Soluble fragment of the receptor tyrosine kinase MERTK as a liquid biopsy biomarker in bladder cancer

Student: Aigerim Nazarbay
PI: Dr. Marina Kriajevskaia, Associate Professor
Co-PI: Eugene Tulchinsky
Submission date: March 18, 2024

ABSTRACT

Aim of the study: The main purpose of the study was to determine the effectiveness of soluble MERTK as a liquid biomarker in bladder cancer. The presence of soluble MERTK in urine samples of bladder cancer patients was analyzed using different laboratory techniques.

Background: MERTK is a member of TAM receptors that are expressed in normal blood cells and cancer cells. MERTK exists in a soluble form which made by proteolytic cleavage, and it is a detectable in urine samples of patients. Currently, the available diagnostic methods for bladder cancer are mostly invasive and expensive, and they used to detect progressive forms of cancer. The main scope of many studies is the utilization of these receptors as potential biomarkers for the early detection of bladder cancer.

Methodology: The small-scale preliminary study was conducted to characterize expression of MERTK in bladder cancer using the several laboratory techniques as ELISA, Western blotting, and immunohistochemistry. Study subjects were adult patients diagnosed with bladder cancer and healthy controls. Biopsy and urine samples of bladder cancer patients were used as samples.

Results and Conclusion: The results of experiments showed the high expression of soluble MERTK in bladder cancer patients' urine samples compared to samples of healthy controls. According to the results immunohistochemistry, MERTK was expressed in bladder cancer tissue of these patients. To conclude, soluble MERTK can be utilized as a liquid biomarker in bladder cancer.

INTRODUCTION

Bladder cancer is a common cancer among males, and its incidence is continuously increasing worldwide. According to the statistics, approximately 549,000 people were positively diagnosed with bladder cancer in 2018, and it accounts for 2.1% of all cancer deaths throughout the world (Saginala et al., 2020). Diagnosis is made using the invasive and expansive procedures such as cystoscopy and CT urography (DeGeorge, Holt & Hodges, 2017). Therefore, it is important to discover cost-effective and non-invasive methods to diagnose bladder cancer at the early stages. Recent studies have been performed to determine the potential biomarkers for bladder cancer to improve cancer diagnosis and treatment. The TAM family of receptor tyrosine kinases (RTKs) are considered as one of the potential biomarkers for bladder cancer. TYRO3, AXL and MERTK are members of the TAM family of receptors which can be used as biological biomarkers. MERTK (myeloid-epithelial-reproductive tyrosine kinase) is a member of the TAM family of RTKs that is abnormally expressed in various types of cancer cells. MERTK is made of Ig-like, fibronectin III and kinase domains extracellularly shown in Fig 1. (Huelse et al., 2020). MERTK binds to its ligands as Gas6 and Protein S to activate downstream signaling cascades (Ohta et al., 2021). The main functions of TAM RTKs are regulation of cell survival, proliferation, migration, adhesion, blood clot stabilization, mediation of efferocytosis, and modulation of inflammation (Chen & Liu, 2021).

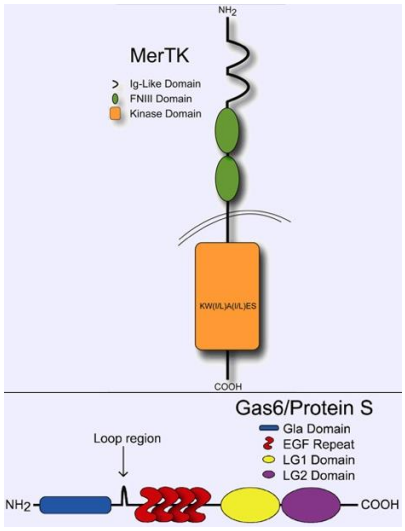

Figure 1. MERTK structure (Keating et al., 2008)

MERTK is expressed in many human cancers such as acute myeloid leukemia (AML), lymphoma, lung cancer, astrocytoma, prostate cancer, gastric cancer, colon cancer, neuroblastoma, and head and neck squamous cell cancer. MERTK plays a role in tumor cell proliferation, invasion migration and metastasis, drug resistance and suppress anti-tumor immunity. Studies showed that signaling pathways downstream of the MERTK kinases involve growth factor-mediated proteins like PI3K, Ras and MAPKs. So, the failure of these signaling pathways may lead to the development of cancerous transformations (Chen & Liu, 2021). This means that MERTK might be a novel target for the treatment of bladder cancer.

According to these studies, the overexpression of AXL, TYRO3, MERTK receptors, and their ligand Gas6 is associated with poor prognosis in various types of human cancers. In general, there are not enough data showing the association between bladder cancer and TAM receptors. Recent studies have shown a close relationship between TAM receptors, especially AXL receptors, and bladder cancer (Akgul et al., 2021). The results of a study by Dufour et al. (2019) have revealed preclinical evidence for the promising therapeutic role of TYRO3 in bladder

cancer. Therefore, it is important to identify the expression of TAM receptors in bladder tumors as potential biomarkers and targets for treatment.

Soluble forms of TAM receptors are made as a result of proteolytic cleavage of extracellular domains by metalloproteases. Human A Disintegrin and Metalloprotease 17 (ADAM17) is one of the proteins which cleave MERTK. ADAM17 cleaves the N-terminal of MERTK, transforming the signal recognition part of the MERTK and making it soluble and incompetent for downstream signaling (Witas et al., 2021). Soluble Mer (sMer) may function as a ligand sink and reduce both MERTK signaling and efferocytosis. According to the study conducted by Witas et al. (2021) the mouse model of Sjögren's Syndrome had impaired MERTK signaling and efferocytosis because of cleavage of MERTK.

To conclude, the role of MERTK as a potential biomarker in cancer diagnosis and as a target for cancer treatment has been investigated in recent studies. The overexpression of MERTK is often found in aggressive tumors which have advanced tumor stage and lymph node involvement. The detection of the MERTK expression will facilitate the improvements in bladder cancer diagnosis and treatment, and it will help to increase the patient survival rate. Therefore, it is important to study the relationship between MERTK expression and bladder cancer progression.

MATERIALS AND METHODS

Cell culture

Standard medium (10% FBS, 1% NEAA, 1% sodium pyruvate, 1% PenStrep) was prepared. Frozen RT4 and RT112 cells in 1 ml cryotubes were quickly thawed and transferred to a 15 ml tube with 8 ml of media. The tubes were centrifuged for 5 minutes at 1000 rpm. The supernatant was discarded, and the pellet was resuspended in a fresh portion of the medium. The cells were transferred to a Petri dish and seeded. The dishes were labeled with the initials, date, passage number, and cell type. The dishes were placed inside the CO2 incubator. The medium was changed every 2-3 days, depending on the cell confluence. The old medium was aspirated, and the dishes were replenished with fresh pre-warmed serum-free medium, a dish with 8 ml. RT4 cells died, and therefore they were discarded. Bladder cancer cell lines were cultured in DMEM medium containing glucose, sodium bicarbonate, then dishes were incubated overnight and were used to make lysates and condition medium.

