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Identification of Protamine 1 as a novel CT antigen in early CLL

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Summary

Early chronic lymphocytic leukemia (CLL) is an ideal disease for immunotherapy. We previously showed that SEMG 1 is a Cancer-Testis (CT) antigen in CLL. In this study, we applied SEMG 1 as the bait in a yeast two-hybrid system of a testicular cDNA library. We isolated seven clones and identified Protamine (Prm) 1 as a novel CT antigen in early CLL. Prm 1 transcripts were detected in 11/41 (26.8%) patients. Prm 1 protein was also expressed but heterogeneously within individual patients. Of the 11 patients expressing Prm 1, four expressed Zap 70 protein and seven did not. These results, therefore, indicate that Prm 1 could potentially be a suitable target for the design of tumor vaccine for patients with early CLL, including for those with poor risk CLL. High titers Prm 1 IgG antibodies could be detected in 20 of these 41 CLL patients but not in any of the 20 healthy donors ($p = 0.0001$), suggesting the presence of Prm 1-reactive immune responses within immune repertoire of patients with early CLL. Further work is warranted, especially in approaches to upregulate Prm 1 expression, to determine the role of Prm 1 as an immunotherapeutic target for early CLL.

Keywords

Protamine 1; CT antigen; CLL; Zap 70

Introduction

Despite advances in modern chemotherapy, CLL remains incurable. Various clinical observations suggest that CLL cells are susceptible to T-cell cytotoxicity. These include 'spontaneous' remission of the disease during increased immunity (Ribera *et al*, 1987), responses to α -interferons (Ziegler-Heitbrock *et al*, 1989) and the graft-versus-leukemia effect following allogeneic stem cell transplant (Ritgen *et al*, 2004). Tumor-reactive T cells have also been demonstrated in some CLL patients (Gitelson *et al*, 2003; Goddard *et al*, 2001). CLL may, therefore, be an ideal disease for immunotherapeutic approaches. Immunotherapy induces cytotoxic mechanisms that may synergize with that by chemotherapeutic agents. Immunotherapy also offers the prospect of inducing immune memory that may be important for long term disease-free survival. However, there are

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obstacles that prevent successful immunotherapy. Tumor-bearing patients are usually immunosuppressed, due to the disease process and also treatment for the disease. Furthermore, antigen expression by tumor cells is generally heterogeneous, opening up the opportunity for the emergence of antigen-negative variant tumor cells. Clinical successes may also be hampered by the relatively high tumor burden, when compared to the antigen-specific effector cells generated by the tumor vaccine *in vivo*. Identification of tumor antigens that are co-expressed within a tumor specimen will overcome some of these problems. These antigens could be used for the design of polyvalent vaccines to produce higher effector:target ratios *in vivo* and reduce the chance for tumor escape by the emergence of variant tumor cells that do not express one particular antigen.

We have previously found SEMG 1 to be a Cancer-Testis (CT) antigen (Zhang *et al*, 2003). SEMG 1 is major protein of semen coagulum that inhibits human sperm capacitation (De Lamirande *et al*, 2001). It plays an important role in sperm clotting and is normally degraded into smaller fragments by prostate-specific antigen. The gene encoding SEMG 1 has been localized to the long arm of chromosome 20 (Ulvback *et al*, 1992), a region of chromosome 20 that is frequently deleted in myeloproliferative diseases and myelodysplastic syndrome (Asimakopoulos *et al*, 1994).

Most intracellular proteins are expressed in conjunction with their interacting ligands. Since SEMG 1 is a CT antigen (Zhang *et al*, 2003), the protein interacting with SEMG 1 is likely to also show expression restricted to normal testis and may be another CT antigen. Identification of this protein provides the opportunity for its application with SEMG 1 in a bivalent tumor vaccine. We have recently applied a CT antigen (Sp17) as the bait in a yeast two-hybrid system of a testicular cDNA library and successfully identified Ropporin 1 as a novel CT antigen (Li *et al*, 2007). In the present study, we have applied SEMG 1 to this system to isolate novel CT antigens for early CLL. We also determined the relationship of this protein to SEMG 1 and Zap 70 expression. The relationship between Prm 1 and Zap 70 was evaluated to determine how applicable a Prm 1-based tumor vaccine would be applicable to CLL patients predicted to have poor survival outcome as judged by Zap 70 expression. The data may provide the rationale for further studies into the design of a bivalent tumor vaccine targeting SEMG 1 and the interacting protein for patients with early stage CLL.

