Establishment and characterization of a highly immunogenic human renal carcinoma cell line

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Abbreviations: RCC, renal cell carcinoma; TAAs, tumor associated antigens; MSI, microsatellite instability; LOH, loss of heterozygosity; 2-DE, two-dimensional electrophoresis; MALDI/TOF, matrix absorption laser desorption ionization/time of flight; MLTC, mixed lymphocytes tumor cell cultures

Key words: renal cancer cell line, immunogenic antigenic format, cell proteome

Abstract. Renal cell carcinoma (RCC) is the most common kidney cancer, and accounts for ~3% of all adult malignancies. RCC has proven refractory to conventional treatment modalities but appears to be the only histological form that shows any consistent response to immunotherapeutic approaches. The development of a clinically effective vaccine remains a major strategic target for devising active specific immunotherapy in RCC. We aimed to identify a highly immunogenic antigenic format for immunotherapeutic approaches, so as to boost immune responses in RCC patients. We established and cloned an immunogenic cell line, RCC85#21 named Elthem, which was derived from a non-aggressive and non-metastatic clear cell carcinoma. The cell line characterization was performed by genomics (real-time PCR, genome instability), proteomics (two dimensional electrophoresis, mass spectrometry) and immunological analysis (mixed lymphocytes tumor cell cultures). Real-time PCR confirmed the RCC85#21 cell expression of tumor antigens and cytokine genes. No difference in microsatellite instability (MSI) in RCC85#21 cell line was found as compared to control, loss of heterozygosity was observed in the RCC85#21 clone, but not in the renal cancer cell lines from which it was generated. The image analysis of RCC85#21 by two-dimensional gels showed 700±26 spots and 119 spots were identified by mass spectrometry analysis. RCC85#21 promoted a significant RCC-specific T cells activation by exhibiting a cytotoxic phenotype after mixed lymphocyte and tumor cell cultures. CD8+ T cells isolated from RCC patients displayed an elevated reactivity against RCC85#21 and efficiently lysed the RCC85#21 clone. The RCC85#21 immunogenic cell line will be suitable for immune stimulation. The identification of novel tumor associated antigens will allow the evaluation of the immune response in vitro and, subsequently, in vivo paving the way for new immunotherapeutic strategies in the RCC setting.

Introduction

Renal cell carcinoma (RCC) is the most common kidney cancer type, accounting for ~3% of all adult malignancies in western countries (1,2). Radical nephrectomy can be curative in early stage disease, but ~30% of patients present with advanced disease, including locally invasive or metastatic RCC at the time of diagnosis, which seems to be resistant to cytotoxic chemotherapies, hormone therapies and radiotherapies (3,4). The most common histological type of RCC is renal clear cell carcinoma (CC), that accounts for ~70-80% of all renal neoplasms and appears to be the only histological subtype that shows any consistent response to immunotherapeutic approaches (5-7). Cytokine-based immunotherapy, such as interleukin (IL)-2 and interferon (IFN)-α, either as single agents or in combination (8), has previously been adopted in the adjuvant setting of RCC, but produced only occasional benefits. The limited success indicates the potential value of optimizing cell-based immunotherapy for RCC with the aim of increasing the number of durable responses, as has already been done with some success in melanoma, in which this approach resulted highly effective for metastatic patients refractory to other treatments (9). One important aspect of cell-based immunotherapy is the in vitro generation of tumo-
the ‘limiting dilution’ technique: 1x10⁵ tumor cells at passage at 37˚C and 5% CO₂ and 10 mM HEPES, and placed in culture flasks incubated (FBS), 20 µg/ml insulin, 10 µg/ml transferrin, 25 nM sodium 1640 medium supplemented with 20% fetal bovine serum cocktail, as previously described (23). The cellular suspension tumoral tissue was minced and digested using an enzymatic matrix absorption laser desorption ionization/time of flight/ of RPMI medium supplemented with 20% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10 mM HEPES, and placed in culture flasks incubated at 37°C and 5% CO₂. The RCC85 cell line was cloned using the ‘limiting dilution’ technique: 1x10⁵ tumor cells at passage 1/100), Ki-67 (dilution 1/100), mitochondria (dilution 1/75), vimentin (dilution 1/2) and the detection system LSAB Plus (DakoCytofilmation). The sections were incubated with primary antibodies for 16 h at 4°C and then with biotinylated secondary antibodies and avidin-peroxidase for 30 min at 37°C. Detection was done with diaminobenzidine chromogen (DAB) for 20 min at 20°C and nuclear contrast was obtained by immersion for 2 min in Mayer's hematoxylin. The sections were finally mounted with glycerine and special coverslips. At least 3 experiment for each sample were performed.

Materials and methods

Ethics statement. The cell line was generated from primary kidney tissue explants, after obtaining written informed consent. The protocol was approved by ethics commission of the medical faculty of the University hospital of Bari, Italy.

