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Original Article

Characterization of C21ORF100 as a Novel Prostate Differentiation Antigen

Fabiana Bettoni*, MSc; Lilian T. Inoue*, USt;# Lilian C. Pires, BS; Raphael B. Parmigiani, PhD; Anamaria A. Camargo, PhD

Laboratory of Molecular Biology and Genomics, Ludwig Institute for Cancer Research, Sao Paulo – Brazil

* These authors contributed equally to this work and are listed in alphabetic order.

Undergraduate Student

Abstract

Differentiation antigens are immunogenic proteins expressed in specific cell lineages or at specific stages of differentiation in a particular tissue. Generally, their expression in normal cells is preserved after neoplasic transformation and this feature has made such molecules potential candidates for cancer immunotherapy. Using alignments between expressed sequence tags (ESTs) and the human chromosome 21 sequence, we have identified a novel gene, named C21orf100, which is exclusively expressed in normal prostate and codes for a putative protein of 55 amino acids. **Objective:** To characterize C21orf100 as a novel prostate differentiation antigen. **Material and Methods:** C21orf100 mRNA expression was determined by RT-PCR in 22 normal tissues and in 65 samples from melanomas and prostate, thyroid, stomach, uterus and breast tumors. The existence of a humoral immune response against C21orf100 protein in prostate cancer patients was evaluated by immunoblotting using a His-tagged recombinant protein. **Results:** As expected for a differentiation antigen, C21orf100 mRNA expression was predominantly detected in both normal and tumor prostate samples. Antibodies against C21orf100 recombinant protein were detected in 1 out of 50 (2%) plasma samples from prostate cancer patients and were not detected in the plasma from healthy blood donors. **Conclusion:** The restricted expression pattern and the detection of antibodies in prostate cancer patients suggest that C21orf100 is a novel prostate differentiation antigen. However, due to the low frequency of antibody response against C21orf100 detected among prostate cancer patients, further analysis is necessary to evaluate its potential for cancer immunotherapy.

Key words: Prostate. Differentiation antigen. Chromosome 21. Immunotherapy.

Introduction

Identification and molecular characterization of tumor antigens recognized by cellular and/or humoral effectors of the immune system is one of the major requirements for the development of tumor immunotherapy. It is also crucial for the development of polyvalent tumor vaccines designed to overcome tumor heterogeneity and immune escape.[1,2]

Differentiation antigens are lineage-specific antigens expressed in tumor cells as well as in normal cells from which the tumors arise.[3] Examples of differentiation antigens include tyrosinase and Glial Fibrillary Acidic Protein (GFAP), which are antigenic in malignant melanoma and glioma, but are also expressed in melanocytes and in normal brain cells, respectively.[4] The restricted expression pattern exhibited by differentiation antigens has made them useful markers in the immunopathological

Correspondence
Anamaria A. Camargo, PhD
Laboratory of Molecular Biology and Genomics
Ludwig Institute for Cancer Research
Rua Professor Antonio Prudente, 109, 4th floor
01509-010 São Paulo, Brazil
Phone number: 55 11 33883248
e-mail: anamaria@compbio.ludwig.org.br

differential diagnosis of malignant neoplasias and also potential candidates for cancer immunotherapy.[5]

During the identification of novel genes located on human chromosome 21 (HC21), we have found a gene (C21ORF100), which is predominantly expressed in normal prostate and codes for a putative protein of 55 amino acids.[6] In this work, we have refined the characterization of C21orf100 by analyzing its expression in samples from different tumors types. We have also evaluated the presence of a humoral immune response against C21orf100 recombinant protein in plasma samples from prostate cancer patients and in healthy control individuals. Our results suggest that C21orf100 is a novel prostate differentiation antigen and a potential candidate for cancer immunotherapy.

