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Rationales for a Multi-Epitope Approach in an Autologous Renal Cell Cancer Tumor Vaccine

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Abstract

Objective: We report about a study according to a detailed characterization of an autologous tumor vaccine already used successfully in the immune therapy of renal cell carcinoma (RCC). The original paper was published in Onco Targets Therapy in 2016. This short communication summarizes the contents of the respective publication as well as shows new results of additional investigations by FACS-analysis.

Method: A total of 133 tumor cell lysates (TCL) were investigated by ELISA, Western blots, topological proteomics and FACS analysis.

Results: A total of 36 tumor-associated antigens (TAA) and cellular marker proteins were considered for analysis, whereof none was detectable in each tumor lysate. Moreover, the coincidental presence of potential danger signals was shown for HSP 60 and 70.

Conclusion: In conclusion the verified tumor heterogeneity indicates the need for a multi-epitope approach for the successful immunotherapy in renal cell carcinoma.

Keywords: Renal cell carcinoma; Tumor associated antigens; Therapeutic vaccine; Potency testing; Multi epitope approach; FACS

Introduction

Renal cell carcinoma is an orphan disease with an incidence of less than 1.6:10.000 [1]. The median age of patients at primary diagnosis is 60 years and the male to female ratio is 3:2. Until now only a 1997 initiated prospective randomized phase-III trial showed a significant effect in overall survival after radical nephrectomy accompanied by treatment with an autologous renal tumor cell vaccine [2]. Furthermore, by comparing data from a compassionate use program with a historical group of patients observed for more than 10 years and treated by radical nephrectomy, May et al. [3] demonstrated the same significant effect on the overall survival (42.3 months) for T3 tumors.

Discussions on common tumor markers or tumor associated antigens (TAA) as potential targets for immunotherapy are ongoing especially since authorities like the EMA and the FDA request additional information about the potency and potential risks of these autologous applied antigens [4,5]. The "best" target for immunotherapy so far seems to be CA-IX [6,7] found mostly on tumors of the clear cell type [8]. Nonetheless, even if a tumor entity is known to express specific markers, like MAGE-antigens in melanoma or Her2/neu, not all tumor cells express these tumor associated antigens [9,10], potentially being caused by an oligoclonality of the tumor [11]. Thus, if only a single antigen or even epitope can be targeted by immune therapies successfully, only the respective tumor cell population will be eliminated, without curing the disease. Moreover, for an induction of an immune response, a second requirement has to be fulfilled: the presence of a danger signal such as heat shock proteins [12]. Calderwood et al. [13] concluded that HSP60 and HSP70 can be processed by antigen-presenting cells and that HSP-derived epitopes subsequently activate regulatory T cells and suppress inflammatory diseases. Recently the combination of CA-IX and HSP110 was described as a tumor vaccine which showed its potential in a tumor prevention model, inhibiting the growth of RENCA tumors in BALB/c mice [14].

In conclusion in a therapeutic setting, the presence of the target antigen and the danger signal has to first be demonstrated on an individual level for tumor vaccines e.g. manufactured according to the Reniale®-scheme [2].

Therefor tumor material was received from 133 patients (58 ± 16.5) who underwent radical nephrectomy, donating the material on a voluntary basis. All patients gave informed consent. TNM classification was performed by the local pathologist [15]. Characteristics of patients highly correlate to typical clinical observations. The median age of patients at primary diagnosis is 60 years and the male to female ratio is 2:1. For vaccine preparation the tumor tissue was prepared according to standard procedures [2,16].

To confirm the heterogeneity of tumor associated antigens (TAA) expressed in a total of 133 tumor tissues (pT1: 43, pT2: 21, pT3: 39,

pT4: 1; unknown: 29), four different approaches like ELISA, Western blotting, topological proteomics and FACS analysis were applied [16].

For ELISA and WB TAAs were initially selected by screening 10 clear cell RCC's to identify antigens which are frequently present in RCC. The preliminary panel of antigens was selected based on the current state of scientific knowledge and the availability of the assays [16]. Topological proteomics was performed on single cells suspension as well as tumor tissue. The antibody panel used includes commercial antibodies against CD3, CD4, CD8, CD10, CD11b, CD20, CD21, CD22, CD34, CD40, CD45RA, CD45RO, CD79, CD90, CD56, cadherin, CA-IX, Cytokeratin (pan), Cytokeratin 19, HIF-1a, hTert, Ki67, Keratin 8, Keratin 18, p53, NSE [16].

