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# **PhD THESIS**

**NUCLEAR MARKERS IN RENAL CELL CARCINOMA**

**A B S T R A C T**

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## ABSTRACT

The current PhD thesis includes a general, theoretical part, and a special part, consisting of three individual original articles, previously published in peer-reviewed scientific journals. The theoretical part focuses on establishing the background, contemporary context and scientific motivation for the retrospective renal cell carcinoma (RCC) immunohistochemistry (IHC) investigations of key novel biomarkers, i.e. Wilm's Tumor 1 (WT1), Thymine Dimers (TDs) and Ki-67, included subsequently in the special part. To achieve this goal, a comprehensive literature review of relevant topics was elaborated, focusing on RCC clinical definitions, semiology and currently available imaging modalities for RCC detection and clinical work-up, RCC differential and definitive pathological diagnosis, subtyping and prognosis assessment. Furthermore, we investigate and provide relevant data regarding emerging clinical tools for RCC screening, risk stratification and therapeutic response prediction, while also identifying existing knowledge gaps and defining promising novel research directions. Subsequently, the special part follows up on these novel research topics, outlined in the general part, being comprised of three retrospective IHC investigations of RCC immunoexpression patterns for the aforementioned key biomarkers, which have been established as being underinvestigated, yet possibly useful for improving RCC clinical management and patient outcomes.

The investigation of WT1 immunoexpression in adult (a)RCCs allowed for the elaboration and publication of 2 original articles, while an additional 3<sup>rd</sup> article, only recently made public, documents, for the first time, novel expression patterns for TDs in RCCs. Our work regarding Ki-67 is currently in the statistical analysis stage and will soon be ready for formal release. Thus, within the current thesis evaluate the presence and expression patterns of WT1 transcripts, as indicators of aRCC epithelial to mesenchymal transition, and TDs, as novel genomic lesions in RCC tumor tissues, respectively.

The global RCC population of the current thesis is comprised of a consecutive series of 134 RCC cases, managed clinically at Arad County Hospital, within the Urology Department, from 2015-2018. All patients had undergone partial or radical nephrectomy directly. Neoadjuvant systemic therapy was not administered in any of

the RCC cases. The resulting native RCC specimens were further processed and paraffin-embedded within the local Morphopathology Department. For this global RCC population, we collected all the available relevant clinical records. However, this RCC specimen library was amassed gradually, throughout the past 4 years, since the debut of the current research initiative. Thus, chronologically, our first IHC investigations, targeting WT1 transcripts and TDs respectively, only assessed 90 RCC samples, from 2016-2017, as the rest of the RCC samples had not yet been obtained. Thus, even though the individual RCC subpopulations defined for each specific biomarker investigation, are derived from the same overall RCC tissue library, they are not homogenous. The available RCC specimens were initially sampled for WT1 staining and then resampled, i.e. sliced repeatedly, for TD staining. Consequentially, even though the same paraffin-embedded RCC specimens were sampled within both investigations, the resulting histological slides showed variability regarding the presence of tumor-adjacent healthy renal tissue, the central study inclusion criteria for both investigations. Implicitly, the final RCC subpopulations included in each investigation overlapped only partially. Later on, an additional 44 RCC cases, treated in the same clinic, in 2015 and/or 2018, respectively, became available and were included in the Ki-67 analysis.

After preliminary deparaffination, the resulting RCC tissue slides were stained with HE, in an automated manner, using the Leica Autostainer XL and then reevaluated morphologically. The main inclusion criteria for WT1 IHC staining was represented by the presence of healthy renal tissue in the immediate proximity of the tumor on HE staining, seeing as the podocyte expression of WT1 in normal renal corpuscles will represent the internal positive control reference. Conversely, for the TD-targeted immunoreaction, control specimens were external, comprised of multiple in vivo healthy skin samples. Even so, inclusion in the TD study still required the same mandatory presence of healthy renal tissue in the immediate proximity of the tumor on HE staining. As in the case of RCCs, TD expression in healthy renal tissue has not been investigated so far. Finally, the study inclusion criteria for Ki-67 IHC analysis were artefact free, viable RCC tumor tissue slides, lacking extensive areas of necrosis. For the Ki-67 investigation, the immunoreaction positive control was also external. We utilized the same protocol as for TD assessment and processed additional in vivo healthy skin samples. Within these samples, the epidermis was constantly positive in the basal and para-basal layers for Ki-67. As

