The protocols of the imaging examinations, histopatholog-ical staining and immunohistochemistry (IHC)

• CT scan (model used and supplier, and a list of imaging parameters)

Author: CT model was Siemens SOMATOM Definition AS 64 slices. CT scan parameters and contrast enhancement were standard for urinary system as follow: native, corticomedullary phase, nephrographic phase and excretory phase, each were taken in 5mm thickness with 1 mm reconstruction, reviewed in axial, coronal and sagittal planes.

• Pathological staining (every stage from fixation to imaging)

Author:

Processing

We fix the specimen for 1-3 days in 10% neutral buffered formalin at room temperature. We then process it using the DiaPath Donatello automated processor through the following stages:

1. Holding in 10% neutral buffered formalin (variable time but average 20 min)
2. Deionized water for 10 min
3. Dehydration in alcohol, starting with a concentration of 70% (1 h) and followed by 95% (1 h), 99% (1 h and 30 min), and another station of 99% (1 h and 30 min)
4. Clearing through 3 stations of xylene (1 h each for a total of 3 h)
5. Infiltration by paraffin wax in three stations (1 h each for a total of 3 h)

The blocks are then embedded in paraffin wax using the Sakura Tissue-Tek embedding center. This is followed by facing the blocks and taking tissue sections using the Sakura Accu-Cut SRM microtome. The sections are floated in a Sakura 1451 water bath at a temperature of 40-50 degrees centigrade and placed on regular glass slides.

The slides are kept in an oven at 60-70 degrees centigrade overnight.

Hematoxylin and eosin staining

The following day, the slides are stained for hematoxylin and eosin using the DiaPath Giotto autostainer through these stages:

1. Xylene in three stations (7, 7, and 5 min)
2. Alcohol at a concentration of 100% (three stations at 7, 6 and 5 min), followed by 90% (4 min), and 70% (3 min) then tap water (2 min)
3. Hematoxylin Gill II (8 min), prepared from Sigma-Aldrish Hematoxylin Natural Black 1
4. Tap water (4 min), ammonia water (1 min), followed by tap water (1 min) and then alcohol at 70% (2 min)
5. Eosin (5 min) prepared from Sigma-Aldrich Eosin Y disodium salt, followed by tap water (1 min)
6. Alcohol starting at a concentration of 70% (15 seconds), 90% (2 min), and 100% (three stations at 3, 3, and 4 min)
7. Xylene in three stations (3, 5, and 4 min)
   The slides are left to dry for 5 min and then covered with the SurgiPath Sub-X mounting medium followed by a coverslip.

• IHC staining (every stage from fixation to imaging, with details of all antibodies used)

Author: The paraffin blocks are sectioned using the microtome and floated in a water bath as described above for routine processing. The sections are, however, placed on charged glass slides in this case. The slides are placed in an oven (Fisher Scientific) overnight at 60-65 degrees centigrade.

The following day, the slides are boiled using the Dako PT Link for antigen retrieval. Each slide is placed in a solution with a pH of either 6 or 9 (both are Dako Envision FLEX target retrieval solutions), depending on the requirement of the target antibody. This device will preheat the slides from 30 degrees centigrade upwards to a boiling temperature of 100 degrees centigrade for 45 min, followed by cooling down to 65 degrees centigrade for a total operation time of 2 h.

The slides are then washed twice (each time for 3 min) using the Dako Wash Buffer solution, followed by welling using the Dako Pen. Endogenous peroxidase is blocked by immersing the slides in hydrogen peroxide for 7-10 min, followed by washing twice (3 min each) in the same buffer solution as above.

The slides are then covered by 50 microns of the primary antibody diluted according to each antibody's manufacturer's recommendations and kept for 45 min. They are then washed again twice (3 min each) with the buffer solution. This is followed by covering the slides with the secondary antibody (Dako Horseradish Peroxidase) for 45 min, then washing twice (3 min each) with buffer solution. Chromogen (diaminobenzidine) is applied for 5-10 min and then washed with running water for 2 min.

Counterstaining with hematoxylin Gill II is performed for 2-5 min, followed by alcohol (at sequential concentrations of 70, 90, and 100%) and xylene (2 stations), each station for 5-10 min. The slides are then covered with mounting medium and coverslip as described above for hematoxylin and eosin staining.

These are the specifications of the antibodies used in our case:

- CK7: Dako, clone OV-TL 12/30, LOT 20038751, pH 9, dilution 1:1
- CD10: Bio SB, clone EP195, LOT 6434XKI28, pH 9, dilution 1:1.5
- GATA-3: Bio SB, clone EP368, LOT 3333PKE11, pH 9, dilution 1:1.3
- AMACR: Bio SB, clone 13H4, LOT 5062JKC24, pH 9, dilution 1:1