Materials and Methods

Clinical materials

Clinical materials consisted of peripheral blood from patients with CLL. All clinical materials were obtained after informed consents and with approval from the local ethics committee. Total RNA and cytospin specimens were prepared from these samples. All RNA samples were cryopreserved at -80°C until being used in experiments.

Screening of a yeast two-hybrid testicular library using SEMG 1

Full length SEMG1 cDNA was amplified by PCR. The primers were: 5' primer: 5'-GCG AAT TCC AAA AAG GTG GAT CAA AAG-3' and 3' primer: 5'-GCC TGC AGG TGT AAA TAA TGG GTT TCG-3'. Sp17 cDNA was amplified by PCR. Successful amplification of the SEMG 1 cDNA was confirmed by sequence analysis and then sub-cloned into pGBKT7 between *EcoR I* and *Pst I* restriction sites. pGBKT7-Sp17 plasmid was transformed into yeast strain AH109 and selected on SD/-Trp plates. Mating was performed between AH109-pGBKT7-Sp17 and pre-transformed human testis cDNA library in yeast strain Y187. Following mating, the culture was first selected on SD/-His/-Leu/-Trp plates and then on

SD/-Ade/-His/-Leu/-Trp /X- α -Gal plates. Yeast plasmids were purified from the positive colonies and subjected to nucleotide sequence analysis.

Reverse transcription-polymerase chain reaction

Total RNA was isolated using an RNeasy kit (Qiagen, Inc., Valencia, CA) according to the manufacturer's recommendation. Reverse transcription-polymerase chain reaction (RT-PCR) was performed. Briefly, all RNA specimens were first treated with DNase I (Ambion, Inc., Austin, TX) to remove genomic DNA contamination. First strand cDNA was synthesized from 1 μ g of total RNA using a random hexamer primer. To amplify Protamine 1 gene segment, the following pair of oligonucleotides was used-5'Protamine1: 5'-CCG CCA GAG ACA AAG AAG TC-3' and 3'Protamine: 5'-TTC TCA GGC AGG AGT TTG GT-3'. They amplify a Protamine 1 gene segment of 149 bp. PCR was performed using 35 amplification cycles at an annealing temperature of 66 °C. To amplify SEMG 1 gene segment, the following pair of oligonucleotides was used-5'SEMG1: 5'-AGC AAG ATG AAG CCC AAC AT-3' and 3'SEMG1: 5'-GAC TTT TTC GGG ACT GGT CA-3'. They amplify a SEMG 1 gene segment of 252 bp. Negative controls in all the PCR reactions contained the PCR reaction mixture except for cDNA that was substituted with water. RNA integrity in each sample was checked by amplification of a β -actin gene segment. Successful removal of genomic DNA contamination was confirmed in each sample by amplification of the RNA without prior RT reaction. PCR products were visualized on an ethidium bromide agarose gel. All results were confirmed on two independent RT-PCRs.

Immunocytochemical staining

Cytospin specimens were prepared and air-dried. The fixed cells were treated with 0.4% Triton X solution before the addition of a diluted murine monoclonal antibody against SEMG 1 (in-house, unpublished) or a rabbit polyclonal antibody against Protamine 1 (Santa Cruz Biotechnology, Inc.) for 2 hours. Following washing, the slides were overlaid with the respective peroxidase-conjugated secondary antibody for 30 minutes. The slides were washed again in PBS and overlaid with diaminobenzidine reagent. Following washing in PBS, the slides were counter-stained in hematoxylin.

Expression of GST-protamine 1 fusion protein

Recombinant GST-protamine 1 fusion protein was generated as described previously (Nikolakaki *et al.*, 2008). Briefly, the human protamine 1 gene was first amplified from normal testis RNA, cloned into pGEM[®]-T Vector System I (Promega) and its sequence was verified. The protamine 1 cDNA was then amplified by PCR using the pGEM[®]-T recombinant vector as template using the following primer pair: 5'-GCG GGA TCC ATG GCC AGG TAC AGA TGC TGT C-3' and 5'-CCC GGA ATT CGT GTC TTC TAC ATC GCG GTC T-3'. The PCR product was purified using the QIAEX[®] II Gel extraction kit (QIAGEN Inc.), digested with *Eco*RI and *Bam*HI, repurified, and ligated into the *Bam*HI/*Eco*RI site of pGEX-2T (construct termed GST-prm1). *E. coli* strains BL-21 were transformed by standard methods. The GST fusion protein was produced in bacteria and purified using glutathione-Sepharose beads according to the manufacturer's instruction (Amersham Pharmacia). The solubility of the recombinant protein was dramatically increased by adding 1 M NaCl to all steps of the purification procedure, except the last step, where GST-prm1 was recovered from the beads by incubation in the normal elution buffer, consisting of 50 mM Tris-HCl pH 8.0 and 10 mM reduced glutathione.