opposed to previous investigations, a positive Ki-67 immunoreaction was considered valid only when the final reaction product was strictly nuclear restricted, without cytoplasmic staining.

Thus, investigation specific, RCC subpopulations emerged and were further processed with immunostaining protocols, as follows: 56 RCCs stained for WT1; 54 RCCs stained for TDs; 120 RCCs stained for Ki-67. After preliminary evaluation, for each individual investigation, the cases which met the inclusion criteria were further classified, based on predominant morphological growth pattern. For the WT1 and TD investigations, the classic 3-tier system for conventional RCC subtypes was used: 1 – clear cell (cc)RCC, 2 – papillary (p)RCC and 3 – chromophobe (ch)RCC, with 4 - sarcomatoid dedifferentiation variants of RCC (svRCC) being reported separately. For Ki-67, an additional morphological distinction was made, within the ccRCC subgroup, between solid and microcystic variants. For all investigations, cellular aggressiveness was assessed, based on predominant nuclear traits, quantified using the WHO/ISUP 2017 grading system (G1/G2/G3/G4).

The targeted immunohistochemical procedures were performed using the Bond Max autostainer (Leica Biosystems) and the primary antibodies used were: for WT1 - ready to use, monoclonal, N-terminus targeted, clone WT49, from Leica Biosystem, Newcastle Ltd, Newcastle Upon Tyne; for TDs - monoclonal mouse antihuman thymine dimer clone (Kamiya Biomedical Company, Seattle, WA, clone KTM53, dilution 1:10.000); for Ki-67 - mouse anti-human Ki67 antigen monoclonal antibody, unconjugated conjugated, clone MM1, from Leica Biosystem, Newcastle Ltd, Newcastle Upon Tyne.

The quantitative WT1 immunoreactivity of each RCC sample evaluated was reported, using the following protocol: 0 - absent WT1 staining; +1 - <2% positive tumor cellularity; +2 - <10% positive tumor cellularity; +3 - >10% positive tumor cellularity. Both nuclear and cytoplasmic WT1 staining were included in the definition of WT1-positivity. Additionally, qualitative labels were attributed, by comparison with the internal positive control (podocytes), regarding color intensity and homogeneity: weak, moderate and high.

For nuclear TD immunoreactions, multiple parameters were documented, starting with the fundamental distinction of positive cellularity localization, i.e. within RCC tumor tissue vs. in the tumor-adjacent healthy kidney tissue. For RCC tumor tissue staining, a simple “yes or no” expression parameter was attributed (labeled

TD+/-), with further nuance being conferred by quantitative (SQ) and qualitative (SI) TD immunoreactivity scores, as well as a cumulative expression score (ST), obtained by the addition of the values of the previous two (ST=SQ+SI). SQ reflects quantitative tumor tissue TD-positivity and its values are defined within a 400x microscopic field, as follows: 0 - negative for TDs; +1 - 1%-10% positive nuclei; +2 - 11%-25% positive nuclei; +3 - >26% positive nuclei. SI reflects qualitative tumor tissue TD-positivity and its values are defined as follows: 0 - negative for TDs; +1 - weak immunoreaction, visibly less intense than external control; +2 - moderate immunoreaction, similar intensity to external control; +3 - strong immunoreaction, visibly more intense than external control. Regarding tumor-adjacent healthy renal tissue, a similar simplified “yes or no” TD expression parameter was attributed (labeled HKE+/-). The specific localization of these TD positive healthy renal cells was also reported, i.e. tubular expression (HTE), and/or stromal/endothelial expression (HSE), in the same simplified “yes or no” manner.