Isolation and cloning of RCC85#21 cell line. The primary tumor was histological type grade I according to the Fuhrman et al classification (22), non-aggressive and did not invade the renal artery or vena cava. The tissue was composed mainly of clear cells with an alveolar/tubular arrangement. The tumoral tissue was minced and digested using an enzymatic cocktail, as previously described (23). The cellular suspension was filtered (100 µm), washed and centrifuged 500 µg for 10 min. The pellet was resuspended in AR5 medium [RPMI-1640 medium supplemented with 20% fetal bovine serum (FBS), 20 µg/ml insulin, 10 µg/ml transferrin, 25 nM sodium selenite, 50 nM hydrocortisone, 1 ng/ml epidermal growth factor, 10 µM ethanolamine, 10 µM phosphorylcholine, 100 nM triiodothyronine, 2 mg/ml bovine serum albumin, 10 mM HEPES buffer, 2 mM L-glutamine and 0.5 mM sodium pyruvate] and incubated for 5 days. In the subsequent step, the cells were resuspended in basal medium composed of RPMI medium supplemented with 20% FBS, 2 mM L-glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin and 10 mM HEPES, and placed in culture flasks incubated at 37°C and 5% CO₂. The RCC85 cell line was cloned using the ‘limiting dilution’ technique: 1x10⁵ tumor cells at passage 39 were diluted in basal medium and plated in 96-well plates; 1x10⁴ ‘feeder cells’ (NIH 3T3) irradiated with 10,000 rad were added to each well to ensure the viability and proliferation of tumor cells. After 16 h of incubation the cells were diluted to obtain 1-10 cells per well. After one week, cell clones presenting cell proliferation were identified by microscopic observation, and the tumor cells were expanded by transferring the plates from 48- and 24-wells. The final result was the isolation of a stabilized, immunogenic clone of renal tumor cells. Epstein-Barr virus (EBV)-transformed lymphoblastoid cell lines (EBV-LCL) were also generated from patient RCC85 PBMC using the B95.8 (type I) virus isolate.

Immunocytochemistry. Samples taken from cell culture flasks were retained in PreservCyt™. Subsequently, cytological preparations were obtained in monolayer apparatus with ThinPrep, the first of which was colored by Papanicolaou staining. The others were used for immunocytochemical staining, performed with the avidin-biotin-peroxidase technique in an automatic immunostainer (DakoCytofilmation, Carpinteria, CA, USA), using the following primary antibodies: cytokeratin AE1/AE3 (dilution 1/5), cytokeratin 18 (dilution 1/2), cytokeratin 19 (dilution 1/100), epithelial membrane antigen (EMA) (dilution 1/100), Ki-67 (dilution 1/100), mitochondria (dilution 1/75), vimentin (dilution 1/2) and the detection system LSAB Plus (DakoCytofilmation). The sections were incubated with primary antibodies for 16 h at 4°C and then with biotinylated secondary antibodies and avidin-peroxidase for 30 min at 37°C. Detection was done with diaminobenzidine chromogen (DAB) for 20 min at 20°C and nuclear contrast was obtained by immersion for 2 min in Mayer's hematoxylin. The sections were finally mounted with glycerine and special coverslips. At least 3 experiment for each sample were performed.

Real-time PCR. Total RNA was isolated from RCC85#21, HeLa (human cervical cancer cells) and HK2 (normal human kidney cells) cell lines with the TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) and cDNA was synthesized with the High Capacity cDNA Reverse Transcription kit (Life Technologies Europe BV, The Netherlands) according to the manufacturer's instructions. The expression levels of the following tumor antigen and inflammatory antigen/cytokine genes were analyzed by real-time PCR on a 7500 Fast Real-Time PCR system (Life Technologies): telomerase reverse transcriptase (TERT), ribosomal protein SA or laminin receptor 1 (RPSA alias OFA/iLRP), carboxylesterase 1 (CES1) and interleukin-6 (IL6).

Real-time PCR reactions were performed using the TaqMan Universal PCR Master Mix and the following TaqMan Gene Expression assays (Life Technologies): Hs00972656_m1 (TERT), Hs01080364_m1 (RPSA), Hs00275607_m1 (CES1) and Hs00985639_m1 (IL-6). Each assay contains two unlabeled PCR primers (each final concentration being 900 nM) and one FAM dye labeled TaqMan MGB probe (final concentration 250 nM). Human ACTB (β actin) was used as endogenous control (VIC/MGB Probe, Life Technologies). Negative controls (samples without reverse transcriptase) were included. Quantitative values were obtained from the Ct (threshold cycle) data determined using default threshold settings. Gene expression data were normalized to human ACTB (β actin) and the relative quantification (RQ) was calculated with the 2-ΔΔCt method. The data are presented as relative quantity (RQ) of target genes, normalized with respect to ACTB and the calibrator sample (HK2). At least 3 experiment for each sample were performed.
Genetics instability. Genomic DNA was extracted from RCC cell lines and lymphocytes with the Blood and Cell Midi kit (Qiagen, Milan, Italy), according to the manufacturer's instructions. Then, DNA samples were evaluated for microsatellite instability (MSI) and loss of heterozygosity (LOH) by polymerase chain reaction (PCR) and a panel of 5 microsatellite markers: BAT25 and BAT26 (mononucleotide repeat), D2S123, D5S346 and D17S250 (dinucleotide repeat) (23). PCR reactions were performed in a final volume of 15 µl with 50 ng of genomic DNA and using the following reaction profile: 2 min initial denaturation at 95˚C followed by 95˚C x 20 sec, 55˚C x 20 sec, 72˚C x 20 sec for 30 cycles and 5' of final extension at 72˚C. The primer set sequences used, the number of GDB/Genebanks and the size of amplified products are shown in Table I. The microsatellite analysis was carried out by SSCP (single-strand conformation polymorphism) using a vertical electrophoresis system in polyacrylamide gel at 10% (acrylamide/bis acrylamide 19:1) containing 8 M urea. The electrophoretic run was performed at 56˚C using the D-GENE System (Bio-Rad, Hercules, CA, USA). Silver staining for visualization of the bands was carried out with 0.2% silver nitrate. Positive MSI results were confirmed by repetition in independent PCR reactions at least twice. Allelic loss (LOH) was determined in cases where one of the normal alleles for a given marker was missing.