Cells were passaged when the confluence was reaching 80-90%. Passaging of cells was performed by aspirating the old medium, washing the flasks with 4 ml of 1 x PBS, incubating cells with 3 ml of Trypsin for 3 minutes, transferring to a 15 ml tube and 7ml of media added, then centrifuging for 5 minutes at 1000 rpm, resuspending the pellet with 2 ml of media and aliquoting cells appropriately into 2 dishes with fresh medium.

Cell lysates and condition medium preparation

Cell lysates were prepared either in 1x Laemmli buffer containing SDS (253J cells) or in buffer, containing 0.5% Triton X100 and 1mM EGTA (UMUC3 cells). Condition media were collected

from a petri dish to 15 ml tubes and a protease inhibitor cocktail was added at a ratio of 1:1000. The dishes were washed with 1x PBS. 1 ml of lysis buffer was added to the dishes and they were incubated on ice for 10 minutes. Then the dishes were scrapped, and the lysates were transferred into 1ml Eppendorf tubes. Eppendorf tubes were centrifuged for 10 minutes, 12000 g, +4 ºC. The purified lysates were transferred to new Eppendorf tubes and were stored at -20 ºC. Collected condition media were centrifuged at 1000 rpm for 5 minutes. The supernatants were aspirated and filtered with 0.4 ul syringe filters for further concentration with Amicon Ultra-4 centrifugal filters. A new Amicon tube was rinsed with 1ml of 1 x PBS and centrifuged for 10 minutes, 3500rpm, 10 ºC. PBS was discarded and 4 ml of condition medium was added to the Amicon tube. Amicon tubes with condition media were centrifuged at 3500 rpm, 10 ºC until the volume of concentrate would be less than 500 ul and more than 250 ul. Then another 4 ml of condition media were added on top of the concentrate to concentrate further to reach the volume of 1 ml. Every time after centrifugation, the solution at the bottom of the larger tube was discarded. 1 ml concentrates were transferred to Eppendorf tubes and stored at -20 ºC.

Urine sample preparation

8 ml of urine samples were taken into a 15 ml tube and spinned at 1500 rpm, 5 minutes, 10 ºC. 4 ul of protease inhibitor cocktail were added. Urine was filtered with a 0.45 um filter unit. 8 ml of unconcentrated urine were aliquoted into the Eppendorf tubes and stored at - 20 ºC.

Western blot

8% resolving gel (30% acrylamide, 1.5 M Tris-HCl (pH=8.8), 10% SDS, 10% APS, 0.04% TEMED) and 5% stacking gel (acrylamide, 10% SDS, 10% APS, 1M Tris-HCl (pH=6.8), 0.1% TEMED) were prepared for the SDS-PAGE using Acrylamide/Bis-acrylamide 30% (w/v)

(Sigma Aldrich, A3574 , USA), 1.5 M Tris solution (pH=8.8) for resolving gel and 1 M Tris solution (pH=6.8) for stacking gel, sodium dodecyl sulfate (74255-250G, Sigma-Aldrich, Kazakhstan), ammonium persulphate (248614-500G, Sigma-Aldrich, Kazakhstan), and TEMED (T9281-25ML, Sigma-Aldrich, Kazakhstan).

500 ul of urine samples (concentrated and unconcentrated) were mixed with 160 ul of 4x Laemmli buffer and 25ul of ME/BB mix and heated for 1 min at 94 °C in the heating block before loading. 25 ul of samples were loaded on gel. 25 ul of lysates and condition media were mixed with 7ul of 4x Laemmli buffer and 1.5ul of ME/BB mix (1 part of 2-mercaptoethanol (M3148-25ML, Sigma-Aldrich, Kazakhstan) and 1 part of 0.4% bromophenol blue (1364332, Fisher Scientific, Kazakhstan), and heated for 1 min at 94°C in the heating block before loading. 25 ul of samples were loaded on gel. The SDS-PAGE apparatus with 1X running buffer (1.0% SDS, 3.0% Tris base, 14.4% glycine) was assembled and protein samples were loaded to the gel. SDS-PAGE electrophoresis was run at 120 V for 1 hour 30 minutes. After SDS-PAGE electrophoresis the transfer apparatus was assembled with 1X transfer buffer (0.3% Tris base, 1.44% glycine), gel with proteins on it, and PVDF Transfer Membrane (Thermo Scientific, 88518, Kazakhstan). The transferring of proteins to the membrane was done at 20 V overnight. SDS-PAGE gel casting and electrophoresis and protein transfer were performed using Mini Trans-Blot Accessories (1703820, BIO-RAD, US) and Mini Trans-Blot Cell Module (1703811, BIO-RAD, China).

The next day, the membranes were stained with the removable dye, Ponceau S Solution, (09189-1L-F, Sigma-Aldrich, Kazakhstan) to check the efficiency of transfer and washed with distilled water. After that, the membranes were blocked in 5% skim milk powder solution in 1X TBTS (0.05 M Tris, 0.15 M NaCl, 0.1% Tween® 20) for 1 hour, washed three times by 5 minutes with

TBST on a shaker and incubated at room temperature in primary antibodies diluted in 3% bovine serum albumin solution (A4503-100G, Sigma-Aldrich, Kazakhstan) for 1 hour at constant shaking. After incubation with primary antibodies, the membranes were washed with 1X TBST three times by 5 minutes on a shaker and incubated with corresponding secondary antibodies (656120, Invitrogen) diluted in 5% skim milk powder in 1X TBST solution for 1 hour at room temperature. After the membranes were washed again an ECL reagent (1:1 of solution A and B) (WBULS0100, MERTK, US) was applied to the membranes. Imaging of the membranes was done using a Bio-Rad ChemiDoc MP system.

Immunochemistry

The slides with bladder cancer tissue were deparaffinized in xylene 3 times for 5 minutes. After that, the slides were rehydrated through ethanol with different percentages for 5 minutes every time (96% ethanol twice, 90% ethanol once, 80% ethanol and 70% ethanol once). The slides were washed with running tap water. Then, antigen retrieval was made by using a citrate buffer (2.94 g of trisodium citrate, 1 liter of distilled water, pH 6.0, 0.5 ml of Tween 20). The buffer was heated until 95°C before use. The slides were placed into the citrate buffer in plastic lab trays and boiled in the microwave plate for 10 minutes, but if it was boiling over, then the microwave was switched off for a few minutes and switched on again. Importantly, the volume of the buffer should be topped up to avoid complete evaporation.

After 10 minutes, the plastic tray with slides was placed under cold deionized water for 10 minutes. Then, the PAP pen was used to make a barrier around a tissue on a slide. Novolink Polymer Detection Systems kit (RE7150K, Leica Biosystems, UK) was used. The endogenous peroxidase was neutralized using the peroxidase block for 5 minutes. After that the slides were

washed in PBS for 5 minutes twice. The slides were incubated with a protein block for 5 minutes. Washing with PBS was repeated for 5 minutes twice. The slides were incubated with a diluted primary antibody overnight at 4°C (52968, Abcam) (3%BSA and 0.1% Triton in PBS). Then the slides were washed with PBS for 5 minutes twice. The slides were incubated with secondary antibodies for 1 hour. Washing with PBS was repeated for 5 minutes twice. The slides were incubated with Novolink polymer for 1hour. Washing with PBS was repeated for 5 minutes twice. Peroxidase activity with DAB working solution (1:20 ratio) was developed for 5 minutes. The slides were rinsed with water and counterstained with hematoxylin for 3 minutes. The slides were rinsed with water for 5 minutes. The slides were dehydrated (70% ethanol once, 80% ethanol once, 90% ethanol once, 96% ethanol twice, xylene twice), cleared, mounted, and visualized on a microscope.