Lastly, the density of Ki-67 positive cellularity, was documented, using the following protocol: 0 - <1% of cells with positive nuclei; +1 - 2-10% of cells with positive nuclei; +2 - 11-25% of cells with positive nuclei; +3 - >25% of cells with positive nuclei. The above-mentioned protocol for Ki-67 immunoreaction quantitative evaluation was utilized on a single representative microscopic field, under 200x magnification. Anisocariasis were also evaluated and noted for each case, using the following labels: absent - 0, mild - 1, moderate - 2, severe - 3. Mitoses observed within Ki-67 positive RCC tumor sections were graded as absent (0), rare (1–2/field), average (3–5/field), or frequent (more than 5/field).

We analyzed the resulting consolidated database of TD stained RCCs, by using the SPSS v.27 software (IBM, Chicago, Illinois, USA) on a Microsoft Windows operating system. The WT1 IHC investigation yielded a very limited number of WT1-positive aRCCs. Therefore, statistical analysis was deemed as being unnecessary. For the Ki-67 positive RCC cohort, the statistical analysis results are currently unavailable, as this assessment is still being elaborated and not yet finalised. It will surely be included in a further research publication on the topic.

Among the 56 viable RCC cases stained for WT1, 38 were ccRCCs, 8 pRCCs, 3 chRCCs and 7 svRCCs. Nuclear grading further classified these tumors: G1 – 21, G2 – 23, G3 – 4, and G4 – 8. The normal renal tissue, adjacent to the tumor, constantly manifested, for all of the evaluated cases, nuclear WT1 staining of

podocytes and, also, of the epithelial cells, constituting the parietal layer of Bowman's capsule. The final reaction product steadily displayed an intense brown coloring, for the aforementioned positive internal control. In 5 cases (8.92%), within the renal capsule, but not in the tumor pseudocapsule, we identified numerous, intensely positive, stromal cells - most likely fibroblasts/myofibroblasts, located at a significant distance from the tumor tissue. For all 5 cases, the tumor tissue was negative for WT1 staining. Moreover, in 3 tumor tissue WT1- negative RCCs, intratumoral endothelial cells manifested positive WT1 nuclear staining.

Out of the 56 cases evaluated, 49 demonstrated negative WT1 staining within the tumor tissue. The 7 positive cases, all manifested intratumoral, exclusively nuclear, WT1 staining and all had a clear cell morphology. Quantitative analysis revealed that only 2 cases had a high density of positive tumor cells (+3). The intensity of staining for the final reaction product from within the positive tumor cells was similar to the control (podocytes) or higher, except for the 2 cases with high positive tumor cell density, which manifested a lower intensity than the control.

The RCC cohort stained for TDs was comprised of 39 ccRCCs, 8 pRCCs, 4 chRCCs and 3 svRCCs. Nuclear grading further nuanced RCC tumor biology and risk stratification: G1 - 15, G2 - 26, G3 - 9, and G4 - 4. Our TD-target immunoreaction was validated by the external control employed, i.e. multiple in vivo cutaneous tissue samples. These samples were not additionally exposed to UVR, outside of their inherent environmental in vivo exposure, before or after obtaining tissue biopsies. As expected, even though some untreated samples were mostly negative, sporadic areas of diffuse nuclear TD staining was encountered, mainly in basal epidermal cells, sweat glands, and stromal endothelial cells. The final reaction product steadily displayed an intense, exclusively nuclear, brown colored immunoreaction stain for the aforementioned external control, with maximum intensity in the epidermis, in the basal and para-basal cellular layers.

