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# Tumor Suppressor Gene P29ing4 is Overexpressed and Induces a CD8 T Effector Cell Response in Human Renal Cell Carcinoma

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## Abstract

**Objective:** ING1 and ING4 are identified as candidate tumor suppressor genes acting in regulation of DNA damage responses and apoptosis through modulation of p53. Their defective function promotes tumor growth in melanoma and breast cancer. Our aim was to determine the overexpression of relevant p33ING1b and p29ING4 isoforms in patients with Renal Cell Cancer (RCC).

**Methods:** Peripheral Blood Mononuclear Cells (PBMCs) from tumor patients (Robson stage I-IV) were stimulated with overlapping peptides of p33ING1b/p29ING4 and results were compared with expression profiles in primary tumors.

**Results:** Early-stage and late-stage tumors demonstrated upregulated ING-isoform gene and protein expression. Early cancers were characterized by increased CD8 and IFN-γ protein and gene expression. Significant p33ING1b and p29ING4 tumor-specific CD8 T effector cell responses from PBMCs of the analyzed tumor patients were observed. Interestingly, peptide sequences p33ING1b (aa259-268) and p29ING4 (aa149-158) elicited significant IFN-γ responses indicative for anti-tumor immune responses while IL-2 responses were detected only for p29ING4 (aa149-158), suggesting inducible T effector cell responses.

**Conclusion:** T effector cell analysis against p29ING4 (aa149-158) suggests a promising candidate for *in vivo* induction of tumor-reactive CD8 T effector cells in patients with renal cell cancer.

**Keywords:** p33ING1b and p29ING4; Tumor antigens; Immune response; CD8 cells; Renal cell carcinoma

## Introduction

Renal Cell Carcinoma (RCC) of the clear cell type accounts for approximately 3% of malignancies in adults, and for 90-95% of neoplasms arising in the kidney. As RCC is usually asymptomatic, most new cases are discovered incidentally. 25%-30% of these tumors are already metastasized. The overall 5-year survival rate is 60%, which drops to less than 10% in those with advanced metastatic disease [1-3]. Radical nephrectomy is the first line of treatment for primary RCC, but half of nephrectomy patients experience metachronous metastasis. There is currently no approved adjuvant treatment after surgery, as RCCs are relatively insensitive to cytotoxic agents and radiotherapy. Besides antiangiogenic drugs, the most promising agents used in the treatment of RCC are biological response modifiers such as interferon (IFN)-α or interleukin (IL)-2, which have response rates of 10%-20%. These data suggest a role for cell-mediated anti-tumor immune responses in RCC. However, such mechanisms may be compromised by various evasion strategies, including down-regulation of tumor-specific antigens, defective tumor-specific antigen presentation, loss or down-regulation of MHC class I and II expression on tumor cells, production of immunosuppressive factors by the tumor, and/or the generation of regulatory T-cells [4-6].

The family of Inhibitor of Growth (ING) genes comprises five members (ING 1-5) including differently spliced isoforms with varying effects on proliferation and apoptosis. The ING4 isoform p29ING4 suppresses tumor growth and angiogenesis in brain tumor [7]. Both ING4 isoform p29ING4 and ING1 isoform p33ING1b play important roles in preventing development of particular tumors through formation of a transcriptional complex with the tumor suppressor gene p53. This is required for the activation of p53-responsive genes that mediate growth arrest, replicative senescence, apoptosis, and DNA repair [8-14].

ING1 and ING4 have been reported as candidate tumor suppressor genes in breast cancer [10,15], thus raising their potential value in vaccine-based anti-tumor therapies. Here, we investigated for the first time the expression of ING isoforms p33ING1b and p29ING4 in RCC, the local and systemic immune environment, and the recognition of ING isoforms by T cells as potential tumor-associated antigens.

## Methods

### Patients and human tissue samples

Tumor and normal tissue samples were obtained from 60 patients mean age 66.1 ± 5.4 years. Clear cell RCC was histologically confirmed after radical nephrectomy at the Department of Urology, University of Wuerzburg, Germany (n=30 Robson stage I/II; n=30 stage III/IV). Histologically confirmed tumor-free tissue from the same patients (n=60) served as control tissue [16]. Data concerning age, gender, level of wall infiltration, and lymph node metastasis were collected in our database. Heparinized peripheral blood from the patients was collected before surgery and Healthy volunteers served as controls (n=15). Blood samples were separated on lymphoprep according to the manufacturer's instructions (Nycomed Pharma, Oslo; Norway). Informed consent was obtained from patients to use their specimens and clinico-pathological data for research purposes. The samples were anonymized before the study. The protocol used was approved by the local medical ethics committee.