Two-dimensional electrophoresis (2DE). RCC85#21 clones were cultured until confluence at the 40th passage. Cells, 3.5x10^6, were resuspended in sample buffer (8 M urea, 4% CHAPS, 40 mM Tris-base, 65 mM DTT, and a trace amount of bromophenol blue). The total protein concentration was measured by colorimetric assay based on the Bradford dye-binding method (Bio-Rad protein assay) and samples were stored at -80˚C until use. Isoelectrofocusing was carried out using a 13-cm immobile DryStrip of pH 3.0-10.0 non-linear range. The IPG strips were rehydrated for 8-10 h at room temperature with 250 ml rehydration solution [8 M urea, 2% (w/v) CHAPS, 0.5% amorphine pH 3.0-10.0, 18 mM DTT, 0.002% (w/v) bromophenol blue]. Proteins (60 mg) were loaded onto rehydrated IPG strips for analysis and 1 g protein was loaded for preparative 2-D PAGE. IEF of the proteins was performed at 40 kVolt hour total produced by overnight run. After IEF, IPG strips were incubated at room temperature for 15 min in 130 mM DTT equilibration buffer [75 mM Tris-hCl, ph 8.8; 6 M urea; 30% (v/v) glycerol 87%; 2% (w/v) SDS; 0.002% bromophenol blue, then for 15 min in 270 mM IAA equilibration buffer]. The second dimension was carried out on in-house polyacrylamide/PDA (12.5% T/2.6% C) lab gels in SDS-PAGE running buffer. Analytical 2-DE gels were stained with the PlusOne silver stain kit. Preparative 2-DE gels were stained with 0.05% (w/v) Coomassie Brilliant Blue R-250. Stained gels were scanned with a flat-bed ImageScanner (Amersham Pharmacia Biotech) to generate digital images. The 2-DE gel images were analyzed using Image Master 2D Platinum software (Amersham Biosciences, Uppsala, Sweden). At least 3 replicate gels for each sample were performed.

MALDI-TOF/MS analysis. The protein spots on 2-DE gels were manually excised, and underwent in-gel tryptic digestion by an adaptation of the procedure by Shevchenko et al (25). Peptide digests were analysed using a MALDI-TOF/MS (Autoflex II, Bruker Daltonics, Bremen, Germany) instrument. Prior to mass spectrometry analysis, the tryptic peptide mixture was desalted and concentrated using ZipTip® Pipette Tips packed with C_{18} resin (Millipore, USA). The peptides were eluted from ZipTip directly onto the Prespotted Anchor Chip™ (PAC,
Bruker Daltonics) a MALDI sample carrier with spotted matrix (α-cyano-4-hydroxycinnamic acid) positioned beside the pre-spotted calibration point. The MALDI mass spectra were acquired on an Autoflex II mass spectrometer equipped with a 337-nm nitrogen laser. All spectra were collected in reflecting mode with a delayed extraction time of 110 ns, except for PSD spectra which were collected without post-ionization delayed extraction. Post source decay (PSD) spectra were externally calibrated using abundant fragment ion peaks derived from angiotensin I, ACTH 1-17 and ACTH 18-39. The selection of precursor ions for PSD analysis was done with an ion gate at a resolution of ~100 FWHM (full width half the maximum). A total of 300-400 laser shots at a 50-Hz repetition rate were collected over different areas of the sample/matrix spot to generate averaged precursor ion and PSD mass spectra. Mass spectra were acquired from each sample in the 400-3500-m/z range. All mass values are reported as monoisotopic masses. The program used to create the 'peak list' from the raw acquired data was FlexAnalysis 2.1 with the default parameters. Protein identification was achieved by database search via Biotools 2.2 and MASCOT search algorithm (http://www.matrix.science.com) against the MSDB, NCBI nr and Swissprot databases using the following parameters: Homo sapiens as taxonomic category, trypsin as enzyme, carbamidomethyl as fixed modification for cysteine residues, oxidation of methionine as variable modification, and one missing cleavage and 100 ppm as mass tolerance for the monoisotopic peptide masses.

**Immunophenotypic analysis.** The following fluorescein isothiocyanate (FITC)-conjugated or phycoerythrin (PE)-conjugated mAbs were used for immunofluorescent staining of the RCC85#21 cell line: anti-HLA class I, anti-HLA-DR, anti-CD54, anti-CD80, anti-CD40 and anti-CD86 (BD Pharmingen). In order to stimulate the expression of costimulatory markers, RCC85#21 cells were incubated with IFN-γ for 48 h at the concentration of 500 IU/ml. Cells were washed and resuspended in FACS buffer (phosphate-buffered saline pH 7.2, 0.2% bovine serum albumin, and 0.02% sodium azide) and incubated with fluorochrome-conjugated mAbs for 15 min at 4°C, then washed with the same buffer before flow cytometric analysis. Data were acquired using an EPICS XL flow cytometer (Beckman Coulter, USA) and analysed using WinMDI Version 2.8 software. The area of positivity was determined using an isotype-matched mAb, and a total of 10^5 events for each sample were acquired. At least 3 experiment for each sample were performed.

**Mixed lymphocyte and tumor cell cultures (MLTC).** Peripheral blood mononuclear cells (PBMCs) were obtained at the time of diagnosis from whole blood of autologous RCC85 patient, after obtaining informed consent, under an institutional review board-approved protocol, and were isolated by Ficoll-Hypaque density gradient centrifugation (Sigma Chemical Co., St. Louis, MO, USA), washed twice in phosphate-buffered saline and used in mixed lymphocyte/tumor cell cultures as described below. The RCC85#21 line was prior incubated with IFN-γ (100 IU/ml) for 48 h. Autologous PBMCs were co-cultured in 24-well plates (Costar, Corning, CA, USA) at 10^6 cells/well with irradiated RCC stimulator cells (10^5 cells/well) in AIM-V medium (Life Technologies, Invitrogen, Italy) supplemented with 10% heat-inactivated pooled human serum [Sigma (medium Mb)]. Recombinant human IL-2 was added on day 3 (250 IU/ml; Proleukin, Chiron, and Emeryville, CA, USA). Responder lymphocytes were restimulated weekly with 10^5 irradiated tumor cells in IL-2-containing Mb medium for a further 2 weeks. On day 21 (T21) CD8+ lymphocytes were selected by immunomagnetic CD8+ microbeads (Miltenyi Biotec, Milan, Italy) and positively-isolated T cells were cultured for an additional 2 weeks. On day 35 of culture, CD8+ T cell responders were used as effector cells in functional and molecular analyses.