Enzyme-linked immunosorbent assays (ELISA)

DuoSet ELISA Development Systems kit by R&D Systems was used to detect MERTK. 1 x PBS solution was made by dissolving 2 tablets of Sigma-Aldrich PBS in 400 mL of deionized water. Wash buffer (0.05% Tween® 20 in PBS, pH 7.2-7.4) was prepared. Capture antibody was mixed with 1 ml of 1 x PBS, detection antibody was mixed with 1 ml of reagent diluent. Standards or recombinant MERTK in various concentrations was used to calibrate the reaction and measure its sensitivity, then they were mixed with 0.5 ml of reagent diluent. The reagents were aliquoted for further storage at -20 ºC. Before usage, all reagents warmed up at room temperature.

The capture antibody was diluted in PBS until the working concentration. Each well of 96-well microplate was coated with 100 µL of the diluted capture antibody. The plate was closed up and then incubated overnight at room temperature. Next day, each well was aspirated and washed

with buffer three times. For washing, each well was filled out with 400 µL washing buffer using a special pipette. After the third wash, any remaining buffer was disposed by aspirating. The microplates were blocked by adding 300 µL of reagent diluent to each well and incubated at room temperature for a minimum of one hour. The aspiration and wash steps were repeated.

100 µL of sample and standards in reagent diluents were added per well. The plate was closed with an adhesive cover and incubated for 2 hours at room temperature The aspiration/wash step was repeated. 100 µL of the detection antibody, diluted in reagent diluent, were added to each well. The plate was covered and incubated for 2 hours at room temperature. The aspiration and washing steps was repeated. 100 µL of the working dilution of Streptavidin-HRP A were added to each well. The plate was covered and incubated for 20 minutes at room temperature. The aspiration/wash step was repeated. 100 µL of substrate solution were added to each well. The plate was incubated for 20 minutes at room temperature. 50 µL of stop solution were added to each well. The plate was gently tapped to ensure thorough mixing. The optical density of each well was identified immediately, using a microplate reader set to 450 nm. A standard curve for MERTK was constructed by plotting the MERTK concentration (pg/ml) against absorbance.

Statistical analysis

The statistical analysis was accomplished with the help of online platforms. The normality of data was calculated with the Shapiro-Wilk Test because of the small sample size. Statistical significance between two groups was assessed with the chi-square test and student t-test . p values less than 0.05 were considered as a significant.

Study subjects

Patients with bladder cancer were recruited from National research oncology center (Table 1). Urine samples collected from healthy volunteers provided by Professor Abduzhappar Gaipov. Bladder cancer cell lines as UMUC3, 253J and RT112 were given by the PI.

Ethical approval

The study is approved by the Institutional Research Ethics Committee of Nazarbayev University School of Medicine. The number and date of ethical approval: # 704/12042023, May 11, 2023.

Table 1. Baseline characteristics of the patients.

CHARACTERISTIC	CASES (N=23)	CONTROLS (N=20)	P-VALUE
MEDIAN AGE (RANGE) — YR	64 (47-84)	37 (27-57)	< .00001
SEX — NO. (%)			
FEMALE	5 (22)	5 (25)	0.530357
MALE	18 (78)	15 (75)	
CANCER STAGE— NO. (%)			
IEN	1 (4)	NA	
I STAGE	11 (48)	NA	
II STAGE	5 (22)	NA	
III STAGE	4 (17)	NA	
IV STAGE	2 (9)	NA	

Values are given as median or number: N (%). IEN: Intraepithelial neoplasia. NA: not applicable. Statistical significance between two groups was assessed with the Chi-Square test and Student t-test. $p < 0.05$ as a significant.

RESULTS

Detection of MERTK in 253J, UMUC3 and RT112 bladder cancer cell lines

Expression of MERTK in different bladder cancer cell lines was analyzed. Bladder cancer cell lines were used as a model system for further analysis of MERTK in urine samples of patients. MERTK-positive 253J and UMUC3 cell lines and MERTK-negative RT112 cell lines were grown, and the phase contrast images of these cell lines are shown on Figure 2.

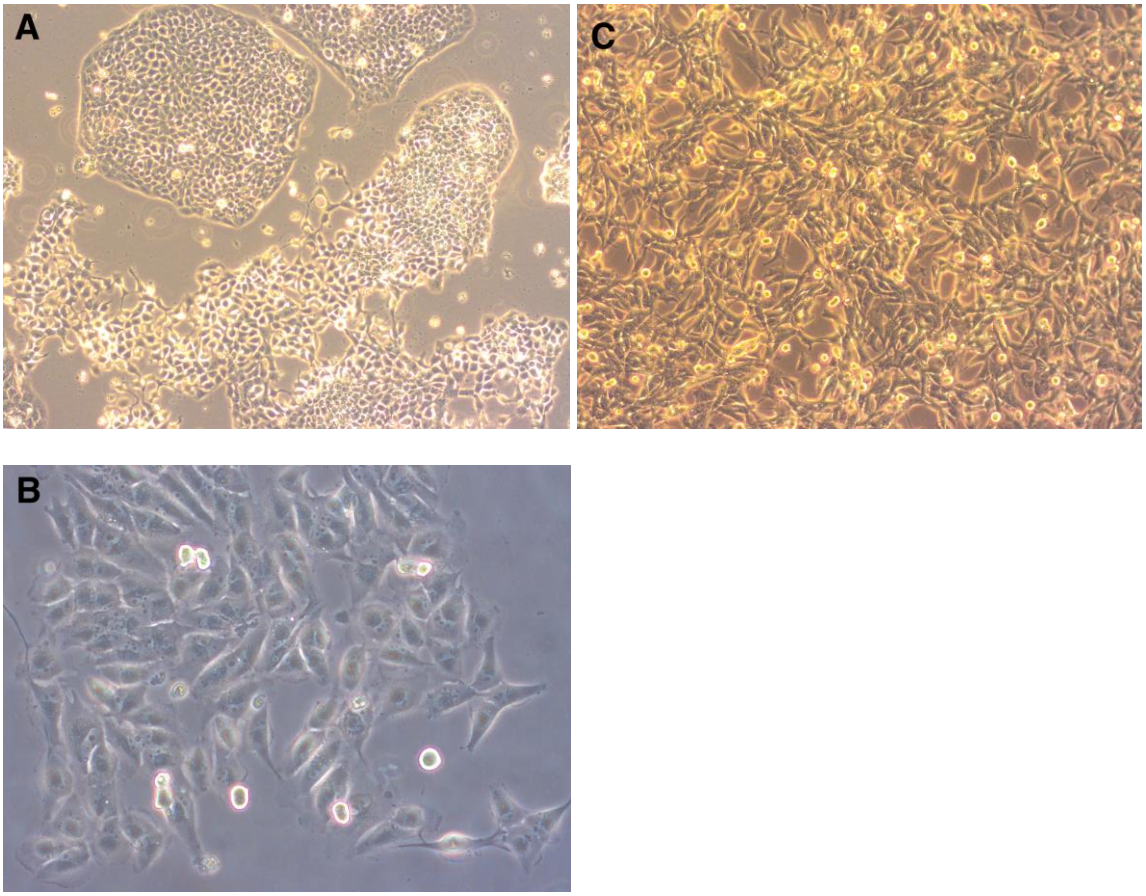

Figure 2. Phase-contrast images of bladder cancer cell lines: RT112cells lines (A). 253J cell lines (B). UMUC3 cells lines (C). Images of cells were obtained using the 20x objective.