### Quantitative RT-qPCR

mRNA expression of the representative surface molecules and cytokines (CD4, CD8, CD25, CTLA-4, Foxp3, IL-2, IL-10, IFN-γ), and *ING1b* and *ING4* genes (MWG Biotech, Ebersberg, Germany) was analyzed in RCC specimens by Real Time Quantitative Reverse Transcriptase-Polymerase Chain Reaction (RT-qPCR) assay. Housekeeping gene Beta-Actin (ACTB), Glyceraldehyde 3-Phosphate Dehydrogene (GAPDH), and TATA Box Binding Protein (TBP), were used for relative quantification and cDNA quality control. Reproducibility was confirmed by independent PCR in duplicate. The average threshold cycle (Ct) value was calculated as the cycle number at which the fluorescence of the reporter reached a fixed threshold. The difference (ΔCt) between the average Ct values of the samples in the target wells and those of the housekeeping gene, GAPDH, was assessed, followed by calculation of the difference between the average ΔCt values of the tumor samples for each target and the ΔCt value of the normal tissues (ΔΔCt). The relative quantification value is expressed as 2-ΔΔCt. Results were normalized to kidney normal tissue and expressed as x-fold difference.

### Immunohistochemistry staining

Sequential staining was performed on serial cryostat sections of snap-frozen RCC (n=60 patients) and normal tissue (n=60 patients). Analysis of single staining was performed for p33ING1 (Santa Cruz Biotechnology, CA, USA), p29ING4 (Rockland/Biomol, Hamburg, Germany) CD4, CD25, Foxp3, CD8, (Abcam, Cambridge, UK), IFN-γ (BD Pharmingen, Heidelberg, Germany), IL-2, and IL-10 (R&D Systems, Minneapolis, USA). The secondary antibodies FITC-, Cy3-, or horseradish peroxidase (HRP)-conjugated F (ab`)2 fragment, donkey anti-goat IgG and Cy3-, HRP-, or Alkaline Phosphatase (AP)-conjugated F (ab`)2 fragment donkey anti-mouse IgG were purchased from Jackson ImmunoResearch, West Grove, USA. Serial cryostat sections (5 µm) were performed. Positively stained cells were scored semi-quantitatively as follows: 0=no positive cells; 1 ≤ 10% positive cells; 2=10-50% positive cells; 3 ≥ 50% positive cells.

Immunohistochemical double staining was performed. Cells that were single positive for CD4 (brown or black, cell surface staining) were distinguished from double positive CD4+CD25+ (red, cell surface staining), CD4+CTLA4+ (red, cell surface staining), and CD4+Foxp3+ (Foxp3+, brick red, nuclear staining) stained cells. Quantification of double staining was performed by counting CD4+ cells in six microscopic fields (400 × magnification) in parallel with CD25+ and Foxp3+ serial cryostat sections. The proportion of CD25+ and Foxp3+ positivity in counted CD4+ cells was expressed in percentages.

To confirm ectopic expression of p33ING1 and p29ING4 (Supplementary Figure S1) by renal cell carcinoma cells we used a renal cell carcinoma (gp200) antibody, Clone 66.4.C2 (Novocastra, Wetzlar, Germany) to perform immunofluorescence double staining.

FITC-conjugated AffiniPure Donkey anti-goat IgG and Cy3-conjugated AffiniPure Donkey anti-mouse IgG (Jackson ImmunoResearch) were used as secondary antibodies.

### Protein extraction and western blot analysis

Total protein extracts were assembled using RIPA lysis buffer. Equal protein amounts (30-50 µg) were electrophoresed using NuPage Novex precast gels (Invitrogen/Life Technologies) and transferred with the iBlot dry blotting system (Invitrogen/Life Technologies). Bands were detected by ECL solution (Thermo Scientific, Waltham, MA, USA).