**Enzyme-linked immunosorbent spot assays (ELISPOT).** CD8+ responder T cells were assessed for specific cytokine production using hIFN-γ enzyme-linked immunosorbent spot (ELISPOT) assays (Mabtech, Mariemont, OH, USA), as previously described (22). Determinations were performed in triplicate and spots were counted using an ELISPOT plate reader (Zeiss-Kontron, Jena, Germany). HLA-restriction of T cell recognition was determined by the addition of blocking antibodies (W6/32, an anti-HLA class I kindly donated by W.J. Storkus) at final concentrations of 100 mg/ml to replicate ELISPOT wells. At least 3 experiments for each sample were performed.

**Cytotoxicity test.** Responder CD8+ T cells stimulated by MLTC assay were evaluated at day 30 + 6 for their ability to kill target cells, including the patient-derived RCC cell line, EBV-LCL, and K562 cells (erythroid cell line) in standard 4-h ^51^Cr-release assays (23).

**Statistical analysis.** The results of quantitative variables are expressed as mean ± SD. All experiments were repeated...
more than three times and similar results were observed. Comparisons between data groups were performed using the nonparametric Mann-Whitney rank sum U test. Values of $p \leq 0.05$ were considered statistically significant.

Results

**RCC85#21 cell line isolation and cloning.** The RCC85 tumor cell line was cultured in complete medium added with 20% FBS and cloned by the scalar dilution method to obtain a single cell per culture plate well. The RCC85#21 clone showed a homogeneous cell shape, being polygonal and multinucleated with nuclei positioned at the center of the cytoplasm, and tended to form cellular clusters. The proliferative rate remained constant with trypsinization to 90% cells confluence every 72 h. The clinical and pathological characteristics of the RCC 85 patients are listed in Table II. RCC85 cells were cloned by the limiting dilution technique at step P39. Through this procedure, several clones were isolated from a single cell placed in culture in a 96-well plate, but only one, the RCC85#21 clone named Elthem, showed morphological and functional characteristics that could define an immunogenic renal tumor cell line.

**Phenotypic characterization of the RCC85#21 cell line.** The tumor phenotype characterization and confirmation of the epithelial origin of RCC85#21 cells were performed by immunocytochemistry and flow cytometry analysis. Trypsin was not used to avoid altering the membrane antigens and subsequent specific binding with the antibody used for immunostaining. Cells were inbedded in paraffin. Cytokeratins 18, a marker of mitochondria, vimentin and Ki-67, were strongly positive (40-90%) and cytokeratins AE1/AE3, cytokeratins 19 and EMA were weakly positive (5-30%) (Fig. 1). Flow cytometry
analysis revealed that RCC85#21 cells expressed a high percentage of HLA-class-I (100%) and a lower rate of CD40 (28%) and CD54 (7.5%) molecules when compared with cells stimulated with IFN-γ (Fig. 2). By contrast, HLA-class-II and
costimulatory CD80 molecules were not detectable under basal conditions nor after stimulation with IFN-γ.

Real-time PCR and genetic instability. The RCC85#21 clone was characterized by real-time PCR to evaluate the expression of tumor and inflammatory biomarkers, such as RPSA alias OFA/iLRP (RQ = 6.3±0.15), TERT (RQ = 2.0±0.28), CES1 (RQ = 6.6±0.05) and IL-6 (RQ = 6.3±0.20). As shown in Fig. 3, the expression levels of RPSA/OFA, TERT and IL-6 genes were significantly upregulated in the RCC85#21 cell line as compared to HK2 cells and HeLa cells (p<0.001). The expression level of CES1 was significantly upregulated in the RCC85#21 cell line as compared to HK2 (p<0.001), while CES1 gene expression resulted decreased as compared to HeLa tumor cells (p<0.001). Genome instability was studied by evaluating microsatellite instability (MSI) and loss of heterozygosity (LOH) with a standard panel of 5 markers, already used to characterize other tumors (24). No difference in MSI in the RCC85#21 cell line was found as compared to control (Fig. 3). LOH was observed at the locus DP1 or D5S346 in the RCC85#21 clone but not in the renal cancer cell lines from which it was generated (RCC1/RCC2/RCC3). (B) No difference in MSI in the RCC85#21 cell line was found as compared to control at the locus D17S250 (lane 1, RCC2; lane 2, RCC3; lane 3, RCC85#21; lane 4, PBMC, peripheral blood mononuclear cells).

2DE and MS analysis. Image analysis of silver stained RCC85#21 gels showed 700±26 spots. An average of 250 spots was selected from two different Coomassie Blue-stained preparative gels representing the total proteome displayed; among them 119 spots were identified, corresponding to 99 different proteins. The proteome map was drawn by identifying protein spots present in at least three out of four analytical gels. Table III lists all the identified proteins corresponding to the protein spots presented on Fig. 5. Their

Figure 4. Microsatellite instability (MSI) and loss of heterozygosity (LOH) of the RCC85#21 clone. Data are representative of three independent experiments. (A) No difference in MSI in the RCC85#21 cell line was found as compared to control at the locus BAT25, BAT26 and D2S123 (lane 1, RCC85#21; lane 2, PBMC, peripheral blood mononuclear cells; lane 3, RCC1; lane 4, RCC3; lane 5, RCC2), but LOH was observed at the locus DP1 or D5S346 in the RCC85#21 clone but not in the renal cancer cell lines from which it was generated (RCC1/RCC2/RCC3). (B) No difference in MSI in the RCC85#21 cell line was found as compared to control at the locus D17S250 (lane 1, RCC2; lane 2, RCC3; lane 3, RCC85#21; lane 4, PBMC, peripheral blood mononuclear cells).