Material and Methods

Generation of C21orf100 full-length sequence

C21ORF100 sequence was obtained by sequencing the full insert of the cDNA clone from which the ESTs aligning to HC21 were derived. The cDNA clone (IMAGE 3289153) was obtained from Research Genetics (http://www.resgen.com) and was sequenced directly using vector's primers as described previously.[6] The cDNA sequence was translated to protein using the Translate Tool from Expasy (http://ca.expasy.org/tools/dna.html) and proteins domains were searched using the Pfam and Prosite databases.

mRNA expression analysis

C21orf100 mRNA expression was determined by nested RT-PCR since the expression level of this transcript was expected to be low. Total RNA derived from 22 different normal human tissues (testis, lung, prostate, small intestine, breast, brain, heart, uterus, bone marrow, placenta, colon, fetal brain, liver, fetal liver, thymus, salivary gland, spinal cord, kidney, spleen, skeletal muscle, trachea and adrenal gland) were commercially obtained from Clontech, Palo Alto, CA. Samples from six different types of tumors (prostate, thyroid, stomach, uterus, breast and melanoma) were collected from patients treated at A.C. Camargo Hospital, after explicit informed consent and with local ethics committee approval. Total RNA from tumor samples was extracted by conventional CsCl-guanidine thiocyanate gradient method.[7]. RNA samples were checked for integrity in 1% agarose gel and 2µg of the sample were used for cDNA synthesis. Reverse transcription was performed with DNA-free RNA using SUPERSCRIPT II Reverse Transcriptase (Invitrogen) and oligo dT. Primers used for expression analysis were designed in distinct exons so that the eventual amplification of genomic DNA could be distinguished from cDNA amplification. The first reaction was performed in a final volume of 25µL using 1µL of first strand cDNA, 1x Taq DNA polymerase buffer (GIBCO/BRL), 0.2mM dNTP, 1.6mM $MgCl_2$, 1 unit of Taq DNA polymerase (GIBCO/BRL) and 0.4µM of the following primers: C21orf100F1 5' CAACGTGACATTGTTTGGAG 3' and C21orf100R1 5' TGTCCATCTGA ATGCCCAC 3'. PCR conditions were as follows: initial denaturation for 4 min at 94ºC followed by 35 cycles of 30 sec at 94ºC, 45 sec at 60ºC and 60 sec at 72ºC and a final extension of 6 min at 72ºC. Nested-PCR was carried out in the same conditions using 1µL of the first reaction as template, 30 cycles of amplification and the following internal primers: C21orf100FN 5'TGGTGTCTGTGAAAAGGGG 3' and C21orf100RN5' TGGTTCATTCAGTA GCTCCAC 3'. PCR fragments were analyzed on 8% silver-stained polyacrylamide gels.

C21orf100 recombinant protein

C21orf100 longest open reading frame was amplified by RT-PCR from normal prostate using the following primers: Forward – 5' CGGAATTCATGCACATTATTTCTTTGAT 3' and Reverse – 5' CCCAAGCTTTCACTCA AGTATGACCTC 3'. Amplification conditions were as follows: initial denaturation for 2 min at 94°C followed by 35 cycles of 30 sec at 94°C, 30 sec at 60°C and 60 sec at 68°C and a final extension of 6

min at 68°C. The amplified C21orf100 cDNA fragment was gel purified, cloned into pGEM-T vector (Promega) and transformed into *E. coli* DH10B cells. Recombinant colonies were screened by PCR and inserts of the expected size were gel purified, digested with *EcoRI* and *HindIII* and cloned into the expression vector pET28a(+) (Stratagene). The recombinant plasmid pET28a(+)/C21orf100 was sequenced using Dynamic ET Terminator Cycle Sequencing (Amersham Biosciences) and ABI 3100 Prism sequencer. *E. coli* BL21-DE3 cells were transformed with the recombinant plasmid and grown to an A_{600nm} of 0.6. After induction with 4μM IPTG for 4 hours at 37°C, the His-tagged C21orf100 recombinant protein was purified by Ni^{2+} affinity chromatography using the NiNTA agarose (Invitrogen). Protein purification was checked by Western-blot using an anti-His tag monoclonal antibody (Amersham Biosciences).