### Peptides and stimulation procedure

Fifty-four PEPscreen peptides spanning the entire length of wt p33ING1b, and 48 peptides of wt p29ING4, were synthesized *via* solid phase Fmoc chemistry (ProImmune Inc., Springfield, USA). The peptides were synthesized as 15-mers overlapping by 5 amino acids and divided into 6 pools of ten peptides for p33ING1b (pool 1 [aa1-58], pool 2 [aa59-108], pool 3 [aa109-158], pool 4 [aa159-208], pool 5 [aa209-268], and pool 6 [aa269-273]), or into 5 pools for p29ING4 (pool 1 [aa1-58], pool 2 [aa59-108], pool 3 [aa109-158], pool 4 [aa159-208], and pool 5 [aa209-248]). To better determine the residues of interest, single epitopes of p33ING1b (residues aa109-118, 119-128, 129-138, 139-148, 149-158, 259-268, 269-273) and p29ING4 (residues aa109-118, 119-128, 129-138, 139-148, 149-158, 209-218, 219-228, 229-238, 239-248) were used.

Peripheral Blood Mononuclear Cells (PBMCs, 5x105, n=30 patients) were added in 100 µl of complete RPMI-1640 medium (Invitrogen, Karlsruhe, Germany) with 10% human serum (PAA, Coelbe, Germany); 100 U/ml penicillin and 100 µg/ml streptomycin

(Biochrom, Berlin, Germany); 5 mM HEPES (Biochrom); 1% non-essential amino acids (Biochrom); 1 mM sodium pyruvate (Biochrom); and 2 x $10^{-5}$ M 2-mercaptoethanol (Invitrogen) to each well with the addition of the relevant peptide pool p33ING1b or p29ING4 (50 µg/ml of each peptide). Control wells contained either unstimulated cells (negative control) or cells with phytohemagglutinin (PHA) at a final concentration of 5 µg/ml without the peptide (positive control). Negative control values were observed as background.

### Measurement of the frequency of p33ING1b and p29ING4 reactive T cells

The Elispot assay used to measure the frequency of the peptides p33ING1b and p29ING4 reactive T cell producing IFN-γ was performed according to the manufacturer's instructions (BD Bioscience, Heidelberg, Germany). The resulting spots representing IFN-γ production were counted on a computer-assisted ELISA Spot Image Analyzer (CTL, Cleveland, OH). Results were expressed in spots/5 x $10^5$ cells.

The human cytokine bead assay kit (Biosource, Nivelles, Belgium) was used for measurement of IL-2, IL-10, IFN-γ in supernatants of PBMCs (5 x $10^5$) in response to p33ING1b- and p29ING4-specific peptides using Luminex analysis. The assay was performed according to manufacturer's instructions. Analysis was carried out in the Luminex 100 instrument (Gurce Nivelles, Belgium). Results were measured in pg/ml and expressed in pg/ml or in stimulation index (%).

### Isolation of CD8+ T cells

CD8+ T cells were isolated by magnetic labeling of CD4+ T cells using Dynabeads (Dynal Biotech/Invitrogen, Karlsruhe, Germany). The *via*bility of the CD8+ T cells was >95%.

### Statistical analysis

Comparisons between groups were made by Student's t-test. Each experiment was repeated at least twice. Significance was assumed if $p<0.05$.

## Results

### Expression of p33ING1b and p29ING4 in RCC

To determine whether p33ING1b and p29ING4 mRNA was differently expressed in tumor tissue, RT-PCR was carried out in 60 tumor samples (stages I/II, n=30; III/IV, n=30). Normal tissues from the same patients were used as controls (n=60). Analysis of tumors from patients at early stages (I/II) and late stage tumors (III/IV) revealed up regulated expression of both *p33ING1b* and *p29ING4* genes (p33ING1b and p29ING4: stages I/II and III/IV *vs.* normal tissue $p<0.001$ and $p<0.0001$, respectively; p33ING1b and p29ING4: stages III/IV *vs.* I/II $p<0.01$ and $p<0.001$, respectively) (Figure 1a).

Differences in mRNA and protein expression between early and late stages were positively correlated for both p33ING1b and p29ING4. In contrast to early stage tumors, stage III and IV tumors demonstrated more than 50% positive cells for both proteins (I/II *vs.* III/IV, p33ING1b: 10 out of 30 *vs.* 18 out of 30 patients and p29ING4: 15 out of 30 *vs.* 22 out of 30 patients) (Figure 1b). These results were confirmed by Western Blot analysis (Figure 1c).