The cells were cultured in growth medium, when they reached the confluence of 85-95%, cell lysates and condition media were prepared. Detailed preparation of cell lysates and condition media is given in methods part. Then, Western blot analysis was performed using cell lysates and condition media of 253J, UMUC3 and RT112 bladder cancer cell lines.

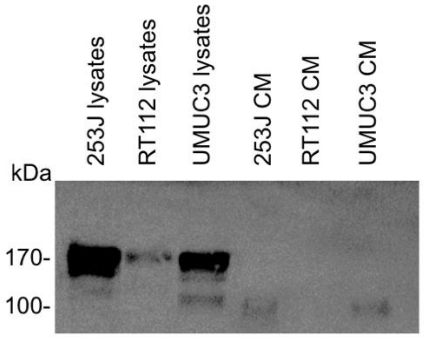

Figure 3. Detection of N-terminal fragment of MERTK by Western blotting. Samples of the protein lysates and conditioned media were analyzed. Full-sized molecular weight MERTK was visualized at 170 kDa and a proteolytically cleaved sMERTK can be seen at 100 kDa.

Based on results of Western blotting, MERTK is detected in cell lysates and condition media (see Fig. 3). N-terminal fragment of MERTK or proteolytically cleaved fragment is identified using Western blotting. So, the presence of N-terminal fragment means that MERTK was proteolytically cleaved, and its soluble fragment was detected in the conditioned medium of the MERTK-positive cells. Figure 3 demonstrates the full-sized molecular weight of MERTK was detected as 170 kDa protein in UMUC3 and 253J cell lysates and a sMer can be visualized at 100 kDa in UMUC3 and 253J condition media. In general, full-sized MERTK is expressed in the UMUC3 and 253J bladder cancer cell lines and sMer is secreted into condition media.

Preparation of MERTK standard curve

The protocol of ELISA kit was used to measure the recombinant human MERTK. Standard curve was used to check for the ELISA sensitivity and as a positive control (see Fig 4.)

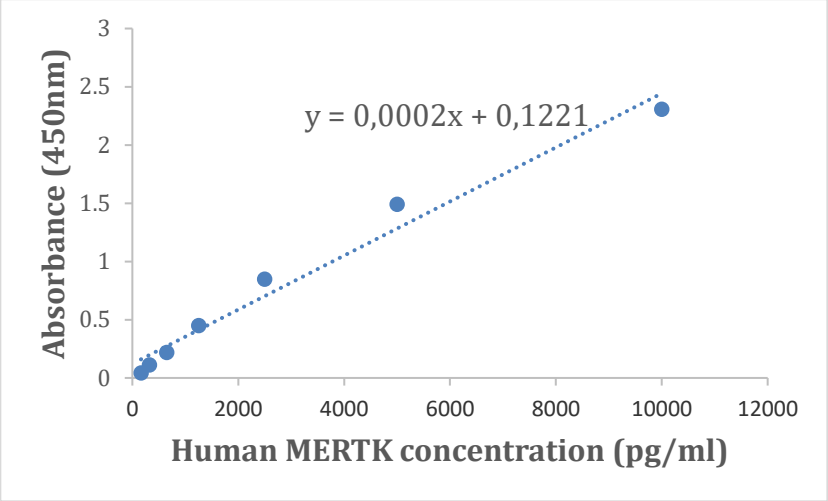

Figure 4. Standard curve for the human MERTK. ELISA was a sensitive, reaching oversaturation levels at concentrations over 10,000 pg/mL.

Next, ELISA was performed to verify the results of Western blotting. The concentration of MERTK was measured in cell lysates and condition media by ELISA, and it is shown on Fig 5.

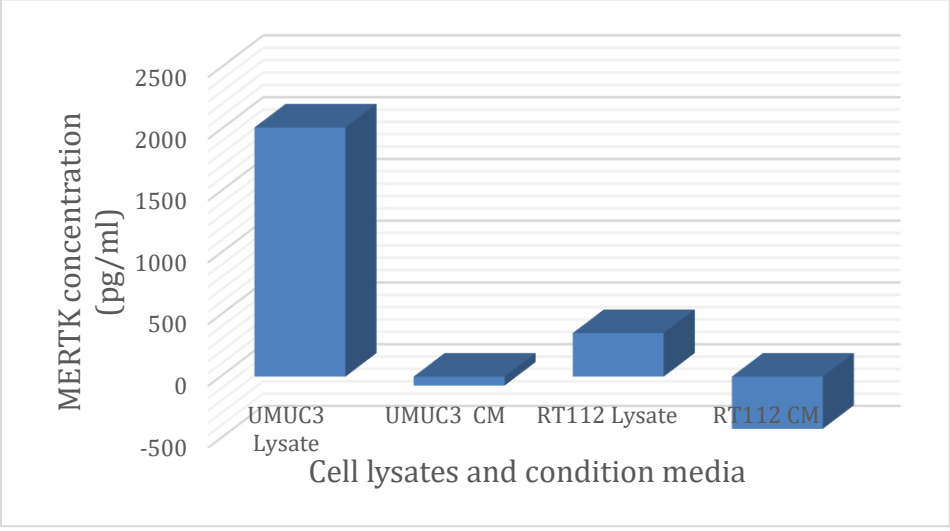

Figure 5. Detection of MERTK in UMUC3 and RT112 cell lysates and condition media by ELISA. MERTK concentration in cell lysates and condition media was calculated using the standard curve.

As a result, MERTK was found in cell lysate of UMUC3. And it is secreted into the condition media of UMUC3 cells. UMUC3 cells were decided to use as a positive control on analyzing the urine samples. The condition medium of RT112 cells showed no expression of MERTK and it was used as a negative control. However, MERTK was also detected in small concentrations in the cell lysate of RT112 cells. They also had the weak band at 170 kDa in Western blot analysis (see Fig. 3).