Antibody response in cancer patients

Plasma samples were obtained from prostate cancer patients treated at the A.C.Camargo Hospital. All samples were collected after explicit informed consent and with local ethics committee approval. In addition, plasma samples from 30 healthy individuals were collected from blood donors at the A.C.Camargo Hospital Blood Bank. Antibodies against the recombinant C21orf100 protein were detected by Western-blot. Four hundred and twenty nanograms of purified recombinant C21orf100 protein were fractioned on 15% SDS-PAGE gel and transferred to Hybond-P PVDF membranes. After blocking with PBS solution containing 5% low-fat milk, the membranes were incubated for 1h at room temperature with plasma from healthy individuals or from cancer patients at a 1:25 dilution. Plasma antibodies binding to C21orf100 recombinant protein were detected by incubation with goat anti-human IgG HRP-conjugate (Amersham Biosciences) and visualized with ECL™ Western Blotting Detection Reagents (Amersham Biosciences).

Results

Gene structure

Using the information generated by alignments between expressed sequence tags (ESTs) and the genomic sequence of the human chromosome 21 (HC21), we have identified a gene, named C21orf100,[6] which is mapped on 21q21.3 and its sequence is divided into two exons. This transcript has two alternative splicing isoforms (Figure 1) and conserved GT-AG splice junctions are present in both isoforms. The isoform C21orf100A (AY063458) has 880 nucleotides and the isoform B (AY063459), derived from an alternative exon usage, has 524 nucleotides. Both isoforms have an open reading frame of 168pb that codes for putative protein of 55 amino acids with no predicted protein domains.

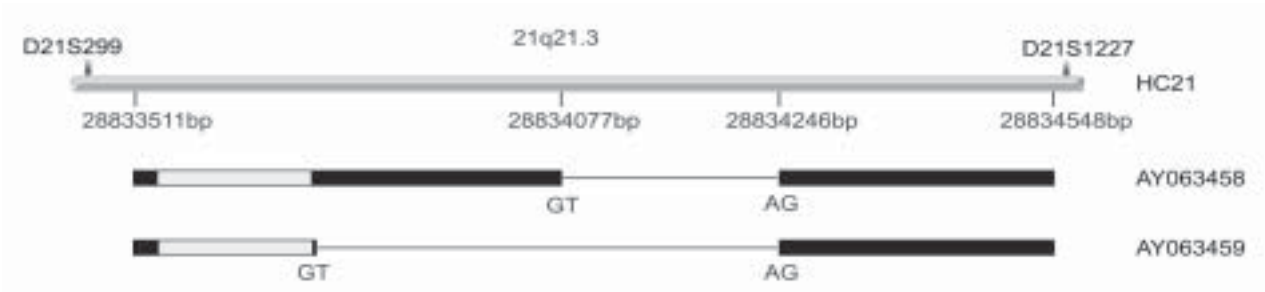

Figure 1 - Schematic representation of the exon/intron structure of C21orf100 gene and splicing isoforms. Grey line represents the genomic sequence of human chromosome 21 (HC21). Exons are represented by black boxes and introns by black lines. The region corresponding to the open reading frame is marked in white

mRNA expression analysis

C21orf100 mRNA expression was first determined by nested RT-PCR in cDNA samples derived from 22 normal tissues. As shown in Figure 2A, C21orf100 expression was found to be restricted to normal prostate among all normal tissues analyzed. C21orf100 expression was then analyzed in 65 samples from six different types of tumors (prostate, breast, thyroid, uterus, stomach and melanoma). As expected differentiation antigen, positive expression was detected in 80% (20/25) of the prostate tumor samples but in only 2 of 40 non-prostate tumors samples (Figure 2B).

Humoral immune response in prostate cancer patients

C21orf100 was expressed and purified as His-tagged recombinant protein (~11kDa) in *E.coli*. As shown in figure 3A, a single band of approximately 15kDa was detected after purification using an anti-His antibody. The difference between the expected molecular mass (~11kDa) of the recombinant C21orf100 protein and that visualized by Western-blot could be attributable to the partial neutralization of the protein charges by SDS, resulting in an altered migration in SDS-PAGE gel.[8]