### Correlation between stage-dependent changes in CD8+ T cell infiltrates and number of regulatory T cells in the tumor

To analyze the effectiveness of the tumor T cell responses within the tumor, we evaluated quantitative changes in the CD8 and *IFN-γ* gene expression profiles between early- and late-stage tumors. Early stage tumors showed increased CD8 gene expression, suggesting the presence of effector CD8+ T cells (Figure 2a). At early stages, gene expression of IL-2 and IFN-γ was also increased compared to normal tissue ($p<0.001$). However, in late stage tumors a sharp decrease in IFN-γ expression was detected (Figure 2a). This correlated with respective protein expression as shown by CD8+cells expressing IFN-γ (Figure 2b). Interestingly, an increased gene profile indicative for regulatory T cells in late stage tumors compared to early tumors was observed, with a minimum 2-fold difference in CD4, CD25, CTLA-4, Foxp3, and IL-10 expression (stages I/II *vs.* III/IV: CD8 $p<0.001$, IFN-γ $p<0.0001$, IL-2 $p<0.01$, CD4 $p<0.0001$, CD25 $p<0.05$, CTLA-4 $p<0.01$, Foxp3 $p<0.001$, IL-10 $p<0.0001$) (Figure 2a). These results are in accordance with protein expression (I/II *vs.* III/IV, CD4+CD25+: 17.8% *vs.* 28.5% and CD4+Foxp3+: 8.6% *vs.* 19.7%).

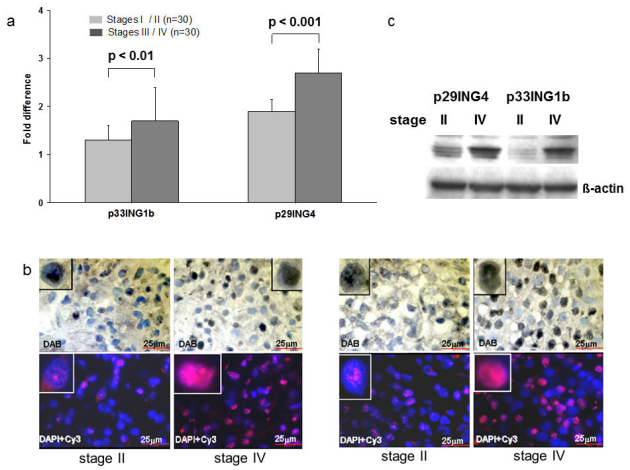


**Figure 1:** Expression of p33ING1b and p29ING4 in renal cell carcinoma. (a) mRNA expression of p33ING1b and p29ING4 was increased at early and late stages of disease with higher expression of both genes at late stages of disease compared to early stages (Robson stages III/IV *vs.* I/II) (p33ING1b and p29ING4: stages I/II and III/IV *vs.* normal tissue $p<0.001$ and $p<0.0001$, respectively; p33ING1b and p29ING4: stages III/IV *vs.* I/II $p<0.01$ and $p<0.001$, respectively). (b) Results of the real time RT-PCR were normalized to kidney normal tissue and expressed as x-fold difference ($2^{-\Delta\Delta Ct}$). Expression of p33ING1b and p29ING4 by renal carcinoma cells was confirmed (Cy3 red; DAPI blue (nuclear counterstaining)). (c) Sections are representative of n=30 patients/group. Representative western blots of p33ING1b and p29ING4 (loading control ß-actin).

### Characterization of T cell subsets and cytokines in peripheral blood mononuclear cells for therapeutic options

Based on these findings within tumor tissue, it can be speculated that the anti-tumor immune response becomes ineffective with tumor progression. Thus, we characterized T cell subsets and cytokine profiles

in PBMCs from the analyzed patients. At the protein level, the number of double positive CD8+ T cells expressing IL-2 or IFN-γ was decreased at all stages compared to controls (Figure 3a). These results are in accordance with those observed within the tumors, suggesting an increasingly ineffective CD8+ T cell response. In contrast, markedly higher amounts of CD4+CD25+, CD4+CTLA4+, CD4+Foxp3+ cells, as well as CD4+ cells expressing IL-10, were observed in PBMCs from late-stage patients compared to early-stage patients and healthy controls (stages I/II *vs.* III/IV: CD4+CD25+ p<0.0001, CD4+Foxp3+ p<0.0001, CD4+CTLA-4+ p<0.0001, CD4+IL-10+ p<0.001, CD8+IFN-γ p<0.001) (Figure 3a and 3b).