Detection of MERTK in urine samples

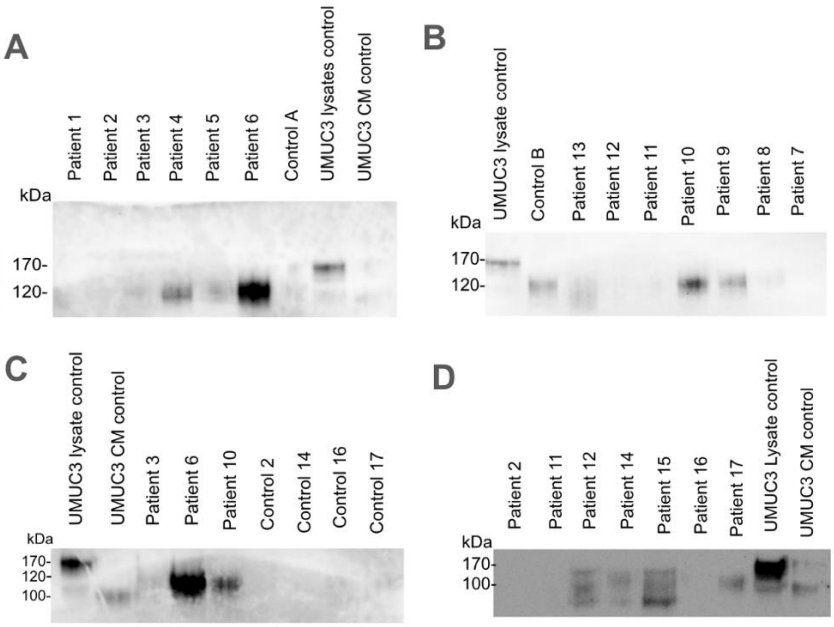

Figure 6. Detection of the N-terminal fragment of MERTK by Western blotting. Urine samples of patients and healthy volunteers, cell lysates (A, B, C and D) were analyzed. Full-sized molecular weight MERTK and proteolytically cleaved sMERTK were detected. Control A and B (A, B) samples were prepared in the PI laboratory.

Table 2. Detection of proteolytically cleaved MERTK in urine samples of patients by Western blotting

	Cases (N=23)	Controls (N=6)	p-value
Patients who had positive urine samples for sMER - No. (%)	15 (65.2%)	1 (16.6%)	0.09517
Patients who had positive urine samples for sMER- No. (%)	8 (34.8%)	5 (83.4%)	

Values are given as number: N (%). Statistical significance between two groups was assessed with the Chi-Square test. $p < 0.05$ as a significant.

Results of Western blot analysis was shown in Table 2 which aimed to detect proteolytically cleaved MERTK in urine samples of bladder cancer patients. In total, sMer was detectable in urine samples in 65.2% (15 out of 23) cases.

Proteolytically cleaved MERTK was detected in urine samples of bladder cancer patients and healthy controls. The concentration of MERTK was calculated using standard curve given in Fig 4. Statistical significance between patients and healthy controls was assessed with the student t-test. However, p-value was higher than 0.05, and the results were not considered as statistically significant.

Table 3. The concentration of MERTK in urine samples

CHARACTERISTIC	CASES (N=23)	CONTROLS (N=20)	P-VALUE
MEAN OF MERTK CONCENTRATION CALCULATED BY ELISA (RANGE)-PG/ML	1595.47(-1342-8907)	294.1 (-1180-9538)	0.29372

Values are given as mean. Statistical significance between two groups was assessed with the Student t-test. $p < 0.05$ as a significant.

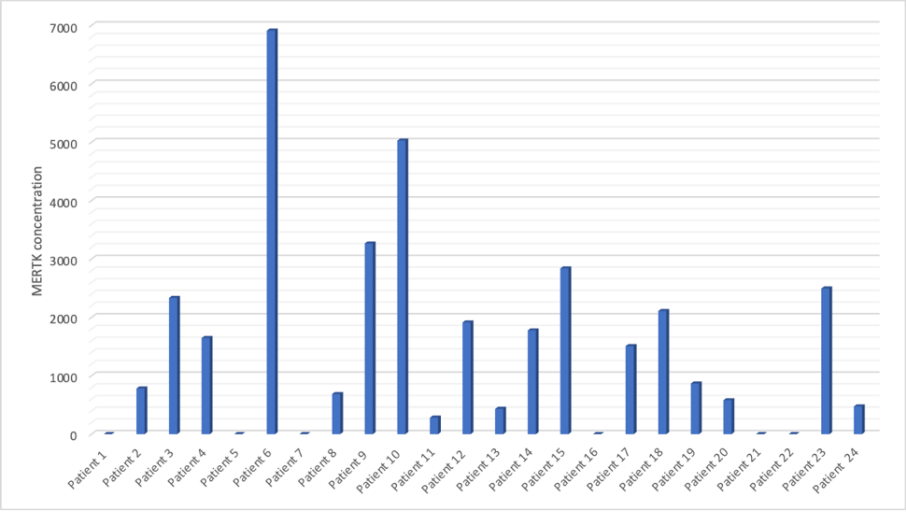

Figure 8. Detection of MERTK in urine samples of patients by ELISA.

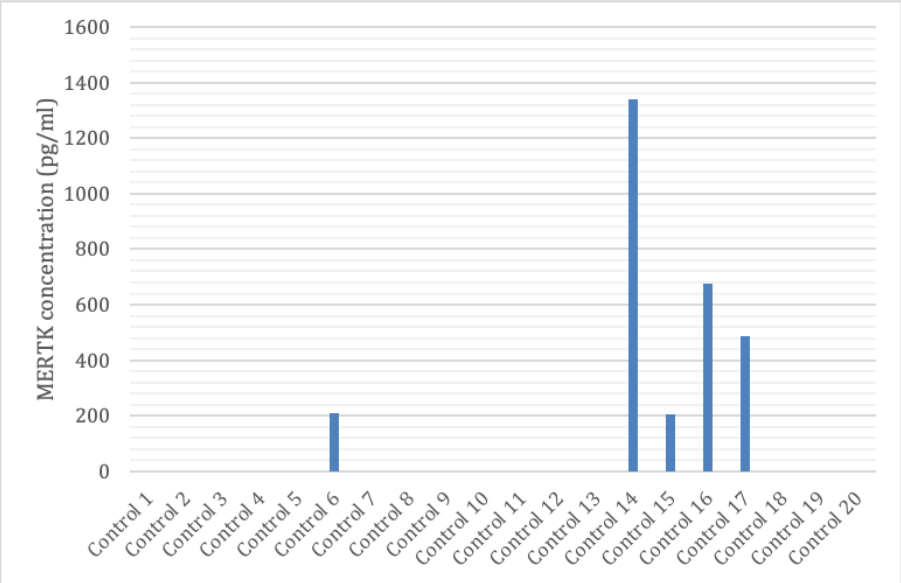

Figure 9. Detection of MERTK in urine samples of health controls by ELISA.

According to the results of ELISA, the concentration of MERTK was higher in urine samples of most of the patients. For instance, there were high concentration of MERTK in urine samples of patients 6,9 and 10 (see figure 8). Also, the corresponding results of Western blotting analysis of these patients showed high expression of MERTK (see figure 6). The concentration of MERTK in some urine of healthy controls 2, 14,16 and 17 was relatively higher than other controls (see figure 9). However, these controls showed no bands on Western blot analysis (see fig 6 C). In general, on panel A (figure 6), proteolytically cleaved sMer can be clearly seen at 120 kDa in urine samples of patients. There are no bands for healthy controls expect for controls A and B (see fig 6) which were provided by PI.