Remarkably, out of the 54 samples evaluated, 42 RCCs (77.8%) were positive, manifesting nuclear expression of TDs within RCC tumor tissue. A complete documentation of TD immunoreaction quantification parameters was reported. During the microscopic evaluation of our TD-positive RCCs, we identified two main distribution patterns for TD immunostaining in tumor tissue, namely: 1) heterogeneous TD expression, with moderate to high density and intensity, concentrated at the level of the proliferation front and/or along the transitional area,



between tumor and healthy renal tissue, yet inconsistent in more central tumor areas; 2) homogenous, diffuse, and high intensity TD expression, with the majority of RCC tumor tissue cellularity being positive for TDs.

These two predominant, apparently organized and recurrent patterns of TD nuclear immunoreactivity amongst the evaluated RCCs, were seen, at least partially, in the tumor tissue of a majority (33 samples) of the 42 TD-positive RCCs analyzed, having a moderate or high global staining score ( $ST \geq 3$ ). An additional, disorganized, seemingly random, low density ( $SQ = +1$ ), variable intensity (mostly  $SI = +1/+2$ ), third TD immunoreactivity pattern distribution model was also encountered, albeit more rarely. In 9 TD-positive RCCs, a weak nuclear TD-stain was encountered, within only a few isolated nests of RCC tumor cells, with mostly negative stromal RCC cellularity, nuclear TD immune-reactivity being distributed in a random manner within the tumor tissue. Adjacent healthy renal tissue is also generally negative in these 3rd distribution model RCCs, with the exception of 2 cases, which showed TD expression in both tumor tissue and adjacent healthy tissue (tubular cells).

The first, heterogeneous, nuclear TD immunostaining distribution pattern, seen in positive RCC tumor tissue samples, is not maintained in the more central tumor areas, nor consistently along the proliferation front, for that matter. Dominant TD-positive RCC cellularity, with moderate staining, can be seen in the more mitotically active proliferation front. Yet, when approaching the actual transitional area, between tumor tissue and adjacent healthy renal tissue, two distribution models have been observed. Either, TD immunoreaction may extend into healthy renal tubules in the proximity of the tumor, or tumor-adjacent healthy tissue loses TD expression altogether.

Regarding RCC tumor tissue TD-positive stroma, there were multiple cell lines which demonstrated nuclear TD-positivity. Usually, TD-positive tumor stroma was encountered in the homogenous tumor tissue TD-staining distribution subgroup and favored the tumor-healthy tissue transitional areas, especially when demonstrating abundant inflammatory infiltrate on HE, at this level. Positive stromal cells (fibroblasts) and diffusely reactive immune cells, mainly macrophages based on morphology, were identified, usually bordering TD-positive RCC cells. As an isolated finding, a TD-positive intratumoral arterial blood vessel was encountered, immunoreactive both at the level of the endothelium, but also in parietal myocytes. Furthermore, we identified a total of 20 RCC specimens (37%), which demonstrated

TD-positive cellularity in tumor-adjacent healthy renal tissue, yet two of these cases did not manifest TD-positive RCC tumor cellularity.

Statistical analysis of our TD investigation RCC cohort was performed. We performed the Chi-Square and Fisher's tests to analyze proportions between the TD positive and TD negative RCC subgroups. The Student's t-test was performed to determine the mean differences of normally-distributed continuous variables. The Spearman correlation coefficient was calculated to determine whether significant associations exist between the variables that were studied. The significance level required was less than or equal to 0.05. No statistically significant differences between the study groups were observed. The majority of tumor tissue TD-positive RCCs, 18 samples (42.8%), had a moderate value attributed in quantitative evaluation (SQ=+2), while the most prevalent staining intensity score value, attributed to 19 samples (45.2%) within the qualitative evaluation, was also moderate (SI=+2). The overall expression score (ST) ranges in value from +2 to +6, with a high prevalence for the mid-range values: 3 (26.2%), 4 (28.6%), and 5 (23.8%).