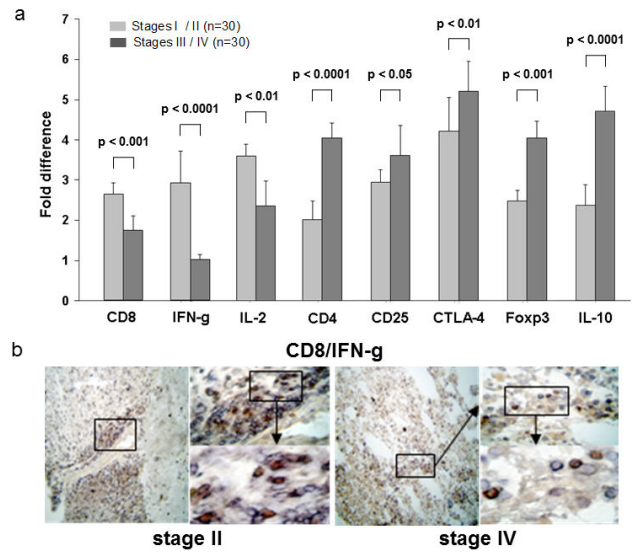


**Figure 2:** Characterization of infiltrating lymphocytes in the tumor. The tumors of patients at stages I/II showed higher expression of CD8, IFN-γ, and IL-2 than those at stages III/IV. Higher expression of CD4, CD25, CTLA-4, Foxp3, and IL-10 was observed at late stages (stages I/II *vs.* III/IV: CD8 p<0.001, IFN-γ p<0.0001, IL-2 p<0.01, CD4 p<0.0001, CD25 p<0.05, CTLA-4 p<0.01, Foxp3 p<0.001, IL-10 p<0.0001). Results of real time RT-PCR were normalized to kidney normal tissue (GAPDH as housekeeping gene) and expressed as x-fold difference (2-ΔΔCt) (a). Early stage tumors as compared to late stage tumors showed comparably more CD8/IFN-γ double positive cells (stage I/II *vs.* III/IV: 18.5 ± 3.9 *vs.* 7.5 ± 2.7, respectively) (b). Representative images for a stage II *vs* stage IV patient (200 x, 400 x magnification).

## Analysis of a p33ING1b- and p29ING4-specific T cell response as a putative therapeutic strategy

To assess putative development of tumor-specific T cell responses in these patients, we further explored p33ING1b- and p29ING4-specific immune responses. Using pools of overlapping p33ING1b and p29ING4 peptides, T cell responses were investigated. Four peptides showed a significant induction of IFN-γ expression in PBMCs derived from RCC patients compared to healthy volunteers independent of the underlying tumor stage (p33ING1b(aa109-118) and (aa259-268), as well as p29ING4 (aa149-158) and (aa239-248) (Figures 4a and b; p<0.001). Peptide-stimulated expression of IL-10 was also clearly observed for peptide p33ING1b (aa109-118) at all stages and for p29ING4 (aa239-248) at late stages (Figures 5a and b). Additionally, a significantly increased IL-2 response was detected for p29ING4 (aa149-158), while other epitopes demonstrated no response (stages I/II *vs.* III/IV p<0.001) (Figure 6). These data indicate that T cells are specifically responding to p29ING4 (aa149-158) in RCC patients.