Detection of MERTK in tumor samples

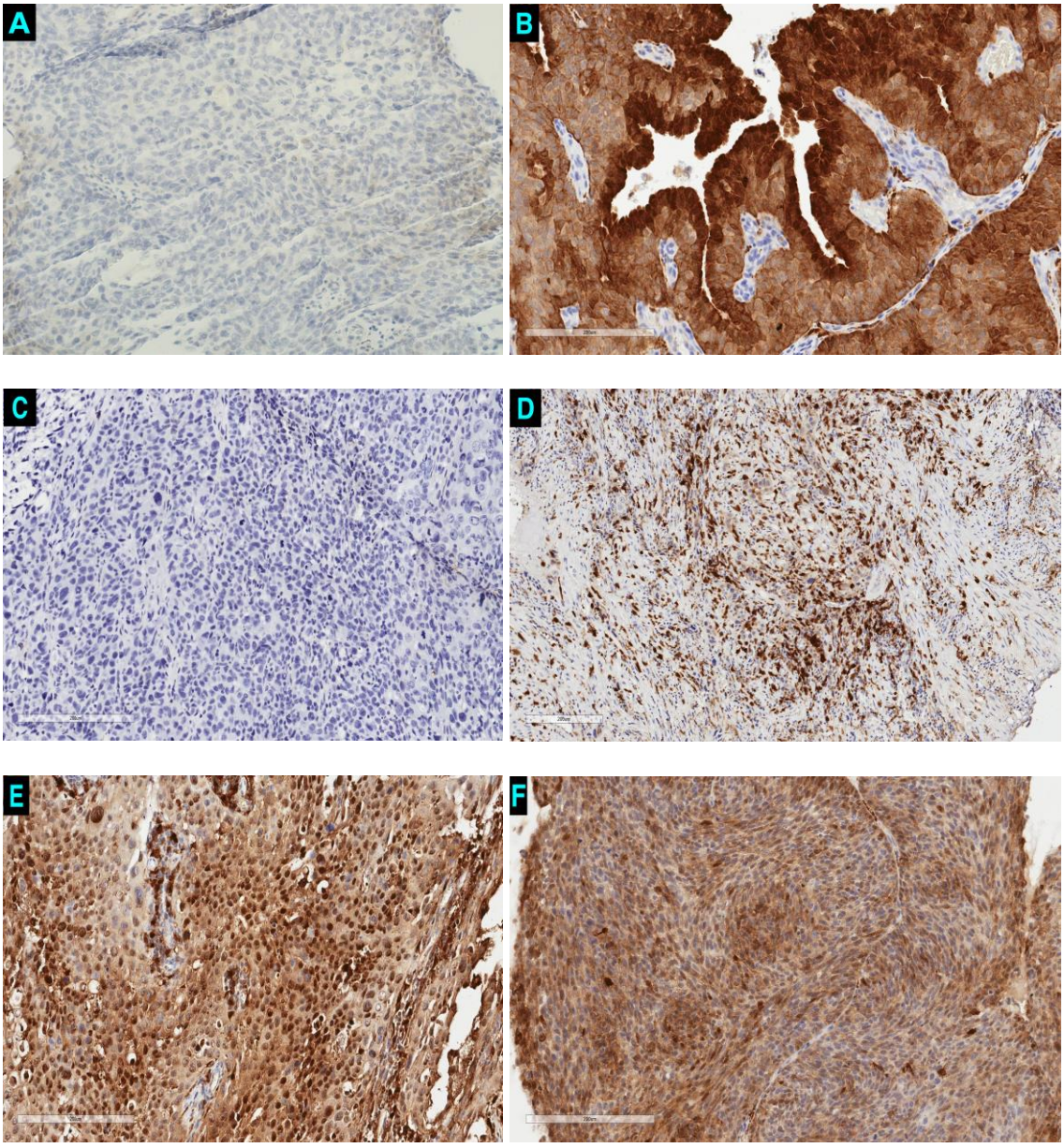

Figure 10. Immunohistochemistry staining of bladder tumor slides against hematoxylin and MERTK. Patient 6 hematoxylin stain (A) and MERTK stain (B). Patient 23 hematoxylin stain (C) and MERTK stain (D). Patient 4 MERTK stain (E). Patient 10 MERTK stain (F). Images were prepared using 10x objective.

The bladder tumor samples were stained against MERTK and counterstain with hematoxylin. It was difficult identify the expression of MERTK because of artifacts and low quality, therefore bladder cancer tissues of 17 patients were analyzed. Negative control was either a negative tissue or staining without primary anti-MERTK antibody. The patient 6 and 10 showed the strongest staining compared to other patients. As shown on panel C and D, patient 23 showed the expression of MERTK compared to negative control. Negative control is used to prove that secondary antibody doesn't give any background staining. Overall, MERTK was detectable in bladder cancer cells in 88% (15 out of 17) cases.

DISCUSSION

The expression of MERTK in bladder cancer tissues was analyzed using different techniques which detected the expression of this receptor in UMUC3 and 253J bladder cancer cell lysate and condition media. ELISA was highly sensitive to MERTK, and it was possible to obtain strong signal. The range of detection MERTK is from 156 pg/mL to 10, 000 pg/mL. The concentration higher than 10, 000 pg/mL was not detectable because of oversaturation. Soluble form of MERTK was detectable in urine samples of patients in ELISA analysis. The mean concentration of MERTK of bladder cancer patients and healthy controls were 1595.47 pg/ml and 294.1 pg/ml respectively. This demonstrates that low expression of sMER in control samples.

The Western blot analysis was performed to investigate whether MERTK is localized in cells and can be detected in a soluble form. Membrane-bound MERTK was detected at 170 kDa in

UMUC3 lysates, and molecular weight of proteolytic cleaved N-terminal of MERTK was approximately 100 kDa in UMUC3 condition medium (see figure 3). Moreover, sMer can be seen at 120 kDa in urine samples of patients. It is proposed that the molecular weight differences of sMer between urine samples and cell lines because of differences in N-linked glycosylation. According to the studies, the N-glycosylation is an important in regulating a carcinogenesis as it provides stability to the MERTK (Liu et al., 2022). Results of studies showed similar molecular weight of MERTK in other cells. For instance, the molecular weight of sMer in J774 and U937 human monocytic cell lines was 140kDA in condition media (Sather et al., 2007). Another study with mantle cell lymphoma (MCL) cell lines has showed that JVM-2 and Z-138 MCL cell lines expressed MERTK at 180 kDa (Shi et al., 2018).

There is some discrepancy in results obtained by Western blotting and ELISA. A few samples were positive in Elisa and negative on Western blotting. It showed that ELISA was more sensitive than Western blotting. For instance, the concentrations of MERTK were high in urine samples of patients 2, 11 and 19. Although, sMer was undetectable in urine samples of these patients by Western blotting, the reason for that might be the protein degradation in urine. It important to consider that these urine samples were not concentrated. Therefore, the detection could be possible if the urine samples will be concentrated in future experiments. Moreover, fresh urine samples should be analyzed to avoid protein degradation.

