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Frequency and prognostic relevance of cancer testis antigen 45 expression in multiple myeloma

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Objective. **This study aims to analyze the expression of cancer testis antigen *45* (*CT45*) in normal tissues and in plasma cell disorders and to identify possible associations with clinical data and prognosis in multiple myeloma (MM) patients.**

Materials and Methods. **Expression of *CT45* was studied in 20 normal tissues (testis, placenta, skeletal muscle, bladder, lung, spleen, heart, brain and fetal brain, thymus, uterus, stomach, mammary gland, pancreas, prostate, small intestine, kidney, adrenal gland, spinal cord, colon, and one pool of 10 normal bone marrow samples) and bone marrow aspirates from 3 monoclonal gammopathies of undetermined significance, 5 solitary plasmacytomas, 61 newly diagnosed MM patients and MM cell line U266 by reverse transcriptase polymerase chain reaction.**

Results. ***CT45*** **was positive in 3 of 20 (15%) normal tissues tested: lung, brain (both fetal and adult), and spinal cord. Among monoclonal gammopathies, *CT45* was positive in 2 of 5 (40%) solitary plasmacytomas bone marrow aspirates, 10 of 61 (16%) MM bone marrow aspirates, and in the U266 MM cell line.**

Conclusions. **We did not find associations between bone marrow histology and *CT45* expression. However, we demonstrated for the first time that positive expression of *CT45* was associated with poor prognostic (International Staging System) and poor outcomes in MM patients, meaning that *CT45*-positive cases presented seven times more chance of worse evolution than the negative ones.** © **2009 ISEH - Society for Hematology and Stem Cells. Published by Elsevier Inc.**

Cancer testis (CT) antigens have become the most extensively studied antigen group in the field of tumor immunology. Recently, Chen and collaborators [1] discovered a new CT antigen by massively parallel signature sequencing, denominated *CT45*. CT45 is a peptide corresponding to the gene product of the newly described gene family MGC27005, located on chromosome Xq26.3, and is expressed in testis and in some tumor cell lines. This gene possesses six members and all are amplified together by reverse transcriptase polymerase chain reaction (RT-PCR). Each gene spans 8 to 9 kb and is located in tandem within a 125-kb region. The three centromeric genes are transcribed in the centromeric to telomeric direction, whereas the three telomeric genes are transcribed in the opposite direction [1].

The monoclonal antibody Ki-A10, an IgG1 generated after immunization of mice with Hodgkin's lymphoma cell line L428, is the first monoclonal antibody that detects CT45 [2]. Especially, in the aggressive variant of nodular sclerosis Hodgkin's lymphomas subtype, there was obvious Ki-A10 positivity, indicating that *CT45*-antigen expression may be associated with a more aggressive disease [2]. In MM, two independent groups described *CT45* expression in 44% (four of nine) of anaplastic plasmacytomas using Ki-A10 monoclonal antibody anti-CT45

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[2] and in 6% of multiple myeloma (MM) cases by microarray analysis [3].

The present study analyzed expression of *CT45* expression by RT-PCR in a panel of 20 normal tissues and in monoclonal gammopathies and showed for the first time that positive expression of *CT45* was associated with poor outcomes in MM patients, meaning that *CT45*-positive cases presented seven times more chance of worse evolution than the negative ones.

Materials and methods

Patients

Expression of *CT45* was studied in bone marrow aspirates from 3 monoclonal gammopathies of undetermined significance, 5 solitary plasmacytomas, and 61 MM samples (including 2 Durie-Salmon stage I, 2 stage II, and 57 stage III; Table 1). Samples were obtained between June 2002 and April 2006 at Hospital São Paulo UNIFESP/EPM, São Paulo, Brazil. All of these patients were newly diagnosed and treatment-naïve at the time of bone marrow harvest. According to the International Staging System (ISS) [4] (based on albumin and β2-microglobulin serum levels at diagnosis), there were nine cases classified as ISS 1, 21 ISS 2, and 26 ISS 3. In the 61 MM patients, bone marrow biopsies presented 10% to 98% of malignant plasma cells infiltration (median 80%). MM cases were classified as mature (70%), intermediate (or immature) (20%), and plasmablastic (10%), according to plasma cell morphology [5], by the same hematopathologist (M.R.R.S.). This study was approved by the Hospital São Paulo/UNIFESP Ethical Committee and all patients provided written informed consent prior to collection of bone marrow samples.

Table 1. Baseline characteristics of patients with MGUS, solitary plasmacytomas, and multiple myeloma

	MGUS	Solitary plasmacytomas	Multiple myeloma
Age range (y)	62–72	48–71	
Gender (n)			
Male	3	3	33
Female	0	2	28
Isotype			
IgA	—	—	15
IgG	2	1	34
NA	1	4	12
Light chain isotype			
Kappa	1	3	38
Lambda	1	1	22
NA	1	1	1
Durie-Salmon stage			
I	—	—	2
II	—	—	2
IIIA	—	—	37
IIIB	—	—	20
ISS			
1	—	—	9
2	—	—	21
3	—	—	26
NA	—	—	5
Plasma cell morphology			
Mature			41
Intermediate (immature)			12
Plasmablastic			6
NA			2
No. of patients	3	5	61

ISS = International Staging System; MGUS = monoclonal gammopathies of undetermined significance.