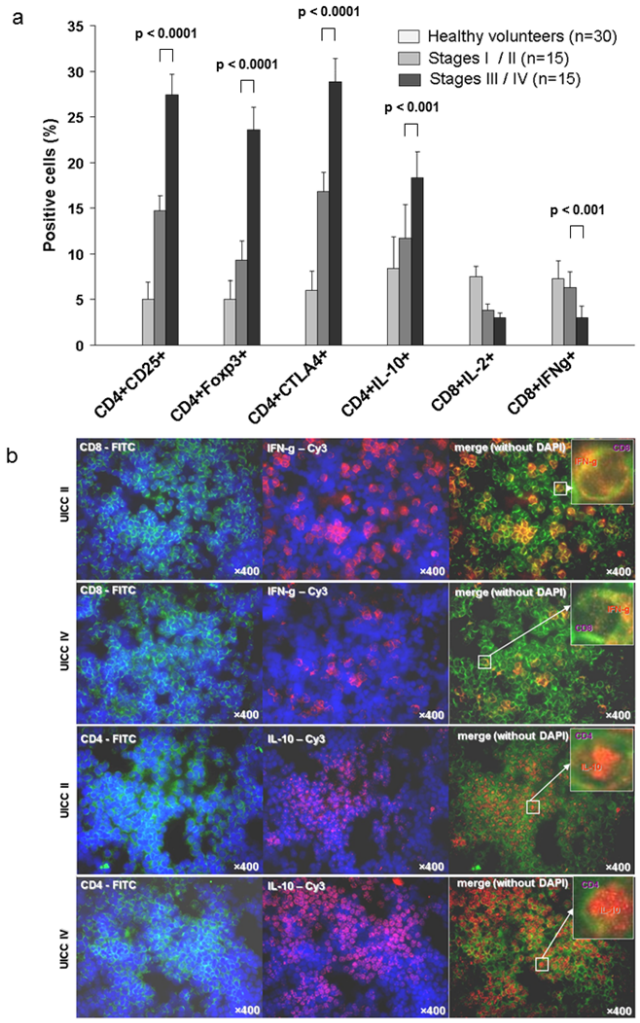


**Figure 3:** Characterization of T cell subsets and cytokines in peripheral blood mononuclear cells (PBMCs). At the protein level, the number of double positive CD8+ T cells expressing IL-2 or IFN-γ was decreased at all stages compared to controls. Higher amounts of CD4+CD25+, CD4+Foxp3+ cells, CD4+CTLA4+, as well as CD4+ cells expressing IL-10, were observed in PBMCs from late-stage patients compared to early-stage patients and healthy controls (stages I/II *vs.* III/IV: CD4+CD25+ p<0.0001, CD4+Foxp3+ p<0.0001, CD4+CTLA-4+ p<0.0001, CD4+IL-10+ p<0.001, CD8+IFN-γ p<0.001) (a). Mean percentage of cells staining positive in six microscopic fields. Representative images for CD8+IFN-γ+ and CD4+IL-10+ expression in stage II *vs.* stage IV patients (400 x magnification; FITC-green; Cy3 red; DAPI blue (nuclear counterstaining).

We investigated whether CD8+ T cells from PBMCs of the patients could be stimulated for a specific tumor cell response. To test these cells, we depleted PBMCs of the CD4+ subpopulation. The CD8+ T cells demonstrated a significant IFN-γ production in response to the peptide p29ING4 (aa149-158) (stimulating index 51.8 ± 5%, n=30) compared to CD8+ T cells from healthy volunteers (3.9 ± 1.5%, n=30), suggesting that an anti-tumor effector (CD8+) T cell response could be induced. This may have implications for the development of clinical anti-tumor immune therapies.

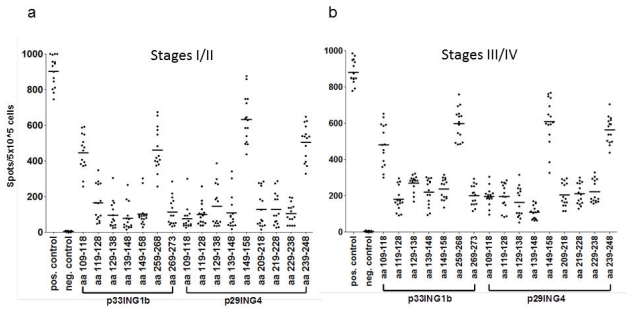


**Figure 4:** p33ING1b- and p29ING4-specific T cell reactivity. Four peptides resulted in significantly increased expression of IFN-γ compared to controls, independent of tumor stage (p33ING1b (aa109-118) and aa259-268, and p29ING4 (aa149-158) and aa239-248; p<0.001) Stages I/ II (a) and Stages III/IV (b). Elispot analysis was measured as spots/5 x $10^5$ cells/patient.

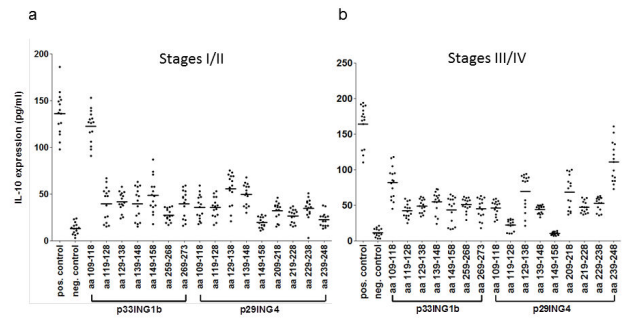


**Figure 5:** p33ING1b- and p29ING4-specific T cell reactivity. Two peptides resulted in increased expression of IL-10 compared to controls; peptide p33ING1b (aa109-118) at all stages and p29ING4 (aa239-248) at late stages (p<0.001). Stages I/ II (a) and Stages III/IV (b). Luminex analysis was measured as pg/ml.

