Research Paper

MAGED4-Expression in Renal Cell Carcinoma and Identification of an HLA-A*25-Restricted MHC Class I Ligand from Solid Tumor Tissue

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MAGE derived HLA ligands have repeatedly been shown to elicit T-cell responses against tumor cells. In renal cell carcinoma (RCC), however, only few T-cell epitopes from cancer testis antigens have been described. To identify potential candidates, we applied a combined approach of microarray/qPCR expression analysis and sequencing of HLA ligands from RCC by mass spectrometry. We analyzed the expression of 21 MAGE genes in ten RCC samples and two glioblastoma samples and could identify the first MHC class I ligand NIGDEALGRW from MAGED4 presented by HLA-A*25 on RCC solid tumor tissue. MAGED4 was expressed in 30% of RCC and both glioblastoma samples. Among the other MAGE family members only MAGEB2 and -C1 and the broadly expressed MAGED1, -D2, -F1 and -H1 were expressed in RCC. Ligands from MAGED4 could thus be interesting tumor-associated antigens in a subset of RCC, even though the identified ligand is presented by a rather rare allele.

INTRODUCTION

In the past, MAGE derived HLA ligands have successfully been used to elicit T-cell responses and more than 20 epitopes of MAGE proteins are known.1 In 1991 van der Bruggen et al identified the first gene of the MAGE family, MAGEA1, which encodes a tumor-specific antigen.2 MAGE-A, -B and -C were found to be exclusively expressed in male germ lines and tumor cells. Demethylation of the promotor region appears to be the cause of activation of these genes in tumor cells.3 Immunotherapy ideally aims at identifying antigens that are either exclusively or preferably expressed on tumor cells and as little as possible in other tissues. Application of such candidates has the potential of overcoming immune tolerance and avoiding cross reactivity. Since germ cells as the classical normal tissues expressing MAGE do not express MHC for antigen presentation on their cell surface,4 ligands from MAGE proteins are thus promising antigens for immunotherapy. In contrast to the exclusive germ line/tumor expression of MAGE-A to -C, members of the MAGE-D, -E, -F and -H-family have been found to be expressed in many normal human tissues.5 Some of them, however, are predominantly found in certain tissues.

MAGED4 (MAGE-E1) expression has only been described for brain, ovary and glioma cells in two splice variants, and additionally in lung, stomach, colon and hepatocellular carcinoma in another variant.6 This implies that some MAGE-D genes could exhibit an expression profile advantageous for immunotherapy.

Importantly, the genes of the MAGE-D family appear to be ancestral MAGE genes, which are especially well-conserved between mouse and man.5 They may thus execute important cellular functions: MAGED3 is identical to trophinin, which is likely to be involved in embryo implantation7 and MAGED1 interacts with the p75 neurotrophin receptor.8 Beside the conserved MAGE domain shared by all MAGE genes, the MAGE-D family is entirely different in its exon-intron structure from other MAGE families: most MAGE genes consist of 3–4 Exons in contrast to MAGE-D genes with 13–14 exons, which probably result from retrotransposition during evolution.5,9,10

Some MAGE genes, MAGEA1-A4, were also found to be expressed in RCC cell lines and solid tumor samples without detection in normal kidney tissue,11,12 yet no MAGE-derived T-cell epitope for RCC is known so far. Overall, few T-cell epitopes from cancer testis antigens are known, e.g., two ligands from RAGE-11,13,14 and four ligands from PRAME15 were recognized by CTLs. Due to the relative radio- and chemoresistance of RCC, there is however a strong need for the identification of tumor-associated antigens for immunotherapy in RCC.

KEY WORDS

renal cell carcinoma, MAGED4, cancer testis antigen, immunotherapy, MHC peptides, glioblastoma, expression profiling, microarray

ABBREVIATIONS

CA cancer
HLA human leukocyte antigen
MAGE melanoma-associated antigen
MHC major histocompatibility complex
RCC renal cell carcinoma
PCR polymerase chain reaction
HPLC high performance liquid chromatography

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Pascolo et al. identified an HLA-A2-restricted MAGEA1 epitope by mass spectrometry.\textsuperscript{16} We used a combined approach consisting of sequencing HLA-presented peptides from RCC by mass spectrometry and microarray gene expression analysis of RCC, autologous normal kidney tissue, and a set of reference samples from other healthy organs. This usually allows us to correlate about 100 identified HLA ligands with the expression data of approximately 20,000 human genes in every tumor. Our criteria for tumor-association are: (1) overexpression in RCC versus normal kidney tissue with (2) possibly low expression in other human tissues.\textsuperscript{17,18} The results for MAGED4 expression in RCC, glioma and reference tissues were confirmed by qRT-PCR.

We report on the identification of the first MHC class I ligand from MAGED4 in RCC from solid tumor tissue. We further examined the expression of MAGED4 in ten RCC samples, two glioblastoma samples and a range of normal tissues. Finally, we analyzed these ten RCC samples for expression of 20 additional MAGE genes.