Figure 5. Representative 2-DE map of proteins extracted from the RCC85#21 clone. The image analysis of three different gels showed 700±26 spots.
Table III. Protein spots identified by MALDI-TOF analysis.

| Spot ID | Accession no. | Protein name | Mascot score | Sequence coverage (%) | N. matched peptides | PI | PM (Da) |
|---------|---------------|--------------|--------------|-----------------------|---------------------|----|---------|
| 1       | Q9Y4L1        | Oxygen-regulated protein precursor | 126          | 20                    | 22                  | 5.16 | 111,266 |
| 2       | P18206        | Vinculin     | 88           | 26                    | 20                  | 5.5  | 124,292 |
| 3       | Q8WUM4        | Programmed cell death 6-interacting protein | 71           | 21                    | 21                  | 6.13 | 95,963  |
| 4       | Q12931        | Heat shock protein 75 | 18           | 18                    | 17                  | 6.09 | 73,971  |
| 5       | P02545        | Lamin-A/C    | 281          | 45                    | 42                  | 6.57 | 74,095  |
| 6       | Q92804        | TATA-binding protein-associated factor 2N | 56           | 20                    | 13                  | 8.04 | 61,793  |
| 7       | P11021        | Glucose-regulated protein precursor | 231          | 37                    | 29                  | 5.07 | 72,402  |
| 8       | P11142        | Heat shock cognate 71 kDa protein | 293          | 43                    | 36                  | 5.37 | 71,082  |
| 9       | P38646        | Stress-70 protein | 330          | 47                    | 43                  | 5.87 | 73,635  |
| 10      | Q59GB4        | Dihydropyrimidinase-like 2 variant | 106          | 27                    | 15                  | 5.85 | 68,142  |
| 11      | P15311        | Ezrin        | 73           | 20                    | 16                  | 5.94 | 69,199  |
| 12      | P49915        | GMP synthase [glutamine-hydrolyzing] | 179          | 42                    | 32                  | 6.42 | 76,667  |
| 13      | P61978        | Heterogeneous nuclear ribonucleoprotein K | 144          | 33                    | 24                  | 5.39 | 51,230  |
| 14      | Q03252        | Lamin-2      | 186          | 53                    | 33                  | 5.29 | 67,647  |
| 15      | P17987        | T-complex protein 1 subunit α | 140          | 33                    | 24                  | 5.8  | 60,306  |
| 16      | P49368        | T-complex protein 1 subunit γ | 174          | 37                    | 27                  | 6.1  | 60,495  |
| 17      | P36871        | Phosphoglucomutase-1 | 91           | 36                    | 19                  | 6.32 | 61,279  |
| 18      | P31948        | Stress-induced-phosphoprotein 1 | 104          | 36                    | 25                  | 6.40 | 62,599  |
| 19      | P31939        | Bifunctional purine biosynthesis protein | 107          | 22                    | 14                  | 6.39 | 64,425  |
| 20      | P14618        | Pyruvate kinase isozyme M1/M2 | 110          | 27                    | 13                  | 7.96 | 58,470  |
| 21      | P07237        | Protein disulfide-isomerase precursor | 300          | 55                    | 38                  | 4.76 | 57,480  |
| 22      | P10809        | Heat shock protein, mitochondrial precursor | 131          | 34                    | 21                  | 5.7  | 61,187  |
| 23      | P48643        | T-complex protein 1 subunit ε | 176          | 37                    | 23                  | 5.45 | 59,633  |
| 24      | P30101        | Protein disulfide-isomerase A3 precursor | 166          | 38                    | 24                  | 5.98 | 57,146  |
| 25      | P00352        | Retinal dehydrogenase 1 | 206          | 37                    | 22                  | 6.29 | 54,696  |
| 26      | O60701        | UDP-glucose 6-dehydrogenase | 189          | 44                    | 22                  | 6.73 | 55,674  |
| Spot ID | Accession no. | Protein name | Mascot score | Sequence coverage (%) | N. matched peptides | PI | PM (Da) |
|---------|---------------|--------------|--------------|-----------------------|---------------------|----|--------|
| 27      | P07437        | Tubulin β chain | 57           | 15                    | 5                   | 4.78 | 50,095 |
| 28      | P08670        | Vimentin      | 369          | 82                    | 43                  | 5.06 | 53,676 |
| 29      | Q969G3        | SWI/SNF-related matrix-associated actin-dependent regulator of chromatin subfamily E member 1 | 93 | 31 | 14 | 4.85 | 46,621 |
| 30      | P06576        | ATP synthase subunit β, mitochondrial precursor | 133 | 25 | 9 | 5.26 | 56,525 |
| 31      | P43686        | 26S protease regulatory subunit 6B | 114 | 31 | 14 | 5.09 | 47,337 |
| 32      | Q15084        | Protein disulfide-isomerase A6 precursor | 64 | 19 | 7 | 4.95 | 48,091 |
| 33      | Q9Y230        | RuvB-like 2  | 99           | 43                    | 19                  | 5.49 | 50,994 |
| 34      | Q13561        | Dynactin subunit 2 | 80⁺ | 42 | 14 | 5.1 | 44,318 |
| 35      | P31943        | Heterogeneous nuclear ribonucleoprotein H | 116 | 31 | 14 | 5.89 | 49,484 |
| 36      | P06733        | α-enolase     | 233          | 60                    | 29                  | 7.01 | 47,481 |
| 37      | Q15293        | Reticulocalbin-1 precursor | 91 | 29 | 7 | 4.86 | 38,866 |
| 38      | P63261        | Actin, cytoplasmic 2 | 79⁺ | 38 | 8 | 5.31 | 42,108 |
| 39      | O60664        | Mannose-6-phosphate receptor-binding protein 1 | 105 | 45 | 12 | 5.3 | 47,189 |
| 40      | P61163        | α-centractin  | 94           | 30                    | 9                   | 8    | 42,701 |
| 41      | P49411        | Elongation factor Tu, mitochondrial precursor | 121⁺ | 45 | 16 | 7.26 | 49,852 |
| 42      | P04075        | Fructose-bisphosphate aldolase A | 66 | 38 | 11 | 8.3 | 39,851 |
| 43      | Q13347        | Eukaryotic translation initiation factor 3 subunit 2 | 117 | 45 | 11 | 5.38 | 36,878 |