## HLA ligands and peptide motifs

The p29ING4 peptide aa149-158 was analyzed with respect to binding characteristics by the SYFPEITHI class I and II epitope prediction algorithm (http://www.syfpeithi.de). The peptide was predicted to bind strongly to both HLA class I (HLA-A*0201, HLA-A*03, and HLA-B*4402) and HLA class II molecules (HLA-DRB1*0101 and HLA-DRB1*0401). Thus, the peptide p29ING4 (aa149-158) can be presented to T cells *via* both HLA class I and II molecules, which supports its role as a potential tumor-associated antigen (Figures 7a and b).

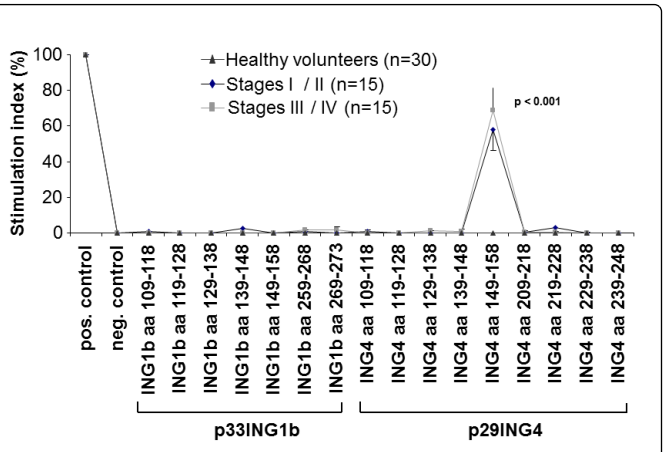


**Figure 6:** p33ING1b- and p29ING4-specific T cell reactivity. Evaluation of IL-2 expression revealed that only a single epitope (p29ING4 (aa149-158)) resulted in high responsiveness of T cells from RCC patients after stimulation with the peptide (stages I/II *vs.* III/IV p<0.001). PBMCs from healthy volunteers (n=30) had no significant IL-2 expression after stimulation with all peptide epitopes. Luminex analysis for IL-2 expression was measured as pg/ml and presented as percent stimulation index.

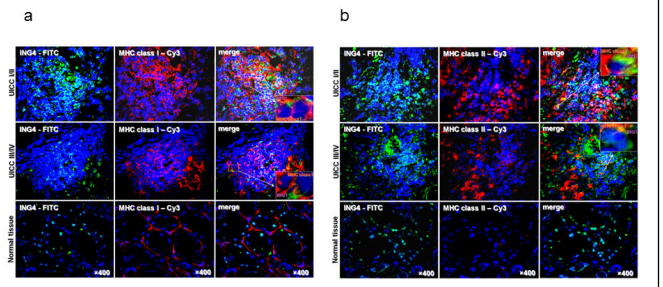


**Figure 7:** p29ING4 presentation by MHC class I (a) and II (b) molecules. p29ING4 presentation by major histocompatibility complex (MHC) class I and II antigens was shown to be increased in RCC but not in healthy kidneys.

## Discussion

We studied two isoforms of ING family members, ING1 isoform p33ING1b and ING4 isoform p29ING4. The identification of tumor immunogenic molecules with increased or exclusive expression in RCC tumor tissue is important in order to develop T cell-mediated strategies against RCC. Our results suggest a stage-dependent expression of p33ING1b and p29ING4 isoforms in the tumor tissue of RCC patients. Late-stage tumors demonstrated higher expression of the two isoforms compared to early tumors and healthy control tissue. Thus both ING isoforms could be potential tumor-associated antigens, and might act as targets for natural or induced immunity. Indeed, p33ING1b is identified as a tumor-associated antigen in breast carcinoma [15]. It is suggested that ING4 plays a significant role in the suppression of breast cancer progression [17]. Interestingly, all major isoforms of ING family members can promote the transactivation of

p53 and the majority of them are shown to directly interact with p53. In addition, ING proteins are thought to interact with and modulate the function of auxiliary members (MDM2, ARF, p300, p21) of the p53 pathway, indicating their widespread involvement in the regulation and function of this prominent tumor suppressor pathway [18].