Further variable correlation analysis, using Spearman's correlation, demonstrated that, in fact, the more simplistic, -"yes or no"- parameter (TD+/-), quantifying the presence of TDs in RCC tumor tissue, manifests a statistically significant, moderate negative association with RCC stage at diagnosis, whereas the more sophisticated, TD-expression scores (SQ,SI,ST), manifest no statistically significant correlations with any of the well-known prognostic clinical parameters for RCC, included in the analysis. Thus, apparently, TD expression associates lower RCC stage at diagnosis, yet this correlation needs further validation, as the current study has a relatively small RCC sample cohort, of which, almost 80% have a low TNM stage at diagnosis (1 or 2).

Interestingly, the simple "yes/no" parameter for tumor adjacent healthy renal tissue TD-expression quantification (HKE), manifested statistically significant, moderate positive correlations, with all the individual RCC tumor tissue TD-targeted immunostaining parameters, except its corresponding "yes/no" variable for tumor tissue TD-expression, TD+/- . Surprisingly, the only statistically significant association identified for HSE, is with cN, namely a moderate positive correlation between these two variables, meaning that stromal/endothelial TD expression in tumor adjacent renal tissue may be a predictive marker for lymphatic dissemination in RCC. This statement obviously represents wishful thinking. Firstly, correlation does not equate

to causation and, secondly, the statistical analysis was done on a small subset of cases, of which only one case showed positive HSE, albeit set case had pathological confirmation of retroperitoneal lymphatic dissemination.

For Ki-67 IHC, out of the 134 RCC cases sampled, we identified the tumor lesion in 120 cases, with only 73 cases including the RCC/normal tissue interface. Thus, only 120 RCCs could be included in the Ki-67 IHC cohort. The majority of cases were ccRCC (98 cases). Among these ccRCCs, a particular subgroup emerged, displaying a microcystic appearance, i.e. multiple microcysts, hemorrhagic suffusions, with tumor cells arranged in islands and nests. Thus, these morphologies constituted a distinct microcystic ccRCC subgroup within the current study (9 cases), as opposed to solid ccRCCs (the remaining 89 cases). Furthermore, we identified 11 pRCCs (type 1 pRCC - 4 cases, and type 2 pRCC - 7 cases), 6 chRCCs, and 5 svRCCs. Nuclear grading ranked 5 RCCs - G1, 58 cases - G2, 50 cases - G3, and 7 RCCs - G4.

Regarding the tumor-adjacent renal tissue, very rare cells with Ki-67 positive nuclei were seen within the mesangium, the tubular system of the nephron and as isolated elements of the inflammatory infiltrate, if present in the stroma. In fact, cells with positive nuclei were below 1% in all tumor-adjacent renal tissues evaluated, regardless of their microscopic subtype. The Ki-67 immunoreaction within the positive RCC tumor cells differed in density and intensity from case to case, and was characterized by marked intratumoral heterogeneity, with some areas being characterized by major agglomerations of positive nuclei. Ki-67 positivity was more evident at the periphery of the tumor, in the immediate vicinity of the proliferation front. Also, a large number of positive cells was observed in the vicinity of neoformation blood vessels. Practically, each case presented defined values of the proliferation index, and thus Ki-67 becomes an individual prognostic marker.

A number of 12 cases were negative in the sense that they did not present cells with Ki-67 positive nuclei in the tumor area. In cases where mitoses were absent and anisokarya was present, the Ki-67 proliferation rate was typically ranked between 2-10%. The presence of marked mitoses and anisokarya was associated with an increased Ki-67 labeling index. We observed a higher density of Ki-67 positive cells in tumor areas located in the vicinity of the proliferation front and in those proximal to chronic inflammatory infiltrates, sometimes present within the tumor area. Tumors with a high density of cells with Ki-67 positive nuclei were mostly

grade G2, G3, and G4. No statistically significant correlation was seen between these two elements. The relationship between Ki-67 expression and RCC histopathological subtype was further assessed and, as expected, the most common cases, which scored +3 on Ki-67 immunoreaction density, were poorly differentiated and/or sarcomatoid carcinomas. Surprisingly, we obtained very low values for papillary carcinomas. Median values of the proliferation index were observed in solid variant ccRCCs and chRCCs. The microcystic variant clear cell carcinoma showed only rare positive cells, scored as +1.