Controls

Expression of *CT45* was studied also in 20 normal tissues (Clontech, Palo Alto, CA, USA) that included testis, placenta, skeletal muscle, bladder, lung, spleen, heart, brain, fetal brain, thymus, uterus, stomach, mammary gland, pancreas, prostate, small intestine, kidney, adrenal gland, spinal cord, colon, and one pool of 10 normal bone marrow samples from Clontech. MM cell line U266 (TIB-196; ATCC, Manassas, VA, USA) was also submitted to analysis with MM cases. Samples of six normal bone marrow aspirates (donors for allogeneic stem-cell transplants) and three normal tonsils (obtained from children submitted to tonsillectomy) were also included.

RT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. RNA was recovered from the aqueous phase by ethanol precipitation and the pellets were dissolved in 30 μL DEPC water. First-strand cDNA synthesis, primed with oligo (dT) and 2 μg RNA template, was reverse-transcribed (Superscript II; Invitrogen) following manufacturer's instructions and diluted 10× in water. After cDNA synthesis, expression of β-actin was determined in all samples. The *CT45*-antigen expression was evaluated by RT-PCR and 2% agarose gel electrophoresis. Normal testis was used as template for positive controls in all RT-PCR reactions. PCR reactions were assembled in 0.2-mL tubes and conducted in a 25-μL total volume containing: 2 μL cDNA template, 1× PCR buffer, 1.5 mM $MgCI_2$, 0.2 μM dNTPs, 10 pmol each forward and reverse primers, 1.25 U Platinum Taq DNA Polymerase (Invitrogen). PCR steps were performed in an Eppendorf MasterCycler Gradient thermocycler (Hamburg, Germany) and primer sequences were *CT45*-F 5′-ctctgccatgtccaaagcaa-3′ and *CT45*-R 5′-aagtcatcaatctgagaatccaattg-3′. Conditions of cycles were: initial denaturation at 94°C for 2 minutes and 35 cycles of 94°C for 45 seconds, 55°C for 45 seconds, 72°C for 1 minute, and single-step elongation at 72°C for 7 minutes.

Statistical analysis

Associations between the studied variables were tested by the Pearson χ^2. Overall survival (OS) time was calculated from the date of diagnosis of MM until death or last follow-up. Actuarial probabilities of OS were estimated according to Kaplan-Meier method and the curves were compared using the log-rank test. The Cox's regression model was also employed to evaluate which variables could be considered independent prognostic factors on OS in this group of patients. The level of significance for all statistical tests was 5%. Statistical analysis was performed using SPSS 8.0 software.

Results and discussion

CT45 was positive in 3 of 20 (15%) normal tissues tested: lung, brain (both fetal and adult), and spinal cord. Among monoclonal gammopathies, *CT45* was positive in 2 of 5 (40%) solitary plasmacytomas' bone marrow aspirates, 10 of 61 (16%) MM bone marrow aspirates, and in the U266 MM cell line. Six of 10 (60%) *CT45*-positive cases were classified as ISS 3. Pearson χ^2 analysis showed that *CT45* expression was significantly found in MM cases classified as ISS 3 ($p = 0.009$, n = 56). Six *CT45*-positive cases were classified as mature and four as intermediate (or immature) plasma cells.

Attempts were made to demonstrate CT45 protein expression in CT45 mRNA-positive MM cases. However, preliminary immunohistochemical analysis using bone marrow biopsy specimens (B5 and formalin-fixed) and a monoclonal anti-CT45 antibody was unsuccessful (Y.-T. Chen, LICR-New York Branch, personal communication). Although this might indicate that CT45 protein is only expressed at low levels even in *CT45*-positive cases, the possibility that the antigen might have been lost during the process of fixation and/or decalcification cannot be excluded. Additional studies using prospectively collected fresh bone marrow aspirates or CT45 mRNA-positive MM would be needed to explore such possibilities, and these experiments are being pursued. We also believe that we could perform bead separation of CD138-positive cells and then perform PCR on both populations (plasma cells vs all other cells) on a positive sample to prove that the *CT45* expression we found was from the plasma cells and not the background cells. Unfortunately, we did not have CD138-positive cells from *CT45*-positive cases to perform such investigation.