In comparison to normal tissue, which had no significant lymphocytic infiltrates, early-stage tumors were characterized by tumor-infiltrating lymphocytes, including CD8+ and CD4+ T cells, while advanced tumors had lower CD8+ T cells. The high expression of CD8+ T cells at primarily early stages suggests the presence of effector CD8+ T cells. Because CD4+CD25+ regulatory T cells (Tregs) have emerged as a crucial subset for regulating immune responses, several groups have analyzed their prevalence in cancer patients. Increased numbers of Tregs have been found in patients with various cancers, including colorectal cancer, where they have been associated with poor prognosis [6,8,19]. Most studies on human tumors focused on CD4+CD25+ Tregs without taking into consideration more specific markers, such as the transcription suppressor protein Foxp3 [20-22]. We investigated Foxp3 expression in PBMCs and tumor-infiltrating lymphocytes from patients with RCC, and demonstrated that Foxp3+ cells were present within the tumor microenvironment. In parallel with these observations we detected an increased expression of specific T cell regulatory genes (CD4, CD25, and Foxp3) at the tumor site in patients with late-stage disease. This indicates that at late stages the population of regulatory tumor-specific T cells is indeed comprised of CD4+CD25+Foxp3+ cells. The relative proportions of the different T cell subsets in individual tumors could possibly influence clinical outcome, as we observed in patients with colorectal carcinoma [19,23].

Upon stimulation of peripheral blood lymphocytes derived from RCC patients, a considerable *in vitro* tumor immune response by the short peptide stimulation step, together with a significantly lower IFN-γ response in p33ING1b and p29ING4-specific T cells from healthy controls, strongly suggest that ING-specific lymphocytes in RCC patients have been primed *in vivo*. Analysis of the cytokines in response to p33ING1b and p29ING4-specific T cells revealed IFN-γ expression in 67% of the patients. More extensive analysis of the cytokine profiles showed that in those patients p33ING1b and p29ING4-specific responses were associated with the production of the immunoregulatory cytokine IL-10. On the basis of our detection of high IL-10 expression in response to most p33ING1b and p29ING4 epitopes in PBMCs derived from RCC patients, it is tempting to speculate that the population of effector T cells may hinder the patient's ability to productively eliminate p33ING1b- and p29ING4-overexpressing tumors.

Peptide p29ING4 (aa149-158) was the only peptide inducing IFN-γ together with IL-2 production in PBMCs from tumor patients. However, IL-10 cytokine production was significantly reduced in PBMCs from tumor patients. Furthermore, we looked for the role of CD8+ T cells in the recognition of p29ING4 (aa149-158) with respect to their IFN-γ expression in PBMCs derived from tumor patients compared to healthy volunteers. In particular, a significant reactivity of CD8+ T cells expressing IFN-γ (effector T cells) in response to the p29ING4 (aa149-158) could be observed. Because the peptide p29ING4 (aa149-158) binds to both HLA class I and class II molecules, it can be speculated that this peptide participates in the tumor immune response as a tumor antigen. Whether the immune recognition of ING proteins in these patients includes immunopotentiation, or whether the repertoire can be harnessed for T cell-mediated immunotherapy, remains to be determined.

Epigenetic associated transcriptional regulatory mechanism has been suggested to play a key role in the controlling of T helper cell specification [24,25]. For instance, recent observation has found histone H3K9 methyltransferase G9A is able to regulate T cell differentiation during inflammation [26]. In addition, G9a has also been found to regulate gene expression during lineage commitment in adult CD4+T cells [27]. In mammals, G9a and its partner molecular GLP function as the main H3K9me1/2 writers at the euchromatin [28]. Moreover, G9a/GLP complex also plays a role in the maintenance of DNA methylation at imprinted loci [29]. As peptide p33ING1b (aa259-268) and p29ING4 (aa149-158) elicited significant IFN-γ responses indicative for anti-tumor immune responses while IL-2 responses were detected only for p29ING4 (aa149-158), suggesting inducible T effector cell responses, investigation the patterns of H3K9me1/2 as well as DNA methylation at the genes that were associated with T effector cell responses in the human renal cell carcinoma after stimulated by p33ING1b and p29ING4 peptides might further reveal the molecular basis that are required for this process.

In conclusion, the peptide p29ING4 might act as a tumor-associated antigen that induces a tumor-directed immune response. Given the potential direct antitumor effector function of CD8+ T cells [24], our data suggest that the peptide p29ING4 (aa149-158) seems to be a promising vaccine candidate for the induction of tumor-reactive CD8+ T cells in patients with RCC.

## Disclosure Statement

The authors have no conflicts of interest to disclose.

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