FACS analytics were performed at the Fraunhofer Institute for Cell Therapy and Immunology (IZI) in Leipzig (Germany). In brief five specimen of normal and malignant renal tissue were studied to evaluate the protein expression of tumor specific markers like CA-IX, EpCAM and Cytokeratine 7,8 (Table 1).

Tissue	Gender	Age	Size of tumor [cm]	Size of normal tissue [cm]	Morphology of tissue	
					Normal	RCC
1	m	69	4 × 1	2,5 × 1	normal tissue of renal cortex, adipose capsule visible	white, soft, normal RCC
2	f	72	1,5 × 1	2 × 2	normal tissue of renal cortex, adipose capsule visible	little connective tissue visible, bloodshot tissue, normal RCC
3	f	77	1,5 × 1	1,5 × 1,5	normal tissue of renal cortex, adipose capsule visible	Soft tissue, normal RCC, erythrocytes,
4	f	66	5 × 3	3 × 2	normal tissue of renal cortex, adipose capsule visible	Collagen-like phenotype, white tissue, strong, solid tissue
5	m	63	2 × 1.5	2 × 1	normal tissue of renal cortex, adipose capsule visible	Solid tissue, normal RCC

Table 1a: Patient and tissue characteristics for FACS analysis. Percentage of cell expression of different antigens in five different tumor lysates.

Tumor Tissue	Age	Gender	Antibody (positive cells) [%]		
			CK 7,8	EpCAM	CA-IX
RCC 1	69	m	11	8	30
RCC 2	72	f	23	0	26
RCC 3	77	f	7	33	68
RCC 4	66	f	0	0	51
RCC 5	63	m	21	14	28
Mean / SD	69.4 ± 4,8		12 ± 10	11 ± 14	41 ± 18

Table 1b: Patient and tissue characteristics for FACS analysis. And respective healthy tissue.

Normal Tissue	Age	Gender	Antibody (positive cells) [%]		
			CK 7,8	EpCAM	CA-IX
Normal 1	69	m	64	50	4
Normal 2	72	f	71	46	0
Normal 3	77	f	72	48	3
Normal 4	66	f	75	47	2
Normal 5	63	m	64	47	2
Mean / SD	69.4 ± 4.8		69 ± 5	48 ± 2	2 ± 2

Table 1c: Patient and tissue characteristics for FACS. E.g for anti-NSE in average 62 % of the tumor cells and 72 % of the normal tissue cells were stained.

According to standard protocols, healthy and malignant renal tissues were mechanically disaggregated, Percoll-separated and washed in order to receive a single-cell suspension. A single as well as a three-color staining with anti-CAIX, anti EpCAM and anti-Cytokeratine 7,8 was performed followed by flow cytometric analysis (Cytomics FC 500 flow cytometer; Beckman Coulter, Krefeld, Germany).

In a first step, an unbiased screening by ELISA and WB utilizing a large panel of 36 antigens was used to investigate tumor heterogeneity [16]. In a second step, the study focused on antigens with highest overall frequencies of occurrence (FOC > 85%) and highest number of tumors investigated (n > 10) in the first step.

The most frequent antigens in tumor tissue were CA-IX (84%; 71 of 85), NSE (80%; 83 of 104), TPS (81%; 83 of 103), TPA (83%; 85 of 102), CYFRA (81%; 82 of 101). As Danger signal HSP60 (73 of 75) and HSP70 (65 of 71) were detected in nearly each tumor (92-98%) and at least one of the HSP's was present in every TCL investigated [16]. Independent of the RCC-entity no distinct change in the TAA pattern could be observed. Only for CA-IX an increase of the FOC from 84 to 91% (68 out of 75) and surprisingly for NSE from 80 to 87% (79 out of 91) could be noticed when analysis was focused on the most frequent entity clear cell carcinoma.