| 44      | P52907        | F-actin capping protein subunit α-1 | 81 | 50 | 9 | 5.45 | 33,073 |
| 45      | Q99439        | Calponin-2    | 64⁺ | 43 | 12 | 6.95 | 34,074 |
| 46      | Q96PM5        | RING finger and CHY zinc finger domain-containing protein 1 | 64⁺ | 28 | 6 | 6.26 | 31,743 |
| 47      | P31942        | Heterogeneous nuclear ribonucleoprotein H3 | 61⁺ | 47 | 12 | 6.37 | 36,960 |
| 48      | Q5T0U0        | Coiled-coil domain-containing protein 122 | 71⁺ | 44 | 12 | 6.54 | 32,415 |
| 49      | Q8TBZ5        | Zinc finger protein 502 | 65⁺ | 15 | 9 | 9.02 | 65,046 |
| 50      | P22626        | Heterogeneous nuclear ribonucleoproteins A2/B1 | 64 | 35 | 12 | 8.97 | 37,464 |
| 51      | P09493        | Tropomyosin α-1 chain | 91 | 26 | 10 | 4.69 | 32,746 |
| 52      | Q15181        | Inorganic pyrophosphatase | 103 | 50 | 9 | 5.54 | 33,095 |
| Spot ID | Accession no. | Protein name | Mascot score | Sequence coverage (%) | N. matched peptides | PI | PM (Da) |
|---------|---------------|--------------|--------------|-----------------------|---------------------|----|---------|
| 53      | Q9HC38        | Glyoxalase domain-containing protein 4 | 65\(^a\)     | 19                    | 9                   | 5.4| 35,170  |
| 54      | P47756        | F-actin capping protein subunit β | 72           | 32                    | 10                  | 5.36| 31,331  |
| 55      | P09525        | Annexin A4 | 227          | 61                    | 28                  | 5.84| 35,860  |
| 56      | P10768        | S-formylglutathione hydrolase | 118\(^a\)  | 37                    | 11                  | 6.54| 31,956  |
| 57      | P63244        | Guanine nucleotide-binding protein subunit β-2-like 1 | 202         | 58                    | 17                  | 7.6 | 35,055  |
| 58      | P24534        | Elongation factor 1-β | 116          | 44                    | 12                  | 4.5 | 24,748  |
| 59      | P06753        | Tropomyosin α-3 chain | 230          | 58                    | 23                  | 4.75| 29,015  |
| 60      | Q13011        | Cathepsin D chain B | 100\(^a\)  | 28                    | 9                   | 5.31| 26,229  |
| 61      | P25786        | Proteasome subunit α type-1 | 173          | 46                    | 16                  | 6.15| 29,537  |
| 62      | Q8WUF0        | α-amidase NIT2 | 131\(^a\)  | 40                    | 9                   | 6.82| 30,561  |
| 63      | Q6P3U9        | 14-3-3 protein β/δ | 115          | 36                    | 15                  | 4.73| 27,899  |
| 64      | P04792        | Heat shock protein β-1 | 133          | 51                    | 11                  | 5.98| 22,826  |
| 65      | Q9NVS9        | Pyridoxine-5'-phosphate oxidase | 95          | 43                    | 9                   | 6.62| 29,969  |
| 66      | P30040        | Endoplasmic reticulum resident protein 29 | 65          | 27                    | 8                   | 6.77| 28,975  |
| 67      | P30041        | Peroxiredoxin-6 | 114          | 39                    | 11                  | 6   | 25,133  |
| 68      | P60174        | Triosephosphate isomerase | 183          | 63                    | 15                  | 6.45| 26,938  |
| 69      | P18669        | Phosphoglycerate mutase 1 | 130          | 60                    | 14                  | 6.67| 28,900  |
| 70      | P00918        | Carbonic anhydrase 2 | 94          | 50                    | 9                   | 6.87| 29,228  |
| 71      | Q15056        | Eukaryotic translation initiation factor 4H | 89          | 35                    | 8                   | 6.67| 27,368  |
| 72      | O15305        | Phosphomannomutase 2 | 70\(^a\)  | 25                    | 10                  | 6.35| 28,406  |
| 73      | P17931        | Galectin-3 | 149\(^a\)  | 40                    | 13                  | 8.58| 26,172  |
| 74      | Q9Y5U2        | Protein TSSC4 | 64\(^a\)  | 24                    | 9                   | 5.02| 34,436  |
| 75      | Q9Y224        | UPF0568 protein C14orf166 | 72          | 40                    | 8                   | 6.19| 28,051  |
| 76      | P25787        | Protasome subunit α type-2 | 116          | 44                    | 11                  | 6.92| 25,882  |
| 77      | P09211        | Glutathione S-transferase P | 95          | 48                    | 8                   | 5.43| 23,569  |
| 78      | P30048        | Thioredoxin-dependent peroxide reductase | 72          | 30                    | 6                   | 7.67| 28,017  |
| 79      | Q92507        | ES1 protein homolog, mitochondrial precursor | 111          | 65                    | 12                  | 8.5 | 28,153  |
function and localization was derived from the databases of NCBI and SWISS-PROT (http://www.ncbi.nlm.nih.gov, http://us.expasy.org/sprot/). Cytoskeleton proteins (structural proteins), chaperones, proteins involved in energy, carbohydrates, amino acids and the basal metabolism were identified. Different enzymes were identified as isomerases, oxidoreductases and proteases, as well as the channel protein family, the proteasome complex, actin and calcium binding proteins and proteins involved in apoptotic and proliferative processes. Most of the identified proteins are cytoplasmic proteins (structural proteins). Several lysosomal enzymes were identified, as well as membrane proteins (protein channels and receptors). The cellular function of each identified protein was searched for in several proteic and bibliographic databases (SWISS-PROT and PubMed) to assess the impact on the biology of the tumor, confirming their role in several pathophysiological mechanisms. Some of these identified proteins were components of the cytoskeleton such as Lamin-A/C, vimentin and the tropomyosin \( \alpha \)-3 chain. Vimentin has already been shown to be abundant in kidney cancer cell lines (27). Cofilin-1, the F-actin capping protein \( \beta \)-and \( \alpha \)-1 subunit, Actin cytoplasmic 2 and Stress-70 protein were essential in the reorganization of actin filaments as a cellular response to various growth factors (28). Enzymes with a different catalytic activity were