In hindsight, WT1 is a complex gene, with a seemingly endless myriad of transcript isoforms, acting upon and being acted upon, equally complex, molecular signaling pathways. The data regarding WT1 protein expression in aRCCs is quite limited and highly inconsistent. Multiple IHC antibodies, targeting different areas of WT1 protein (C-/N-terminus), have shown different immunoreactivity rates and staining patterns. In our investigation, we used, for the first time, the recent, N-terminus targeted, WT1 IHC antibody, clone WT49, to evaluate WT1 protein expression, in the largest aRCCs cohort to date. In contrast with existing data, despite including cytoplasmic staining in the definition of WT1 positivity, we report exclusively nuclear WT1 staining with clone WT49, but also the highest rate of nuclear WT1-positivity in aRCCs to date (12.5%). Additional, comparative IHC analyses of the many commercially available antibodies are essential in order to standardize the quantification of WT1 protein expression, in aRCC and its many subtypes.

Representing the very first investigation of its kind, our second IHC analysis focused on evaluating the expression of TDs in RCC tumor tissue and tumor-adjacent healthy renal tissue, using a TD-targeted IHC monoclonal anti-body, clone KTM53. Our results illustrate a novel and pronounced association between RCCs and a mutagenic genomic lesion of great biological significance, previously thought to occur exclusively during UVR exposure. Out of the 54 RCC specimens evaluated, 77.8% showed nuclear TD-expression in RCC tumor tissue and 37% in the tumor-adjacent healthy renal parenchyma. A comprehensive report regarding quantitative and qualitative TD-targeted nuclear immunostaining parameters was elaborated. We also report the identification of two main distribution models for TD-expression within RCC tumor tissue. Further rigorous molecular analysis is required in order to fully

comprehend/validate the biological significance of this newly documented expression of TDs in RCC.

In our most recent investigation, we studied proliferation rate RCC tumor cells, using morphological assessment and Ki-67 immunoexpression comparatively. Preliminary results show a relatively low rate of cellular proliferation within the RCCs evaluated. Conversely, microcystic morphological variants of ccRCCs and pRCCs consistently associated increased Ki-67 expression patterns. However, Ki-67 immunoreactivity did not correlate with the degree of RCC differentiation. It is becoming increasingly clear that Ki-67 should be viewed as an individual and independent prognostic marker for RCCs. Additionally, Ki-67 immunoreactivity based treatment response assessment, as an objective indicator of the effectiveness of systemic humanized growth factor inhibition therapy, should be further investigated. So far, our data supports the integration of Ki-67 IHC quantification in routine clinical practice, constituting a relevant addition to the currently approved panel diagnostic/prognostic IHC antibodies. Most likely, Ki-67 will at one point become a part of the standard pathological evaluation of all renal tumors.

In conclusion, beyond the acknowledged limitations and validation issues of the current thesis, the investigations encurred have allowed for thought provoking and wildly provocative incursions into a novel, albeit still speculative, fundamentally redefined molecular univers, with important deviations from the conservative definitions of RCC carcinogenesis. The shifts in perspective provided must naturally be verified by further rigorous genetic analysis, yet more importantly, they offer invigorating and conceptually novel directions within the on-going scientific struggle to identify the ideal clinical applications for these promising molecular instruments, namely emerging RCC biomarkers. If nothing else, these thought experiments regarding RCC pathogenesis inherently contribute their unique perspective, stimulating scientific debate, while wholeheartedly acknowledging the still unvalidated, yet wildly exciting, novel molecular premises they are rooted in.