Isolation of Human Prostatic Epithelial Plasma Membranes for Proteomics Using Mirror Image Tissue Banking of Radical Prostatectomy Specimens

Kanishka Sircar,1 Louis Gaboury,3 Lydia Ouadi,3 Melanie Mecteau,3 Eleonora Scarlata,1 Fred Saad,4 Armen Aprikian,2 Simon Tanguay,2 Steven Lapointe,4 Christian Lussier,3 Tina Miletti,5 and Joel Lanoix5

Abstract

Purpose: To isolate human prostatic epithelial plasma membranes for the identification of cell surface proteins in the therapeutic targeting of cancer cells while permitting the retrieval of banked samples for clinical purposes.

Experimental Design: Radical prostatectomies from 84 patients (median, 61 years; prostate-specific antigen, 5.9; 66% nonpalpable) were processed with alternate, mirror image slices submitted for histology and tissue banking. Benign and malignant foci were macrodissected from the banked sections using the pathologically mapped, mirror image histology sections as a guide. Epithelial plasma membranes were isolated using novel immunomagnetic purification and their purity was assessed. Tissue homogenates were probed by Western blot for malignant (AMACR) and benign (p63) markers to test the accuracy of this protocol. Selected banked tissue slices were retrieved, thawed, and compared pathologically to their corresponding routinely processed alternate slices.

Results: Plasma membrane preparations showed the enrichment of epithelial plasma membrane markers (prostate-specific membrane antigen and epithelial-specific antigen) with minimal marker expression from nonepithelial cells or intracellular organelles. Cancer homogenates showed up-regulated AMACR and down-regulated p63, whereas benign homogenates showed up-regulated p63 and down-regulated AMACR. There was 30% benign (p63+) contamination in cancer slices and 6% cancer (AMACR+) contamination in benign slices. Retrieved tissues showed the retention of immunoreactivity while their histology was always adequate for diagnosis.

Conclusions: We have successfully isolated purified epithelial plasma membranes from benign and malignant human prostates and provided validation data for the accuracy of our protocol in a prostate-specific antigen-screened cohort. Our method also enabled the retrieval of banked tissues for clinical purposes with the retention of good histologic and immunohistochemical quality.

Prostate cancer is the most common cancer diagnosed in North American men and is a major cause of cancer-specific mortality. Current treatment, even when successful, carries significant morbidity and many patients will fail standard regimens and die of their disease (1). Therapeutic targeting of cancer cells using monoclonal antibodies is a novel but promising form of selective, nontoxic treatment. In the U.S., there are currently five such antibodies approved for use in cancer, but none for prostate cancer. The first step in developing anticancer monoclonal antibodies is to identify antigenic targets—plasma membrane proteins—that are overexpressed by the neoplastic cells relative to benign cells. Studies should ideally be carried out on fresh primary human tissues. Unfortunately, the isolation of purified epithelial plasma membranes cannot be done on routinely processed, formalin-fixed tissues because formalin induces conformational changes in the proteome through the formation of intermolecular cross-links (2) which renders such tissues unsuitable for organelle purification and proteomic analysis.

In the past, standard protocols to isolate plasma membranes have used the principle of differential centrifugation coupled to isopycnic centrifugation to generate a fraction enriched in plasma membrane (3–5). However, these protocols suffer from two drawbacks that limit their use for the proteomic identification of immunotherapeutic targets. The first is the overlapping density between different organelles that results in the presence of various contaminants in the plasma membrane fraction. The second is the inability to purify plasma membranes of specific cell types from primary tissue. To overcome these problems, the velocity/isopycnic centrifugation method needs to be coupled with another purification
Epithelial Plasma Membrane Purification from Human Prostates

Materials and Methods

Patient data. Tissues used in this study were drawn from 84 patients who underwent radical prostatectomy for prostate cancer at two different institutions between 2004 and 2005. Informed consent was obtained from all patients as part of an institutionally approved protocol and after approval from the institutional review boards of the two participating universities. The patient population showed a median age of 61 (range, 45-72), median PSA of 5.9 (range, 1.4-18.4) with 34% showing clinically palpable disease and 66% nonpalpable (T1a). At prostatectomy, patients had prostate cancer of Gleason scores 6 (44%), 7 (48%), and 8 (8%) with pathologic stages (according to the American Joint Committee on Cancer tumor-node-metastasis 2003 staging system) of pT2 (82%), pT3a (13%), and pT3b (5%), and positive surgical resection margins in 20% of the cases.