| Spot ID | Accession no. | Protein name | Mascot score | Sequence coverage (%) | N. matched peptides | PI | PM (Da) |
|---------|---------------|--------------|--------------|-----------------------|---------------------|----|---------|
| 83      | Q99714        | Hydroxyacyl-CoA dehydrogenase type-2 | 60           | 22                    | 6                   | 7.66 | 27,134  |
| 84      | P30043        | Flavin reductase (NADPH-dependent diaphorase) | 127          | 58                    | 12                  | 7.13 | 22,105  |
| 85      | O75947        | ATP synthase D chain, mitochondrial | 77\*         | 65                    | 11                  | 5.21 | 18,537  |
| 86      | P07741        | Adenine phosphoribosyltransferase | 68\*         | 32                    | 6                   | 5.78 | 19,595  |
| 87      | P30086        | Phosphatidylethanolamine-binding protein 1 | 100\*        | 55                    | 7                   | 7.01 | 21,044  |
| 88      | Q06830        | Peroxiredoxin-1 | 84           | 24                    | 5                   | 8.27 | 22,096  |
| 89      | P37802        | Transgelin-2 | 76           | 35                    | 6                   | 8.41 | 22,377  |
| 90      | P30626        | Sorcin | 72           | 49                    | 11                  | 5.32 | 21,947  |
| 91      | P00441        | Superoxide dismutase [Cu-Zn] | 57           | 38                    | 5                   | 5.7  | 16,154  |
| 92      | Q86XQ2        | Nucleoside diphosphate kinase A | 131          | 49                    | 9                   | 5.42 | 19,641  |
| 93      | P23284        | Peptidyl-prolyl \( cis-trans \) isomerase B | 97           | 42                    | 12                  | 9.33 | 22,785  |
| 94      | P23528        | Cofilin-1 | 117          | 71                    | 11                  | 8.22 | 18,719  |
| 95      | P14550        | Alcohol dehydrogenase [NADP\(^+\)] fragment | 118          | 42                    | 16                  | 6.32 | 36,892  |
| 96      | P62937        | Peptidyl-prolyl \( cis-trans \) isomerase A | 90           | 53                    | 12                  | 7.68 | 18,229  |
| 97      | P49773        | Histidine triad nucleotide-binding protein 1 | 62           | 75                    | 8                   | 6.43 | 13,907  |
| 98      | P09382        | Galectin-1 | 109          | 47                    | 7                   | 5.34 | 15,048  |
| 99      | P60660        | Myosin light polypeptide 6 | 68           | 47                    | 10                  | 4.56 | 17,090  |

The first column shows the number of spots corresponding to Fig. 4, the second the accession number of each protein, the third the name of the protein, the fourth the mascot score, in the fifth and the sixth the percentage of coverage and the number of peptides matched are shown, while the last two columns show the isoelectric point (pI) and molecular weight expressed in dalton. \(^{\text{*100 pm.}}\)
Figure 6. ELISPOT test for IFN-γ release (A) and cytotoxic T cell responses using ¹⁰⁶Cr-release assays (B). (A) Frequencies of responder CD8⁺ T cells reactive against RCC85#21, EBV-LCL and K562 cell lines after MLTC stimulation (day 35). Results represent the average (± SD) of triplicate wells and are the mean (± SD) of values obtained from three independent experiments (**p<0.001, RCC85#21 vs EBV-LCL and K562). (B) CD8⁺ T cells stimulated by MLTC assay were evaluated at day 35 for their ability to kill target cells including patient-derived RCC cell lines, EBV-LCL cells and K562 (erythroid cell lines) in standard 4-h ¹³⁵Cr-release assays. ¹³⁵Cr-release assays indicated that MLTC responder CD8⁺ T cells efficiently lysed the RCC85#21 clone (60%, E/T ratio 30:1), while the erythroid K562 line, used to assess non-specific cytolysis, showed a low percentage of lysis (<20%, E/T ratio 30:1, p<0.03) (Fig. 6B).