Similar results were received for Neuron-specific enolase (NSE) which has already been detected in patients with tumors, like neuroblastoma, small cell lung cancer, medullary thyroid cancer, carcinoid tumors, endocrine tumors of the pancreas, and melanoma. Ronkainen et al. (2010) [17] evaluated neuron-specific enolase (NSE) in renal cell carcinoma by immunohistochemistry. 48% of the tumors were positive for NSE and tumors with an immune-positivity for NSE had a shorter (but insignificant) RCC-specific survival. Our findings showed the expression of NSE in about 87% of the tumor cell lysates investigated. Therefor they partly contradict the results of Ronkainen. Nonetheless, both studies reveal that NSE is a highly relevant TAA in all entities of renal cell carcinoma.

Investigations by topological proteomics support these findings. TAA heterogeneity in tumor tissue and single cell suspension was

demonstrated for a total of 26 different tumor associated antigens including CA-IX, NSE and different Cytokeratin's. The analysis revealed that a number of tumor cells showed no CA-IX but did show other typical TAA's [16].

Consecutively FACS analysis was applied to confirm the tumor heterogeneity. Five different normal renal tissues as well as tumor cells derived from five different RCC-samples were used (2 male, 3 female; mean age 69.4 ± 4.8; Table 1, Figures 1a and 1b). Normal tissue displayed a strong positive staining for Cytokeratine 7, 8 in 64 to 75% of the cells (average 69%) compared to tumor cells which displayed a significantly ($p=0.0006$; ($\alpha=5$ % 0.05)) lower expression in the area from 0 to 23% (average 12%). In addition the investigated normal tissues revealed a positive staining for EpCAM in 0-33% (average 11%) whereas the average expression of EpCAM in normal tissue was significantly higher ($p=0.004$; ($\alpha=5$ % 0.05)) with about 48%.

The expression of CA-IX was between 26-68% of the tumor cells with an average expression of 41%. By contrast, the CA-IX positive cells in non-tumor tissue were about 0-3.5% (average 2%) and therefor significantly lower ($p<0.009$; ($\alpha=5$ % 0.05)). These values are comparable to existing data from Li et al (2001), who described an average expression of CA-IX in RCC of 27% ± 24% in 13 specimens [18,19]. In contrast to these tissue data, established RCC-cell lines are known for higher staining of CA-IX. Li et al. described a positive staining for CA-IX in 85 to 95% for the RCC cell lines SKRC-10 and SKRC-52 [19]. These findings could be confirmed in our study by expression rates of 74% in SKRC-10 and 85% in SKRC-52. Overall the cell line data for CA-IX are promising, but not comparable with the heterogeneous situation in tissue sample obtained by a cancer patient.

The additional of a three-color analysis supports these results and indicates the existence of oligoclonal and heterogeneous cell populations within the tumorous environment of RCC-samples investigated (Table 2, Figures 1c and 1f). E.g. normal tissues (Figure 1c) were double positive for Cytokeratine 7, 8 and EpCam in 59 to 71% of the cells (average 65%) whereas only an average of 9% of the tumor cells (Figure 1d) showed both expressions (range 0-23%).

Three RCC samples were double-positive for Cytokeratine 7, 8 and CA-IX in 6 to 18% of the cells. The same samples were double positive for CA-IX and EpCAM. Double Cytokeratine 7, 8 and CA-IX positive normal tissues were not detected.

Three RCC-samples were triple positive for CA-IX, EpCAM and Cytokeratine 7,8 (10 to 20 % of the cells), but no normal tissue samples expressed all three antigens together on the same cell (Table 2).