Tissue processing. Prostatectomy specimens were processed under the supervision of a dedicated pathologist as follows: fresh specimen was collected from the operating room as soon as feasible, usually within 5 to 10 minutes after removal of the gland. After weighing and measuring the gland, prosection was carried out in a cool environment (5°C). We inked the anterior prostatic moiety in red and the posterior half in black. A hair dryer exuding cool air was used to dry the ink rather than immersion in the standard Bouin’s fixative or acetic acid. Shave margins of the bladder neck were taken and the seminal vesicles were amputated and submitted for histology.

The prostatic urethra was cannulated with a probe and the prostate was cut into right and left halves. Each half was then sectioned into anterior and posterior quadrants by cutting along the junction of the red (anterior) and black (posterior) ink. We were thus left with four quadrants: right anterior, right posterior, left anterior, and left posterior. Each quadrant was then sectioned similarly from apex to base using a dissecting blade. The first 4 mm representing the apical portion of each quadrant was amputated, cut sagitally and entirely submitted for histology. The quadrants were then cut at 2 mm intervals with the odd-numbered slices submitted for histology and the even-numbered slices submitted for tissue banking (Fig. 1). Because the pathology sections were embedded and processed with the cut surface facedown, we dotted the “mirror image” face of the subsequent tissue-banked slice with green dye for correct orientation later. The final 4 mm of prostate representing the base of each quadrant was cut sagitally and entirely submitted for histology.

The tissue-banked sections from each quadrant were given a designation so they could be later correlated to their corresponding routinely processed histology block. The banked tissues were then placed in test tubes containing physiologic media (DMEM; Wisent, St.

Fig. 1. Scheme illustrating the identification of tumor areas within a prostate specimen that is prossected for histopathologic diagnosis and tissue banking. T, tumor area.
Bruno, Canada), 10% v/v fetal bovine serum (heat-inactivated; Wisent), 10% v/v DMSO and rotated for 15 minutes to ensure adequate penetration of the reagent. They were frozen and stored at -80°C. The time from removal of the prostate to placement in physiologic medium was ~25 minutes.

Those tissue slices sent for pathology that were mirror images of the tissue-banked slices were embedded flat and the first full face was sectioned with minimal trimming of the block. Areas of prostate cancer and benign tissue were mapped on H&E stained sections. Digital images of the mapped sections were taken using a scanner and the files transferred onto a compact disc. These digitized slices could then be correlated to their mirror image banked slices (Fig. 2). Tissues were only released for research once the case was signed out by the pathologist.

**Macrodissection of cancer tissues from banked sections.** Whole tissue slices containing a high proportion of glands but displaying no sign of invasive cancer, high-grade prostatic intraepithelial neoplasia, or proliferative inflammatory atrophy were used as a source of benign tissues. When selecting cancer sections, only slices displaying sufficiently large malignant areas (>5 x 5 mm focus) underwent a macrodissection procedure as follows.

 Tubes containing tumor sections were thawed in a 37°C water bath. Immediately after thawing, tubes were transferred on ice. Each tissue slice was washed by dipping it into a beaker containing DMEM. Tissue slices were then transferred onto a weighing dish and matched with their corresponding digital images. Posterior (black) and anterior (red) margins, and the upper side of the slice (green dot), were used to help with orientation. Tumor areas were then macrodissected and put into a beaker containing DMEM.

**Tissue homogenization and crude plasma membrane generation.** Benign and tumor tissues were cut into small pieces (cubes, ~3 mm in size), and homogenization buffer [250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4)], 100 units/ml of DNase I (Roche, Laval, Canada), 5 mmol/L MgCl₂, Complete protease inhibitor EDTA-free cocktail (Roche) was added at a concentration of 10 mL/g of tissue. Tissues were homogenized twice for 20 seconds using a polytron (Kinematica, Newark, NJ) set at speed 8 (~20,000 rpm).