The results of immunohistochemistry showed the strong expression of MERTK in most of the patient's bladder tissue samples. The strong expression of MERTK was observed in patient 4, 6 and 10 (see fig. 10). These patients have different stages of bladder tumor; therefore, we can

conclude that there is no association between cancer stage and immunostaining. However, large tissue samples should be studied to reject or accept this hypothesis. The results of Western blot and ELISA also showed high expression of MERTK in urine samples of these patients. So, it shows the potential cleavage of the receptor expressed by cancer cells. The patient 1 showed expression of MERTK in bladder cancer tissue, but the results of ELISA and Western blotting was negative. Therefore, the storage of urine samples should be considered because the protein could be degraded because of improper storage. Study conducted by Schlegel et al. (2013) has aimed to determine the MERTK expression in melanoma cells which was metastasized to brain. The results of their immunohistochemistry study demonstrated that MERTK was observable in 28% (8 of 29) cases (Schlegel et al., 2013). Compared to my data, the MERTK was detectable in bladder cancer cells in 88% (15 out of 17) cases. According to IHC analysis taken from the Human Protein Atlas database, MERTK expression was detected in bladder cancer cells, and the localization of MERTK was cytoplasmic and membranous in patients' tissue samples (The Human Protein Atlas database, 2024). The results of our study also showed the cytoplasmic and membranous localization of MERTK in cancer cells. Moreover, Kaplan-Meir analysis showed the correlation between high expression of MERTK and worst prognosis of bladder cancer (The Human Protein Atlas database, 2024). Wu et al. (2018) used dual immunofluorescence staining to identify the cell population which expresses MERTK in human glioblastoma samples. Results of experiment have showed the expression of MERTK in cancer cells. This method also can be used to show the expression of proteolytically cleaved MERTK in bladder cancer cells. Overall, it is important to conclude that MERTK is expressed in bladder cancer tissue.

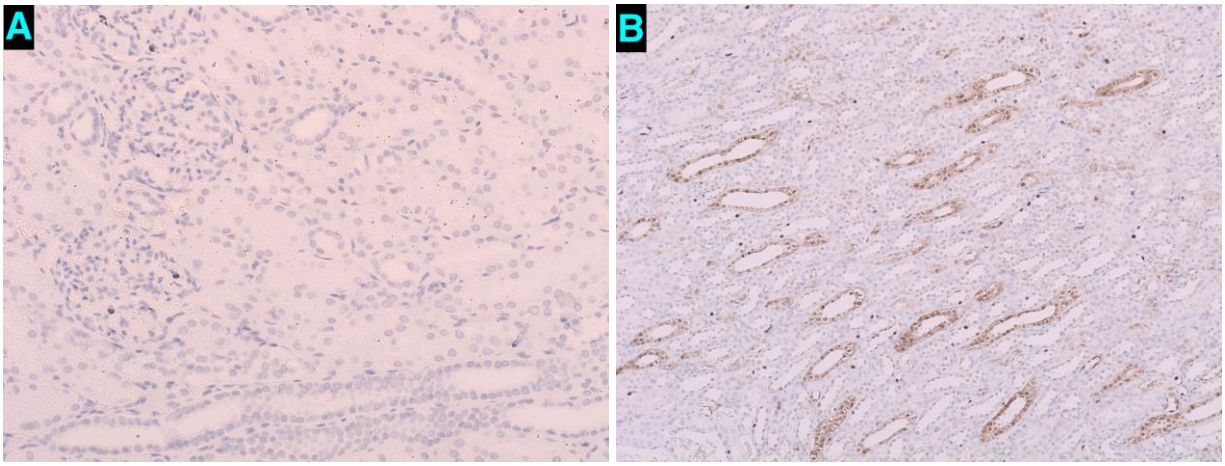

Figure 11. Immunohistochemistry staining of normal kidney tissue sample against hematoxylin (A) and MERTK (B).

The normal kidney tissue sample was stained against MERTK. The results of IHC showed the expression of MERTK in tubular cells of kidney (see fig. 11). This demonstrates that MERTK is expressed in other tissues as kidney. However, MERTK was not detected in the urine samples of most of the healthy controls in Western blot and ELISA studies. It is important to find out that why MERTK is not proteolytically cleaved in these cases. Therefore, it is recommended to consider this point in further study. ELISA kit was made for MERTK detection in serum, but it was used in urine. However, sMer was successfully detected in the patient urine samples and it was confirmed using the Western blotting despite being the small-scale preliminary study.

There were several limitations in this pilot study. The protocol of the study was not fully constructed. One of the limitations was the small number of patient samples and control samples. Therefore, the statistical analysis could not be performed appropriately. The urine samples were collected at different times and prepared by different lab members. They also stored differently and there were many refreezing and defrosting steps. It could negatively impact on the stability

of the MERTK in the urine samples. In general, the staining of slides was not good in quality because the tissue slides were not prepared properly by medical personal, therefore larger number of freshly samples should be analyzed to detect MERTK in samples. For instance, the quality of tissue samples from patient 4 and 10 was not good, they were unsuitable for analysis with hematoxylin as a control. It will be very informative if there will be chance to compare the MERTK staining with hematoxylin staining of these samples. sMer was successfully detected in the patient urine samples and it was confirmed using the Western blotting despite being the small-scale preliminary study.

In further study, the experiments should be done with larger size of samples and appropriate statistical analysis. The storage and handling with urine samples should be well controlled. Lastly, the slides of tumor samples should be prepared properly.

CONCLUSION

In conclusion, for the first time MERTK was expressed in bladder cancer and its proteolytically cleaved form is present in urine samples of patients with bladder cancer. Soluble MERTK can be used as a potential biomarker in unconcentrated form of the freshly collected urine samples of patients as a diagnostic tool in bladder cancer. It might be a potential non-invasive, fast, and accessible method for diagnosis of bladder cancer in future. It is recommended to consider the miscorrelation between the immunohistochemistry, ELISA, and Western blot analysis in identifying the expression of MERTK in larger scale studies. Further investigations are recommended to perform with larger number of participants to prove that sMer can be a potential biomarker for bladder cancer.

ACKNOWLEDGEMENTS

I would like to express my thanks of gratitude to Professors Marina Kriajevskaia and Eugene Tulchinsky who helped me in performing the laboratory experiments and writing the project. I am also grateful for support of Yuriy Pak in doing WB and IHC.

REFERENCES

Aehnlich, P., Powell, R. M., Peeters, M. J., & Rahbech, A. (2021). TAM Receptor Inhibition–Implications for Cancer and the Immune System. Cancers, 13(6), 1195.

Akgül, M., Baykan, Ö., Çağman, Z., Özyürek, M., Tinay, İ., Akbal, C., ... & Türkeri, L. (2021). Gas6 expression and Tyrosine kinase Axl Sky receptors: Their relation with tumor stage and grade in patients with bladder cancer. Archivio Italiano di Urologia e Andrologia, 93(2), 148-152.

Babu, S. S., Thandavarayan, R. A., Joladarashi, D., Jeyabal, P., Krishnamurthy, S., Bhimaraj, A., ... & Krishnamurthy, P. (2016). MicroRNA-126 overexpression rescues diabetes-induced impairment in efferocytosis of apoptotic cardiomyocytes. Scientific reports, 6(1), 1-12.