Tumor Tissue (a)	**Ck 7,8/EpCAM (positive cells)**			**Ck 7,8/CA-IX**			**CA-IX/EpCAM**		
	EpCAM/Ck 7,8	Ck 7,8	EpCAM	Ck 7,8 CA-IX	Ck 7,8	CA-IX	CA-IX/EpCAM	EpCAM	CA-IX
RCC 1	13	0	0	14	0	12	17	0	8
RCC 2	0	37	0	0	35	0	0	0	6
RCC 3	10	0	5	6	0	34	16	0	23
RCC 4	0	0	0	0	0	21	0	0	20
RCC 5	23	0	0	18	3	9	19	0	7
Mean/SD	9 ± 10	7 ± 17	1 ± 2	8 ± 8	8 ± 15	15 ± 13	10 ± 10	0	13 ± 8
Normal Tissue (b)	**Ck 7,8 / EpCAM (positive cells)**			**Ck 7,8 / CA-IX**			**CA-IX / EpCAM**		
	EpCAM/Ck 7,8	Ck 7,8	EpCAM	Ck 7,8/CA-IX	Ck 7,8	CA-IX	CA-IX /EpCAM	EpCAM	CA-IX
Norm. 1	64	0	0	0	69	0	5	57	0
Norm. 2	71	0	0	0	73	0	8	55	0
Norm. 3	59	8	0	0	67	0	0	59	0
Norm. 4	66	8	0	0	75	0	0	66	0
Norm. 5	67	6	2	0	67	0	0	60	0
Mean/SD	65 ± 4	4 ± 4	0 ± 1	0	70 ± 4	0	3 ± 4	59 ± 4	0

Table 2: Percentage of marker-positive cells for Cytokeratine 7,8 (Ck 7,8), EpCAM and CA-IX for tumor tissue (a) and respective healthy tissue (b).

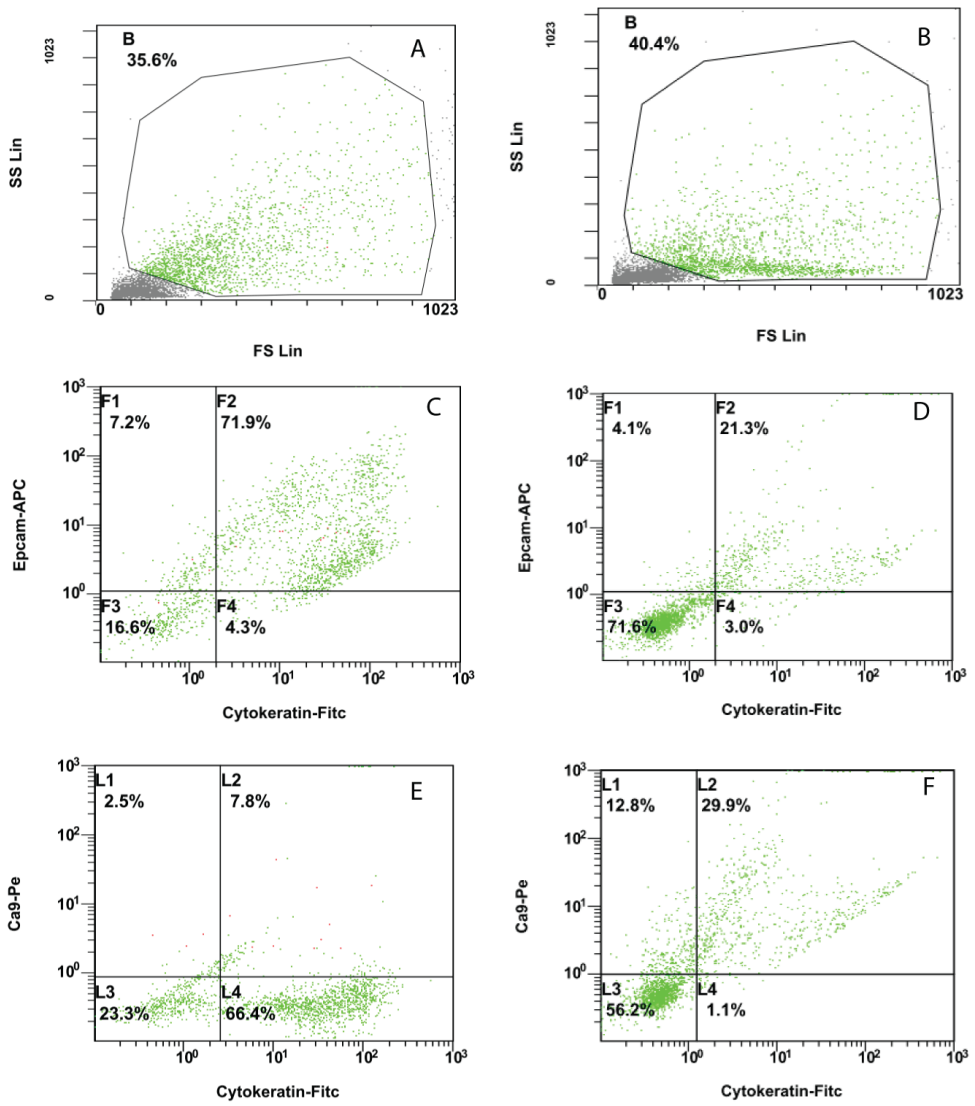

Figure 1: Flow cytometic analysis of normal renal tissue and malignant cells from renal cell carcinoma exemplarily shown for one sample (Normal 1/RCC 1).