Enzyme-Linked Immunosorbent Assay (ELISA)

Antibodies directed at Prm 1 protein were detected in the patients' sera using an in-house ELISA system. Briefly, ninety-six well flat-bottom microtiter plates were coated with the

purified recombinant Prm protein at a concentration 5 µg/ml. After 4 hours adherence of the antigen to the plate at 37 °C, the wells were washed and then blocked with 3% bovine serum albumin in phosphate buffer saline (PBS) at 37 °C for 2 hours. Patients' sera were diluted 1:1000 with the blocking buffer and then dispensed into the wells in triplicates and allowed to bind overnight at 4 °C. Goat anti-human IgG alkaline phosphatase conjugated (Sigma, St. Louis, MO) was then added to each well (1:1000 dilution in the blocking buffer). After 2 hours of incubation at room temperature, p-nitrophenylphosphate solution was added to each well and incubated at room temperature for color development. Twenty five µl of 2N NaOH was added to stop the reaction. Color intensity was measured on a microplate reader (Molecular Devices, Sunnyvale, CA) and analyzed using the Softmax data analysis program. In each experiment, the controls consisted of wells coated with PBS only prior to the addition of the blocking buffer. All experiments were carried out in triplicates and results were confirmed in 2 independent experiments.

Results

Protamine 1 is a novel CT antigen in CLL

We first applied SEMG 1 as the bait in a yeast two-hybrid system of a testicular cDNA. Following plating on selection plates, a total of seven positive colonies were isolated. These colonies were expanded and nucleotide sequence analysis of the clones was performed to determine the identity and sequence homology of the cDNA using the BLAST software on US National Molecular Biology Laboratory and GenBank data bases (Table 1). Following an initial RT-PCR screening of RNA from a small sample of CLL patients, Prm 1 was selected for further study because it was found to be expressed in a proportion of CLL samples.

Using a pair of sequence-specific primers in RT-PCR on total RNA derived from a panel of normal tissues, we demonstrated the normal tissue expression of human Prm 1 that showed a very restricted normal tissue expression; transcripts encoding the Prm 1 gene could not be detected in any of the normal tissues except in testis (Figure 1). We next determined the frequency of Prm 1 expression in a cohort of CLL patients with early stage disease. Prm 1 transcripts were detected in 11/41 (26.8%) of these patients (Figure 2a), suggesting that Prm 1 is indeed a CT antigen in CLL. In contrast, Prm 1 was not detected in all 20 healthy donors (data not shown) (χ^2 ; $p < 0.01$). Using specific antibodies in ICC, we demonstrated that Prm 1 gene expression was associated with Prm 1 protein synthesis in the CLL cells (Figure 2b). However, Prm 1 protein expression within individual CLL patients was heterogeneous (Figure 2b). The median % CLL cells expressing Prm 1 protein within individual CLL patients was 10 (range 5-90), suggesting that if Prm 1 is to be used as a target for immunotherapy of CLL, approaches that upregulate Prm 1 expression are needed before immunotherapy to increase the number of leukemia cells susceptible to killing by Prm 1-specific cytotoxic T cells.

In order to establish whether Prm 1 and SEMG 1 is a suitable pair of targets for a bivalent tumor vaccine development, we then determined whether these two antigens were co-expressed within individual CLL patients. SEMG 1 was expressed in 19/41 (46%) of these patients. Eleven of the 41 patients (26.8%) co-expressed Prm 1 and SEMG 1 (Figure 3). Interestingly, although all the specimens that expressed Prm 1 also expressed SEMG 1, SEMG 1 expression did not necessarily predict for Prm 1 expression. Only 58% of CLL expressing SEMG 1 also expressed Protamine 1.

Zap 70 expression in CLL is associated with poorer survival outcome of the patients. If a tumor vaccine is going to be used as first line therapy for patients with early stage CLL, who normally would not need any specific therapy, it may be more acceptable clinically if it is

applied to patients predicted to have poorer survival outcome, as determined by Zap 70 expression. When the relationship between Prm 1 expression and Zap 70 expression was evaluated, we did not find any difference in the frequency of Prm 1 expression between Zap 70+ CLL and Zap 70- CLL. Four of 15 (27%) patients with Zap 70+ CLL expressed Prm 1 while 7/26 (27%) patients with Zap 70- CLL expressed Prm 1.

