered without adjuvant. Spleen cells from immunized mice were fused with SP2/0 mouse myeloma cells. Hybridomas were cultured in RPMI 1640 medium (Gibco/Invitrogen, Carlsbad, CA, USA) and screened against the immunizing protein by solid phase ELISA. Positive hybridomas were subcloned four times by limiting dilution, and then screened again by ELISA. Positive clones were expanded in 24-well and 75-cm² flasks (Nunc, AGA, Roskilde, Denmark), or as ascites fluid after i.p. injection of hybridoma cells. The Ig isotype was identified using an isotype kit (Sigma, St. Louis, MO, USA, ISO-2).

#### Antibody purification

Monoclonal Abs were purified from supernatant or mouse ascites by protein G affinity chromatography as described by the manufacturer (Pierce, Rockford, IL, USA)
Western blot analysis

Human cancer cell lines were lysed with 0.5% NP-40 in 0.15 M NaCl, 0.01 M MgCl₂, 0.01 M Tris-HCl, pH 7.5 containing Halt Protease Inhibitor Cocktail (Pierce, Rockford, IL, USA). Samples of lysates containing 25 µg total protein and recombinant CT10/MAGE-C2 antigen (0.05 µg) were resolved on 4-12% Bis-Tris SDS-PAGE gels (Invitrogen, Carlsbad, CA, USA) under reducing and nonreducing conditions. Proteins were blotted onto polyvinylidene difluoride membranes (Immobilon-P, Millipore, Billerica, MA, USA) and incubated overnight with 1 µg/ml primary antibody, followed by incubation with HRP-labeled goat-antimouse polyclonal antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Specific binding was visualized by chemiluminescence (Western Lightning Plus, Perkin Elmer Life Sciences, Boston, MA, USA).

IHC staining

Clones with anti-CT10/MAGE-C2 reactivity in ELISA and Western blot assays were then analyzed by IHC. The analysis was initiated using formalin-fixed, paraffin-embedded (FFPE) tissues to ensure applicability to standard archival material. Testis known to be CT10/MAGE-C2 mRNA-positive was used as the initial test tissue. Each supernatant was analyzed at different dilutions using several heat-based antigen-retrieval methods. Five micron cuts of testis were applied to slides for IHC, deparaffinized, rehydrated in a series of graded alcohols, and then incubated in 3% H₂O₂ for 20 min to block endogenous peroxidase reactivity. Antigen retrieval was done using a vegetable steamer and various buffer solutions (citrate buffer, 10 mM, pH 6.0; EDTA buffer, 1 mM, pH 8.0; DAKO hipH buffer). The tissue sections were then incubated with the primary antibody in a humid chamber at 4°C overnight. Detection of the primary antibody was performed with a biotinylated horse-antimouse antibody (Vector, Burlingame, CA, USA) followed by an avidin-biotin-complex system (ABC-Vector, Elite, Vector, Burlingame, CA, USA). Diaminobenzidine tetrahydrochloride (DAB, BioGenex, San Ramon, CA, USA) was used as chromogen. The clones with superior staining were then titered again on testis tissue to achieve optimal results. Clones that were immunopositive on FFPE slides were then tested on frozen testis tissues. Additionally, these clones were tested on a limited number of tumor tissues pretyped by RT-PCR for CT10/MAGE-C2 expression. Finally, a tissue microarray of normal tissues was tested for CT10/MAGE-C2 staining. All tissues were obtained in compliance with the legal regulations of the Department of Pathology of the Xijing Hospital, Xi’an, China and the Ludwig Institute of Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, USA.

Contact
Address correspondence to:

Dr. Boquan Jin
Tel.: + 86 29 8477 4598
Fax: + 86 29 8325 3816
E-mail: immu_jin@fmmu.edu.cn