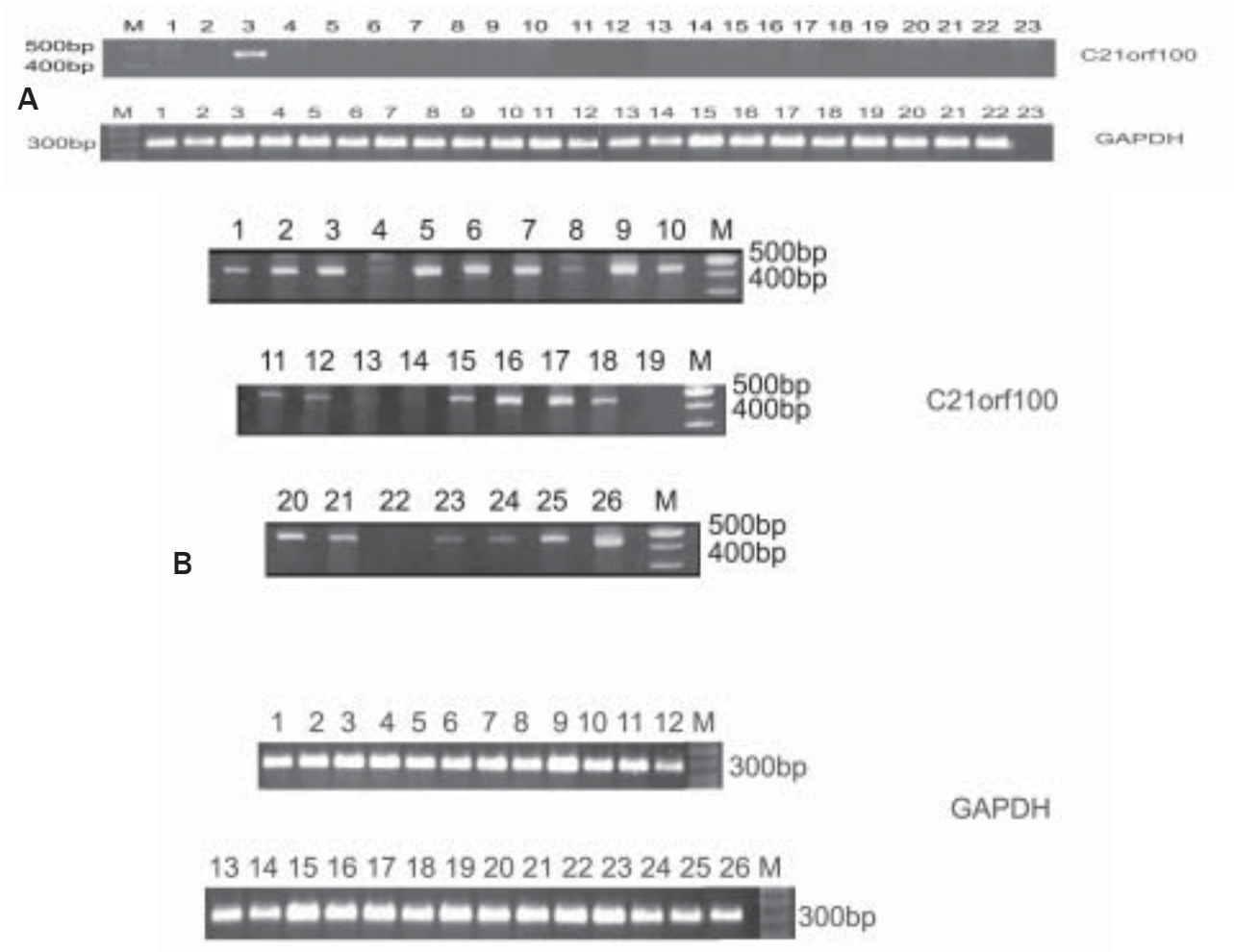

Figure 2 - C21orf100 mRNA expression pattern in normal tissues and prostate tumors (A) Normal cDNA samples analyzed by RT-PCR were: 1. testis, 2. lung, 3. prostate, 4. small intestine, 5. breast, 6. brain, 7. heart, 8. uterus, 9. bone marrow, 10. placenta, 11. colon, 12. fetal brain, 13. liver, 14. fetal liver, 15. thymus, 16. salivary gland, 17. spinal cord, 18. kidney, 19. spleen, 20. skeletal muscle, 21. adrenal gland, 22. trachea, 23. no cDNA negative control. (B) cDNA samples from prostate tumors analyzed by RT-PCR (1-25). Normal testis cDNA was used as positive control (26). GAPDH amplification was used as positive control for cDNA synthesis. Molecular weight markers are indicated in base pairs (bp)