Median OS of the MM group was 21 months (Fig. 1A). Nine patients were submitted to autologous stem cell transplantation. All of the transplanted cases were *CT45*-negative. Univariate analysis showed that Durie-Salmon Staging System (Durie-Salmon IIIA: n = 35, median OS = 40 months; Durie-Salmon IIIB: n = 19, median OS = 12 months; log-rank $p = 0.0139$), β2-microglobulin (β2-microglobulin ≤ 5.5 mg/L: n = 27, median OS = 40 months; β2-microglobulin > 5.5 mg/L: n = 24, median OS = 12 months, log-rank $p = 0.0520$, Breslow $p = 0.0352$, Tarone-Ware $p = 0.0399$), plasma cell morphology (mature: n = 38, median OS = not reached; intermediate (or immature): n = 11, median OS = 12 months; plasmablastic: n = 5, median OS = 1 month; log-rank $p = 0.0037$), transplantation proceedings (transplanted patients: n = 9, median OS = not reached; nontransplanted patients: n = 47, median OS = 14 months; $p = 0.0064$), and *CT45* expression (*CT45* expression negative: n = 46, median OS = 25 months; *CT45* expression positive: n = 10, median OS = 3 months, log-rank $p = 0.038$ for all patients and *CT45* expression negative: n = 37, median OS = 19 months; *CT45* expression positive: n = 10, median OS = 3 months, $p = 0.0245$, only nontransplanted patients)

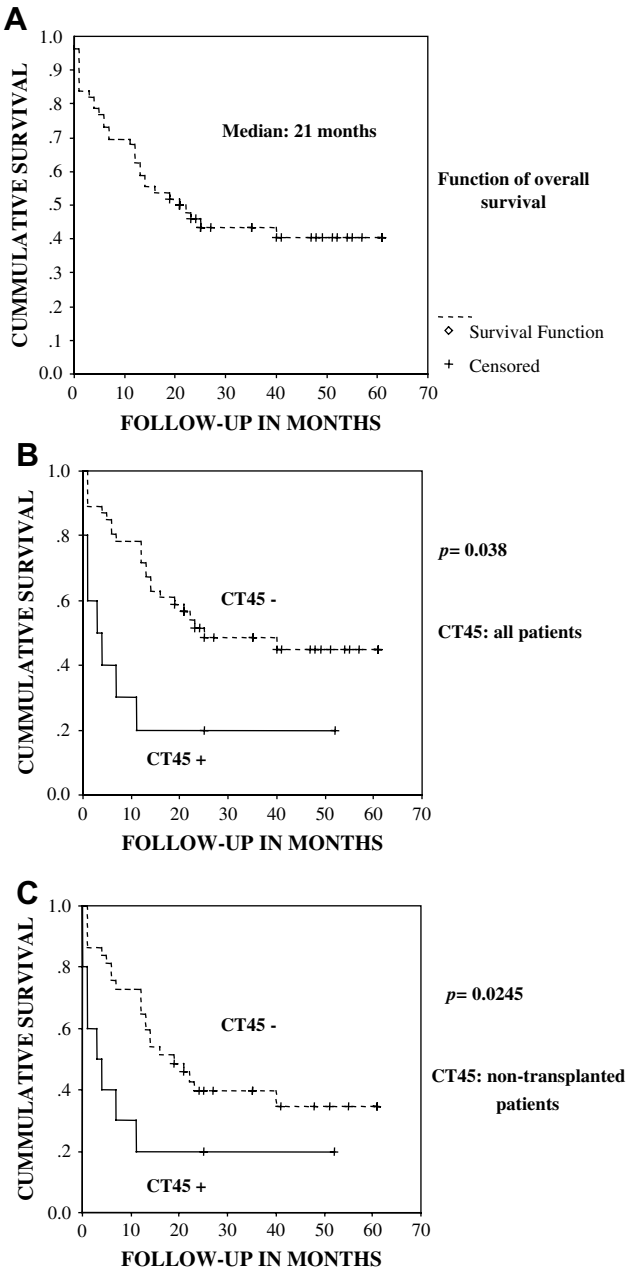

Figure 1. Function of survival. (**A**) Overall survival; (**B**) cancer testis antigen *45* (*CT45*)−positive expression (all patients included); (**C**) *CT45*-positive expression (only nontransplanted patients).

(Fig. 1B and 1C, respectively) had an impact on OS. Cox regression model showed that only plasma cell morphology ($p = 0.029$; relative risk = 5.288; confidence interval, 1.77704−15.7988), transplantation proceedings ($p = 0.0742$; relative risk = 0.1582; confidence interval, 0.0209−1.1976) and *CT45* expression ($p = 0.0016$; relative risk = 7.0403; confidence interval, 2.0978−23.6278) were independent prognostic factors in MM patients survival. *CT45*-positive cases were associated with poor outcomes

and presented seven times more chance of worse evolution than the negative ones.

Our results showed low frequency of *CT45* expression by RT-PCR in advanced-stage MM patients (16%, 10 of 61). However, *CT45* expression was associated with high ISS scores and shortened survival in MM.

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