Bourn, J., & Cekanova, M. (2018). Cyclooxygenase inhibitors potentiate receptor tyrosine kinase therapies in bladder cancer cells in vitro. Drug design, development and therapy, 12, 1727.

Bellan, M., Quaglia, M., Nerviani, A., Mauro, D., Lewis, M., Goegan, F., ... & Pitzalis, C. (2021). Increased plasma levels of Gas6 and its soluble tyrosine kinase receptors Mer and Axl are associated with immunological activity and severity of lupus nephritis. *Clinical and Experimental Rheumatology*, *39*(1), 132-138.

Bellan, M., Pirisi, M., & Sainaghi, P. P. (2016). The Gas6/TAM system and multiple sclerosis. *International Journal of Molecular Sciences*, *17*(11), 1807.

Chen, C. J., & Liu, Y. P. (2021). MERTK Inhibition: Potential as a Treatment Strategy in EGFR Tyrosine Kinase Inhibitor-Resistant Non-Small Cell Lung Cancer. Pharmaceuticals, 14(2), 130.

Cook, R.S.; Jacobsen, K.M.; Wofford, A.M.; DeRyckere, D.; Stanford, J.; Prieto, A.L.; Redente, E.; Sandahl, M.; Hunter, D.M.; Strunk, K.E.; et al. MerTK inhibition in tumor leukocytes decreases tumor growth and metastasis. J. Clin. Investig. 2013, 123, 3231–3242.

Cummings, C. T., DeRyckere, D., Earp, H. S., & Graham, D. K. (2013). Molecular pathways: MERTK signaling in cancer. Clinical Cancer Research, 19(19), 5275-5280.

DeGeorge, K. C., Holt, H. R., & Hodges, S. C. (2017). Bladder cancer: diagnosis and treatment. *American family physician*, *96*(8), 507-514.

Dufour, F., Silina, L., Neyret-Kahn, H., Moreno-Vega, A., Krucker, C., Karboul, N., ... & Bernard-Pierrot, I. (2019). TYRO3 as a molecular target for growth inhibition and apoptosis induction in bladder cancer. British Journal of Cancer, 120(5), 555-564.

Huelse, J. M., Fridlyand, D. M., Earp, S., DeRyckere, D., & Graham, D. K. (2020). MERTK in cancer therapy: Targeting the receptor tyrosine kinase in tumor cells and the immune system. Pharmacology & therapeutics, 213, 107577.

Huang, H. (2021). Proteolytic Cleavage of Receptor Tyrosine Kinases. *Biomolecules*, 11(5), 660.

Kazakhstan. Source: Globocan 2020. (2020). From https://gco.iarc.fr/today/data/factsheets/populations/398-kazakhstan-fact-sheets.pdf

Keating, A., Linger, R., Graham, D. (2008). MERTK (c-mer proto-oncogene tyrosine kinase)

Lee, Y. H., Lee, M. M., De Silva, D. M., Roy, A., Wright, C. E., Wong, T. K., Costello, R., Olaku, O., Grubb, R. L., 3rd, Agarwal, P. K., Apolo, A. B., & Bottaro, D. P. (2021). Autocrine

signaling by receptor tyrosine kinases in urothelial carcinoma of the bladder. *PloS one*, *16*(7), e0241766. https://doi.org/10.1371/journal.pone.0241766

Lemke, G., & Rothlin, C. V. (2008). Immunobiology of the TAM receptors. Nature Reviews Immunology, 8(5), 327-336.

Linger, R. M., Keating, A. K., Earp, H. S., & Graham, D. K. (2010). Taking aim at Mer and Axl receptor tyrosine kinases as novel therapeutic targets in solid tumors. Expert opinion on therapeutic targets, 14(10), 1073-1090.

Liu, Y., Lan, L., Li, Y., Lu, J., He, L., Deng, Y., ... & Lu, B. (2022). N-glycosylation stabilizes MerTK and promotes hepatocellular carcinoma tumor growth. Redox Biology, 54, 102366.

Ochodnicky, P., Lattenist, L., Ahdi, M., Kers, J., Uil, M., Claessen, N., ... & Roelofs, J. J. (2017). Increased circulating and urinary levels of soluble TAM receptors in diabetic nephropathy. *The American Journal of Pathology*, *187*(9), 1971-1983.

Saginala, K., Barsouk, A., Aluru, J. S., Rawla, P., Padala, S. A., & Barsouk, A. (2020). Epidemiology of bladder cancer. Medical Sciences, 8(1), 15.

Sather, S., Kenyon, K. D., Lefkowitz, J. B., Liang, X., Varnum, B. C., Henson, P. M., & Graham, D. K. (2007). A soluble form of the Mer receptor tyrosine kinase inhibits macrophage clearance of apoptotic cells and platelet aggregation. Blood, 109(3), 1026-1033.

Sanli, O, Dobruch,, J, Knowles, MA orcid.org/0000-0002-9363-8657 et al. (4 more authors)

(2017) Bladder Cancer. Nature Reviews Disease Primers, 3. 17022. ISSN 2056-676X

https://doi.org/10.1038/nrdp.2017.22

Shi, C., Li, X., Wang, X., Ding, N., Ping, L., Shi, Y., ... & Zhu, J. (2018). The proto-oncogene Mer tyrosine kinase is a novel therapeutic target in mantle cell lymphoma. *Journal of hematology & oncology*, 11, 1-13.

Schlegel, J., Sambade, M. J., Sather, S., Moschos, S. J., Tan, A. C., Winges, A., ... & Graham, D. K. (2013). MERTK receptor tyrosine kinase is a therapeutic target in melanoma. *The Journal of clinical investigation*, 123(5), 2257-2267.

The Human Protein Atlas. MERTK. Retrieved March 7, 2024, from https://www.proteinatlas.org/ENSG00000153208-MERTK/pathology

Verma, A., Warner, S. L., Vankayalapati, H., Bearss, D. J., & Sharma, S. (2011). Targeting Axl and Mer kinases in cancer. *Molecular cancer therapeutics*, *10*(10), 1763-1773.

Wang, K. H., & Ding, D. C. (2021). Dual targeting of TAM receptors Tyro3, Axl, and MerTK: Role in tumors and the tumor immune microenvironment. Tzu Chi Medical Journal, 33(3), 250.

Witas, R., Rasmussen, A., Scofield, R. H., Radfar, L., Stone, D. U., Grundahl, K., ... & Nguyen, C. Q. (2021). Defective Efferocytosis in a Murine Model of Sjögren's Syndrome Is Mediated by Dysfunctional Mer Tyrosine Kinase Receptor. *International journal of molecular sciences*, *22*(18), 9711.

Wu, J., Frady, L. N., Bash, R. E., Cohen, S. M., Schorzman, A. N., Su, Y. T., ... & Miller, C. R. (2018). MerTK as a therapeutic target in glioblastoma. *Neuro-oncology*, 20(1), 92-102.

Zangouei, A.S., Barjasteh, A.H., Rahimi, H.R. et al. Role of tyrosine kinases in bladder cancer progression: an overview. Cell Commun Signal 18, 127 (2020). https://doi.org/10.1186/s12964-020-00625-7