To evaluate the presence of a humoral immune response against C21orf100 protein in plasma from prostate cancer patients we have established an immunoblotting assay using the recombinant His-tagged protein. Reactivity against the recombinant protein was detected in 2.0% (1/50) of the samples. Plasma samples from 30 healthy blood donors were used as negative controls and none of them was reactive to C21orf100 (Figure 3B).

In the current study, we have demonstrated that the C21orf100 gene codes for a new prostate differentiation antigen, being predominantly expressed in both normal and tumoral prostate tissue. Antibodies against C21orf100 recombinant protein were specifically detected by immunoblotting in

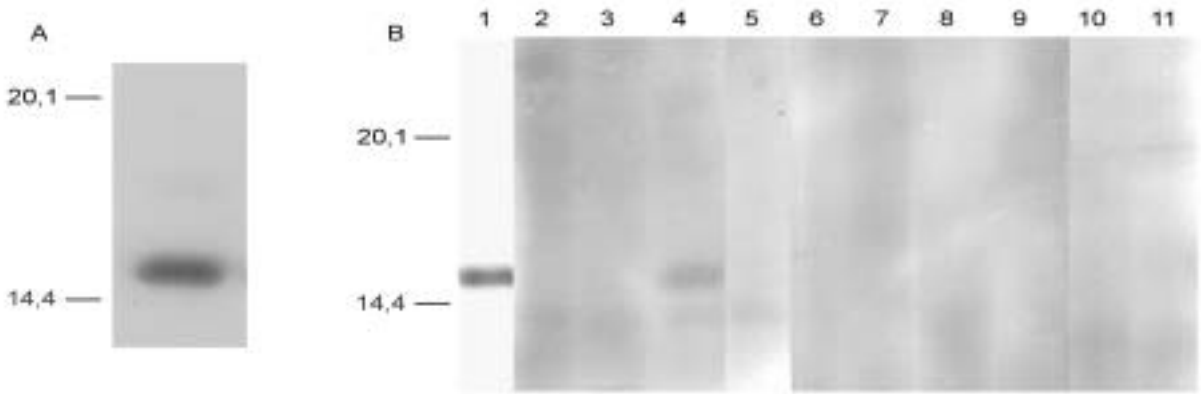

Figure 3 - Purification of the C21orf100 recombinant protein and detection of antibodies in plasma from prostate cancer patients. (A) Western-blot using an anti-His tag antibody and the purified recombinant protein. (B) Western-blot using plasma samples from prostate cancer patients (lanes 2-6) and from healthy individuals (lanes 7-11). An anti-His tag antibody was used as positive control for antibody detection (lane 1). The experiments were repeated twice. Molecular weight markers are indicated in kilodaltons (kDa)

Discussion

Prostate cancer is the most frequently diagnosed noncutaneous cancer and the second leading cause of cancer deaths in men.[9] Identification of prostate cancer molecular targets, which can be used as early detection markers or as target for alternative therapies, is of great importance since there is no current curative therapy for the metastatic disease.

Prostate differentiation antigens are potential targets for immunotherapy and their identification is essential for the development of polyvalent cancer vaccines designed to overcome obstacles associated with tumor heterogeneity and antigen loss, thus increasing the number of cancer patients eligible for vaccination[1]. Differentiation antigens are not tumor-specific and their use as targets for cancer immunotherapies may result in an autoimmune toxicity towards the normal tissue which expresses the protein. However, this autoimmunity may not be a major concern when the organ is not vital or even when it has already been surgically resected in the course of cancer therapy.[10]

2.0% (1/50) of the plasma samples from prostate cancer patients, suggesting that this protein could be target by immunotherapy. However, due to the low frequency of antibody response in patients with prostate cancer, further immunogenicity analyses in an extended number of patients are required to consider C21orf100 as a promising candidate for prostate cancer immunotherapy.

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