A, B: Representative picture of all normal cells (a) and RCC cells (b) using side scatter (SS) and forward scatter (FS).

C, D: Two fluorescences of the three-colour staining of cytokeratine, EpCAM and CA-IX. Quadrant down left dislays Cytokeratine 7,8 and EpCam negative cells. Quadrant down right represents Cytokeratine 7,8 positive and EpCAM negative cells. In the quadrant top-left cells negative for Cytokeratine 7,8 and positive for EpCAM are shown. Cytokeratine 7,8 and EpCAM positive cells are located in the quadrant top-right.

E, F: Two fluorescences of the three-colour staining of cytokeratine, EpCAM and CA-IX. The quadrant down to the left displays Cytokeratine 7,8 / CA-IX negative cells, the quadrant down to the right represents cells, positive for anti-Cytokeratine 7,8 and negative for CA-IX. CA-IX and Cytokeratine positive cells are shown in the quadrant top-left. Anti-Cytokeratine and CA-IX positive cells are located in the quadrant top right.

The numbers within the figures show the percentage of positive stained cells in all cases.

Finally to address regulatory purposes according to potency the immune response in an animal model (mice) was induced [16] by use of five batches of tumor cell lysates. Immune sera were tested for specific IgG antibodies against CA-IX, NSE and cytokeratins by ELISA. Sera of non-treated mice were used as controls. All five TCLs induced specific IgG antibody titers despite of the low concentrations (ng/mL) of each individual antigen in the multi-epitope vaccines. Here, either CA-IX, NSE and/or a cytokeratin epitope plus at least one of the danger signals was present. As expected, the immunization (anti-CA-IX, anti-NSE and anti-CK) did not correlate to the antigen levels in the TCL since the Mode of Action of tumor vaccines prepared according

to the Reniale® scheme does not show a dose/response relationship *in vivo* [20].

Conclusion

In summary, a heterogenic mixture of a subset of five TAA's (CA-IX, NSE, TPA, TPS, CYFRA) and an additional two typical stress/danger proteins, the heat shock proteins hsp60/70 were found in a total of 133 tumors. Concluding from this highly individual antigen patterns, the artificial composition of an individualized tumor vaccine seems to be impossible. A multi-epitope-approach could therefore be appropriate in the fight against (micro) metastasis as can be concluded from antigenic heterogeneities found in several tumors [3,20-22]. Therefore, the use of the autologous composition could be an appropriate opportunity for the manufacturing of a tumor vaccine in RCC patients. When different regions of the tumor tissue are used for the manufacturing process, a probability is given to include as many epitopes as possible in a "multi-epitope-vaccine".

Furthermore the study design addressed regulatory requirements on potency testing in medicinal products by comparing the TAA distribution in tumor lysates and initiated immune response in mice respectively. The potency of a tumor vaccine bases on its immunogenicity. Thus, the proven prevalence of both, antigens and danger signals, could be the key for a successful immunization. To demonstrate the co-existence of both antigen and danger signals an animal model was selected despite of the general antigenicity of a human TCL in mice due to the species barrier. All five test TCL's were immunogenic without the addition of adjuvants and induced specific IgG antibody titers (CA-IX, NSE, etc.) despite of the low concentrations (ng/mL) of each individual TAA in the multi-epitope vaccines.

This leads to the conclusion that all components needed for an (broad) immune response were present in all TCL's in an immunological active form. Since the presence of both (a mixture of TAA and Danger signal) was proven in the animal model used, this could define a minimal requirement for a tumor vaccine, autologous as well as artificially composed. In summary, the immunogenic potency of the TCL's was proven and the mouse model used could be capable to address the requirements by the FDA and EMA for potency testing [23].

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