**MATERIALS AND METHODS**

**Patient samples.** Our investigation comprised ten RCC and corresponding normal kidney tissue samples provided by the Department of Urology, University of Tübingen and two glioblastoma samples (NCh) from the Department of Neurosurgery, University of Heidelberg. Eight RCC samples are classified as clear cell RCC, RCC44 and RCC75 are chromophilic RCC.

The tissue was surgically removed and immediately shock frozen. Patients’ informed consent and the approval of the local ethical committee was given. Tumor staging and grading was performed by the Department of Pathology and HLA typing was done by the Department of Transfusion Medicine, University of Tübingen.

**Peptide isolation and sequencing.** Frozen tumor tissue was processed as described previously.\textsuperscript{19} Peptides were isolated according to standard protocols \textsuperscript{20} using the HLA class I specific antibody W6/32.

HLA-extracted peptide pools were separated by reversed phase HPLC (Ultimate Dionex, Amsterdam, Netherlands) and analyzed online by nano-ESI MS on a hybrid quadrupole orthogonal acceleration time-of-flight mass spectrometer (Q-TOF; Micromass, Manchester, UK) as described.\textsuperscript{21} Fragment spectra were analyzed manually and database searches (National Center for Biotechnology Information) were carried out using Multiple Alignment System for Protein Sequences Based on Three-way Dynamic Programming (MASCOT, http://www.matrixscience.com).

**Peptide synthesis.** The MAGED4 control peptide was synthesized in an automated peptide synthesizer EPS221 \textsuperscript{22} (Abimed, Langenfeld, Germany) following the 9-fluorenylmethyl-oxycarbonyl/tert-buty (Fmoc/tBu) strategy as described.\textsuperscript{19} Gene expression analysis by high-density oligonucleotide microarrays. Frozen fragments of tumors RCC44, RCC68, RCC70, RCC73, RCC75, RCC98, RCC103, RCC112, RCC115, RCC116, NCh359 and NCh361 were homogenized by mortar and pestle under liquid nitrogen. Total RNA was prepared from these samples using TRIZol (Invitrogen, Karlsruhe, Germany) according to the manufacturer’s protocol, followed by a cleanup with RNase (Qiagen, Hilden, Germany). Total RNA from healthy human tissues was obtained commercially (Ambion, Huntingdon, UK; Clontech, Heidelberg, Germany; Stratagene, Amsterdam, Netherlands). The RNA from several individuals (between two and 62 individuals) was pooled in a way that RNA from each individual was equally weighted. Quality and quantity were assessed on an Agilent 2100 Bioanalyzer (Agilent, Waldbronn, Germany) using the RNA 6000 Pico LabChip Kit (Agilent).

Gene expression analysis of all RNA samples was performed by Affymetrix Human Genome U133A oligonucleotide microarrays (Affymetrix, Santa Clara, CA). All steps were carried out according to the Affymetrix manual (www.affymetrix.com/support/technical/manual/expression_manual.pdf).
Relative expression was calculated against the reference sample kidney (=1). T, tumor sample; N, autologous normal kidney tissue; P, present detection (black bars); A, absent detection (white bars).

Figure 2. Microarray analysis of MAGED4 expression of RCC, glioblastoma (NCh), and normal tissue samples.

RESULTS

Peptide characterization. By mass spectrometric analysis of HLA ligands, we could identify the first ligand NIGDEALIGRW from MAGED4 on RCC103 (HLA-A*11; -A*25; -B*15; -B*44) solid tumor tissue (Fig. 1A). According to the peptide motif, the ligand was assigned to HLA-A*25, which asks for hydrophobic amino acids in position 2 and tryptophan at the C-terminal position of ligands. It cannot, however, be excluded that the ligand is also presented by an HLA-C allotype. The sequence was verified by analyzing synthetic NIGDEALIGRW under identical conditions (Fig. 1b).

MAGED4 expression by microarray analysis. MAGED4 expression was examined in ten RCC and two glioblastoma samples (Fig. 2). Among the normal tissue samples, the specific detection of MAGED4 mRNA according to the detection algorithm reached significance only in whole brain, as expected, and uterus. The expression in the uterus sample lies within the expression range of all other healthy tissues, for which no specific detection was reported by the array (‘absent’ call). This indicates a difficulty of analyzing this gene with microarrays: specific detection may be obscured by cross-hybridization of “mismatch probes” with other mRNA targets, espe-
cally from other genes of the highly homologous MAGE-D family. Such disturbing factors depend on the specific mRNA composition of each sample and are more relevant if the actual gene is expressed at a low level. Therefore, the detection in the uterus sample may be specific, whereas for another sample with similar signal intensity (relative expression) like small intestine a possibly higher amount of crosshybridizing mRNA may cause an "absent" call.

Nevertheless, MAGE4 was unambiguously overexpressed in both glioblastoma samples, NCh359 and NCh361, even if compared to whole brain, which also showed high MAGED4 expression. Among the RCC samples, the detection of MAGED4 was significant in only three tumors: RCC68, 75, and 103. While the RCC68 tumor showed a prominent overexpression compared with pooled and autologous normal kidney, the situation for RCC75 and RCC103 was similar to the uterus sample. However, in support of the specific detection in RCC103 is the fact that the HLA ligand described here was found on this sample.