Discussion

Immunogenicity is the principal aspect to be considered in the isolation and characterization of cancer cells, being this feature not always present in cancer cells cultivated in vitro over the past 30 years. In this report, we describe a new tumor cells clone derived from renal primary lesions of ccRCC, that is capable of eliciting a tumor-specific T cell response in vitro. We characterized the RCC85#21 clone derived from a RCC patient with histological grade T3aN0MO. The cell line was called Elthem, patented and properly licensed. This cell line has a potential range of benefits in somatic therapy for the treatment of patients affected by RCC. The RCC85#21 cell line, obtained by limiting dilution, is a cell clone that is morphologically similar to the tissue of origin, namely multinucleated and polygonal cells with a characteristic cluster growth. The RCC85#21 cell line showed a typical tumor cell phenotype given its positivity for the characteristic tumor markers of epithelial origin (cytokeratin CAM 5.2, mitochondrion markers, vimentin, cytokeratin AE1/AE3, cytokeratin 19, EMA and Ki-67). Following tumor cell expansion, antigenic characteristics of RCC cell lines were studied and confirmed. We found that RCC85#21 lacks the costimulatory molecules CD80 and CD86, suggesting that T cell priming against the RCC85#21 cell line could be activated in the absence of costimulation. Other groups have previously analysed the capacity to induce CTL responses of B7.1 (CD80) or B7.2 (CD86) in modified tumor cells (31). In melanoma cell lines, B7 expression appeared to be necessary to induce allogenic responses, whereas this was not found in the RCC85#21 line. In fact, based on its immunogenic potential, the RCC85#21 cell line was selected as a well-characterized human renal cell carcinoma line that is capable of inducing autologous and allogeneic CD3⁺CD8⁺ tumor-associated responses by MLTC.

In addition, the expression levels of some tumor (RPSA/OFA and TERT) and inflammatory (CES1 and IL-6) biomarkers were evaluated by real-time PCR to confirm the tumorigenic and immunogenic capacity of the RCC85#21 cell line. The expression levels of RPSA/OFA, TERT and IL-6 genes were significantly upregulated in the RCC85#21 cell line as compared to HK2 and HeLa cells, while CES1 gene expression was increased in RCC85#21 cell line when compared to obtain and expand RCC-specific CD8⁺ T cells. The degree of immunogenicity was evaluated by testing the release of IFN-γ by responder CD8⁺ T lymphocytes with the ELISPOT assay (Fig. 6A). CD8⁺ T cells isolated from PBMC patient significantly displayed an elevated (HLA class I-restricted) reactivity against RCC85#21, but they failed to react against autologous EBV-LCL cells and the K562 target cell lines (p<0.001). These CD8⁺ cytotoxic lymphocytes (CTL) recognized the RCC85#21 cell line in a predominantly class I-restricted manner, based on the ability of the anti-HLA class I mAbs (W6-32) to inhibit responses by 91%. Analysis of cytotoxic CD8⁺ T cell responses using ¹³⁵Cr-release assays similarly indicated that MLTC responder CD8⁺ T cells efficiently lysed the RCC85#21 clone (60%, E/T ratio 30:1), while the erythroid K562 line, used to assess non-specific cytolysis, showed a low percentage of lysis (<20%, E/T ratio 30:1, p<0.03) (Fig. 6B).

In vitro evaluation of the immunogenic property of the RCC85#21 cell line. The RCC85#21 clone immunogenicity was evaluated after 35 days of MLTC stimulation, where autologous PBMCs were co-cultured with irradiated RCC85#21 cells. After three weeks of culture CD8⁺ T cells were isolated and restimulated for two further weeks, in order
with HK2 control cells, but decreased when compared to HeLa tumor cells. These data confirmed the tumorigenicity of the RCC85#21 cell line. MSI and LOH were also evaluated, no differences being observed in MSI, while LOH was identified at locus D5S346 on chromosome 5q. LOH on 5q was previously described in 7/42 (17%) sporadic RCC patients (26). The minimum region of deletion on 5q to account for LOH was mapped to 5q31.1 (interferon regulatory factor-1; IRF-1 locus), suggesting that LOH on 5q could play an important role in the pathogenesis of RCC. However, recent data have highlighted the low percentage of tumors showing LOH on 5q and this seems to suggest that LOH does not occur sequentially but independently (32). In this study, the RCC85#21 cell proteome was characterized by 2DE combined with mass spectrometry analysis (MALDI-TOF/MS). Among an overall total of 250 protein spots, 119 spots were identified corresponding to 99 different proteins (not redundant). Multiple spots on the gel identified the same protein, suggesting that different isoforms for the same protein were present, probably due to post-translational protein modifications. In literature, several proteomic maps of kidney tumor cell lines have been drawn (33-36), but none for an immunogenic cell line. The results obtained in this study show that several of the proteins identified have already been described in the literature as characteristic of RCC proteins (37,38). However, several others have still to be defined. Protein analysis using NCBI and SWISS-PROT functional annotation showed enrichment of many cancer-related biological processes and pathways such as oxidative phosphorylation and glycolysis pathways. Functional analysis by IFN-γ-ELISPOT assay confirmed that the RCC85#21 clone immunogenicity was able to induce high CD8+ T cells reaction in a predominantly class I-restricted manner. The cytotoxicity tests showed that activated CD8+ lymphocytes have a high capacity to lyse the autologous cell line RCC85#21. In vitro experiments demonstrated a high immunogenicity of the RCC85#21 clone, although the tumor antigens expressed by renal cells have not yet been identified.

The RCC85#21 cell line represents an immunogenic cell line suitable for immune stimulation. The identification of novel TAAs by the proteomic approach will allow the evaluation of the immune response in vitro and, subsequently, in vivo, paving the way for new immunotherapeutic strategies in the RCC setting.

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