Generation of Prm 1 recombinant protein

Recombinant Prm 1 protein was generated from *E. coli* as a fusion protein with GST. Successful generation of the purified fusion protein was first demonstrated by Coomassie blue staining of SDS-polyacrylamide gel (Figure 4A) showing a protein band of an expected molecular weight of 25 kD. The identity of the protein band was then confirmed in Western blot analysis using a polyclonal rabbit anti-human Prm 1 antibody (Figure 4B).

Antibodies against Prm1 can be detected in patients with early stage CLL

Although Prm 1 is expressed in early stage CLL patients, it remained to be determined if immune responses against Prm 1 were intact within the immune repertoire of these patients. To investigate this, we determined the presence of Prm 1 antibodies in the sera from these patients using the purified recombinant Prm 1 protein in an ELISA system. We first established the basal signals in the ELISA system using sera from 20 healthy donors (Mean $\pm$ SD at OD_{405nm} = 0.087 $\pm$ 0.03). Using the mean + 3 SD from these 20 sera as the cut-off signal intensity at OD_{405 nm}, we found that high titer IgG antibodies against Prm 1 protein were frequently detectable by ELISA in patients with early stage CLL (Figure 5). IgG antibodies against Prm 1 protein was detected by ELISA at a serum dilution of 1:1000 in 20/41 (49%) of these patients, supporting the notion that Prm 1 reactive immune responses were intact within the immune repertoire of patients with early stage CLL. Interestingly, the mean level of Prm 1 antibodies in patients with Prm 1-negative CLL was higher than that in Prm 1-positive CLL. Among the 20 patients with Prm 1 antibodies detected, 7 were Zap 70+ and 13 Zap 70-. The mean OD_{405 nm} was significantly higher in Zap 70+ than Zap 70- CLL patients (mean OD_{405 nm} of 0.383 $\pm$ 0.14 vs 0.231 $\pm$ 0.06) ($p < 0.002$). When we analyzed the intensity and frequency of Prm 1 protein expression within individual specimens against the corresponding levels of Prm 1 antibodies, we did not observe any obvious correlation between levels of Prm 1 antibodies in the serum and the intensity and frequency of Prm 1 protein expression within the CLL cells. These sera were tested in high dilution of 1:1000 in order to improve the specificity of the antibodies. In contrast, none of the sera from the 20 healthy donors were positive for Prm 1 antibodies. The binding of the antibodies from these sera to Prm 1 protein was specific since no signal was observed in any of the samples in the control wells in the ELISA system.

Discussions

CLL is an indolent disease. Leukemia cells in CLL are susceptible to the cytotoxic activity of T cells. We previously found that SEMG 1, a CT antigen, is expressed in a proportion of patients with CLL (Zhang *et al*, 2003). Immunotherapy targeting SEMG 1 may, therefore, be a logical therapeutic option for these patients. Unfortunately, CLL patients are generally immunosuppressed even before any therapy is given (Ravandi and O'Brien, 2006; Orsini *et al*, 2003; Norris *et al*, 1980; Foa *et al*, 1986). The immunosuppression increases as the disease progresses, supporting the notion that successful immunotherapy is most likely to be achieved if the therapeutic approach is used to patients with early stage disease, even prior to use of any immunosuppressive chemotherapy. This could be argued for in CLL since it is presently recommended that patients with early stage CLL should follow a watchful waiting strategy because early treatment with chemotherapy has not improved survival. It is with this in mind that we set out to study a cohort of patients with early CLL.

Through the application of SEMG 1 as the bait in a yeast two-hybrid system of a cDNA library, we isolated Prm 1 in one of the positive clones. We showed that Prm 1 is a novel CT antigen in CLL, suggesting that SEMG 1 and Prm 1 may be a suitable pair of tumor antigens for the design of a bivalent tumor vaccine for early CLL. A bivalent tumor vaccine of SEMG 1 and Prm 1 will be expected to produce a higher *in vivo* effector:target ratio at an individual leukemia cell level to improve the cytotoxicity of the leukemia cells. Furthermore, Prm 1 is expressed in the CLL cells irrespective of Zap 70 expression, indicating that a Prm 1-based tumor vaccine could be applicable even to CLL patients with poor risk disease.