MAGED4 expression by real-time quantitative RT-PCR (qPCR). In order to substantiate the microarray data, MAGED4 expression for some of the samples was additionally assessed by qPCR (Fig. 3). In general, this method turned out to be more sensitive than the microarray measurements for this gene. We considered MAGED4 to be expressed if the specific signal was at least 10-fold over the background for the respective sample, defined by the no template control, and if the product had the correct melting point. Samples not fulfilling these criteria were set to 0 in (Fig. 3).

According to this, among normal tissue samples MAGED4 could not only be detected in brain and uterus, as with the microarrays, but also in prostate, small intestine and testis. The qPCR results confirm the high expression levels in the glioblastoma samples and RCC68. The two other RCC samples declared positive for MAGED4 by microarray analysis, RCC75 and RCC103, showed the highest expression in qPCR as well. Thus, three out of ten investigated RCC samples exhibited a clear expression of MAGED4. Altogether, apart from its higher sensitivity, qPCR analysis was in good agreement with microarray results.

Expression of members of the MAGE family in RCC determined by microarray analysis. Table 1 summarizes the expression of 21 MAGE genes in RCC and glioblastoma samples compared to normal kidney and other healthy tissues, and the existence of T-cell epitopes. We analyzed ten RCC samples for expression of MAGEA1-A6, -A8-A12, -B1-B4, -C1, -C2, -D1, -D2, -D4, -F1 and -H1 in RCC compared to normal kidney and other human tissues. Expression was defined as "present" detection of the gene as described above (quantitatively above the expression of all normal tissues or exclusive expression). As expected MAGED1, -D2, -F1 and -H1 were expressed in most normal tissues (Table 1) and in almost all RCC samples. Interestingly, further MAGE expression in RCC could only be detected for one case of MAGEB2, two cases of MAGEC1 and three cases of MAGED4 out of ten RCC samples. This indicates that MAGE-derived epitopes may only be relevant in few cancer patients. MAGED4 was only expressed in brain among normal tissues and showed some expression in uterus as mentioned above. All other MAGE members were only or most strongly expressed in tests with the exception of MAGEA8 and -A10, which were most strongly expressed in placenta, and MAGED1, -D2, -D4, -F1 and -H1 which showed the highest expression in brain. In both glioblastoma samples MAGED1, -D2 and -F1 were expressed at a similarly high level as in normal brain and MAGED4 was 1.5–2.5-fold overexpressed. Brain showed only marginally elevated expression of MAGED1, -D2 and -F1, but high expression of MAGED4. Our literature search identified eight MAGE genes for which T-cell epitopes have been described.

DISCUSSION

In our combined analysis of qPCR and microarray analysis we found MAGED4 expression in three out of ten RCC cases (RCC68T, RCC75T, RCC103T). This observation corresponds to the expression rate of some MAGE family members in other tumors: 10–30% of RCC,11 10% of Breast-CA,22 and 10–20% in Esophagus-CA.23 In addition to the MAGED4 expressing tumors initially mentioned by Sasaki et al,6 RCC constitutes according to our results another tumor species with cases of MAGED4 expression. The results of qPCR and microarray analysis were in good agreement. We could further identify the first MAGED4 ligand from RCC103 solid tumor tissue by mass spectrometry. In the past, this technique has successfully led to the identification of tumor-associated antigens some of which have proven to be T-cell epitopes.16,18,24,25 The ligand was identified from one of the samples that actually showed MAGED4 expression. Unfavorably, the identified ligand is most probably presented by the rare allele HLA-A*25 and can thus only be investigated for in vivo relevance in a limited number of patients.

We confirmed the limited tissue expression of MAGED4 in contrast to the broad tissue expression of other MAGE genes and found MAGED4 only expressed in brain6 and uterus. Along with previous observations we also found no expression of MAGED4 in tests and placenta.6 Accordingly, MAGED4 represents a member of the broadly expressed MAGE family with an expression pattern more favorable for immunotherapy. Both glioblastoma samples showed very high expression levels of MAGED4 as indicated by
Sasaki et al.\textsuperscript{6} and both also expressed MAGED1, -D2 and -F1. Analysis of further glioblastoma samples for expression of these genes will thus be interesting. Our analysis did not show expression of MAGEA8 and MAGEA10 in testis as stated in the literature but confirmed the expression in placenta.\textsuperscript{26}

Further expression analysis of 20 MAGE genes in ten RCC samples only showed expression of MAGED2 and -C1 and the broadly expressed MAGED1, -D2, -F1 and -H1. Ringhoffer et al. also found no expression of MAGEA1 in RCC.\textsuperscript{12}

According to our analysis and in contrast to other tumors with a broad expression of MAGE genes, only few members of the MAGE family seem to be overexpressed in a limited number of RCC cases. Only a small group of MAGE genes, among them MAGED4, MAGEA9, MAGEA8 and MAGEA10 in testis as stated in the literature but confirmed the expression in placenta.

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