Prm 1 is a small highly basic, arginine-rich, protein of 51 amino acids that, together with Prm 2, replace histones during spermatogenesis (Oliva and Dixon, 1991; Oliva, 2006). The result of this progressive replacement is the formation of a highly compact chromatin structure devoid of any transcriptional activity. Prm 1 is present in all species of vertebrates studied, while Prm 2 is only present in some mammalian species including human and mouse (Oliva, 2006). The human gene encoding Prm 1 is localized to chromosome 16p13.2. Data from Gene Expression Atlas databank (<http://expression.gnf.org/cgi-bin/index.cgi>) as well as our RT-PCR analysis on total RNA derived from a panel of normal tissues clearly demonstrate that the expression of Prm 1 is restricted in normal testis. The restricted normal tissue expression of Prm 1 suggests that any tumor vaccine targeting Prm 1 will not induce toxicities in normal tissues except testis, although the clinical relevance of this remains debatable since most CLL patients are older patients and the blood-testis barrier inhibits contact between differentiating germ-line and immune cells.

Unfortunately, the expression of Prm 1 is heterogeneous, even within individual patients. Theoretically, any bivalent tumor vaccines based on these two antigens may induce the emergence of antigen-negative variant tumor cells, although it is possible that the stronger immune responses induced by a bivalent tumor vaccine may result in cross-priming of other tumor antigens that would induce specific immune responses against SEMG 1/Prm 1-negative variant tumor cells. The observation of heterogeneity of antigen expression highlights the likelihood that any future tumor vaccine protocol may have to incorporate the pharmacologic upregulation of the targeted tumor antigens, for example, using DNA hypomethylating agents for antigens regulated through promoter methylation (Wang *et al*, 2004; Wang *et al*, 2006).

The fact that Prm 1 expression predicted for SEMG 1 expression and that only 58% of SEMG 1-positive samples expressed Prm 1 suggests the following points. First, it is very likely that Prm 1 is only one of a number of proteins interacting with SEMG 1. This notion is supported in our studies by the findings of more than one distinct positive clones isolated from the cDNA library. It is, therefore, possible that SEMG 1 in Prm 1-negative CLL cells interact with the products from the other clones. Second, it is likely that the expression of Prm 1 is coordinated through specific intracellular mechanisms to the expression of SEMG 1 so that Prm 1 expression only occurs in the presence of SEMG 1. Third, if the function of one protein (either SEMG 1 or Prm 1) is dependent on the presence of its ligand, then these individual molecules expressed within the tumor cells are likely to be of some functional significance in the tumor cells from most patients.

We also demonstrated that B-cell responses, in the form of high titer IgG, against Prm 1 protein, occurred frequently in CLL patients and not in healthy donors. This finding supports the notion that Prm 1-reactive immune responses are still intact in patients with early CLL. The reasons for the detection of Prm 1 antibodies in Prm 1-negative CLL patients remain speculative. It is possible that the observed immune responses were a reflection of autoimmunity induced by immune dysregulation associated with the disease. However, the

observation that the mean level of Prm 1 IgG antibody was higher in Prm 1-negative than in Prm1-positive CLL patients suggests the possibility that the *de novo* anti-Prm 1 immune responses in this group of patients were stronger and hence more successful in eradicating Prm 1-positive CLL cells, leaving only Prm 1-negative variant leukemia cells.

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Figure 1.

RT-PCR showing the restricted expression of Protamine 1 gene in a panel of normal tissues (M = molecular marker; 1 = Testis; 2 = Placenta; 3 = Heart; 4 = Brain; 5 = Thyroid; 6 = Trachea; 7 = Salivary; 8 = Fetal brain; 9 = Uterus; 10 = Colon; 11 = Stomach; 12 = Spleen; 13 = Small intestinal; 14 = Bone Marrow)

Figure 2.

Expression of Protamine 1 in CLL cells. (a). RT-PCR showing the expression of Protamine 1 gene from six of the 11 Prm 1-positive specimens (M = molecular marker; 2, 3, 4, 5, 6 and 7 = Positive amplification showing the expression of Protamine1 gene segment from CLL patients; 1 = Negative amplification). (b). Immunocytochemical staining for Protamine 1 protein in CLL cytopsin specimens (A = negative control; B = Specimen with low % Protamine 1 expression; C = Specimen with high % Protamine 1 expression).

Figure 3.

RT-PCR detection for the co-expression of SEMG 1 and Protamine 1 genes in individual CLL specimens from eight patients, one expressing both SEMG 1 and Prm 1, one expressing only SEMG 1 and six expressing neither SEMG 1 or Prm 1 (M = molecular marker; 1, 2, 3, 4, 6 and 7 = negative amplification; 5 = specimen co-expressing SEMG 1 and Protamine 1; 8 = specimen expressing only SEMG 1) (a = amplification for the SEMG 1 gene segment; b = amplification for the Protamine 1 gene segment; c = amplification for the β -actin gene segment).

Figure 4. Successful generation of GST-Prm1 fusion protein, as demonstrated by Coomassie blue staining (A) and in Western blot analysis (B).