 Homogenates were filtered through a 180 μm nylon mesh and centrifuged at 900 x g (2,000 rpm) for 10 minutes at 4°C. Supernatants were collected, brought to 12.5 mL with the homogenization buffer and transferred into 12.5 mL Ultraclear centrifuge tubes (Beckman Coulter, Mississauga, Ontario, Canada). For each tube, a cushion made of 100 μL of 50% v/v sucrose was placed at the bottom. Samples were then centrifuged at 35,000 rpm (100,000 x g) for 60 minutes at 4°C to pellet the membranes. Membrane pellets were resuspended at 1 mL of homogenization buffer per gram of tissue and incubated with 500 units/ml of micrococcal nuclease (US Biologicals, Swampscott, MA) and 1 mmol/L of CaCl₂ for 15 minutes at 4°C. To the resuspended membranes, 2.55 mol/L of sucrose solution was added to obtain a final sucrose concentration of 1.7 mol/L. To isolate crude plasma membranes, isopycnic centrifugation using discontinuous sucrose gradients was done as follows: on top of the 1.7 mol/L sucrose fraction containing membranes, the 1.5, 1.3, and 0.5 mol/L sucrose layers were overlaid and samples were then centrifuged at 35,000 rpm (100,000 x g) for 18 hours at 4°C. After centrifugation, the crude plasma membrane fraction located at the 0.5 to 1.3 mol/L sucrose interface was collected. The amounts of protein were determined using the bicinchoninic acid assay according to the manufacturer’s instructions (Pierce, Rockford, IL). Following bicinchoninic acid assay, the crude plasma membrane fractions were snap-frozen in liquid nitrogen and stored at -80°C.

**Immunomagnetic purification of epithelial plasma membranes from benign and malignant prostate tissues.** Crude plasma membranes were thawed and incubated with mouse antiepithelial plasma membrane antibody cocktail for 60 minutes at 4°C. For 1 mg of crude plasma membranes, 2 μg of CD164 (BD Pharmingen, San Jose, CA), 100 μg of epithelial-specific antigen (Neomarkers, Fremont, CA), and 100 μg of epidermal growth factor receptor antibodies (Neomarkers) were added and the incubation was done in 10 mL of isolation buffer [PBS, 0.5 mg/mL PVP-40T (Sigma, St. Louis, MO), 0.5 mg/mL skimmed milk, and Complete protease inhibitor EDTA-free cocktail]. Samples were then transferred into 12.5 mL ultracentrifuge tubes. A cushion of 100 μL of 50% sucrose was placed at the bottom of the tubes and samples were centrifuged at 40,000 rpm for 60 minutes at 4°C to pellet the membranes. Membranes were resuspended in 2 mL of isolation buffer per milligram of crude plasma membrane and incubated for 30 minutes at 4°C with goat anti-mouse MACS immunomagnetic beads (Miltenyi Biotech, Auburn, CA) at a ratio of 1 μL of beads/μg of crude plasma membrane in a total volume of 10 mL isolation buffer. To reduce cytoskeletal protein content associated with plasma membrane, potassium iodide was added to the samples to obtain a final concentration of 600 mmol/L, and then incubated for 30 minutes at 4°C. In a cold room, samples were applied on magnetic LS columns according to the manufacturer’s instructions (Miltenyi Biotech). Columns were washed twice with 8 mL of isolation buffer containing 600 mmol/L of potassium iodide and once with 8 mL of 250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4) buffer. Columns were then removed from the magnet and purified epithelial plasma membranes were eluted with 3.5 mL of 250 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4) buffer into 15 mL tubes. To determine the amount of protein using the micro-bicinchoninic acid assay, 350 μL of the eluted plasma membrane fraction was reserved. The remaining eluate was transferred into a 4 mL Ultraclear centrifuge tube. A cushion of 50 μL of 33% sucrose was placed at the bottom of the tube and samples were spun at 50,000 rpm (337,000 x g) for 30 minutes at 4°C to pellet the plasma membranes. Plasma membrane pellets were snap-frozen in liquid nitrogen and stored at -80°C.

**SDS-PAGE and Western blotting.** Tissue homogenates from benign tissues and from macrodissected tumor areas were probed by Western blotting for the expression of p63 (1:100 dilution; US Biologicals) and AMACR (1:100 dilution; Chemicon, Temecula, CA). Antibodies known to be expressed by benign and malignant prostate tissues, respectively (8, 10). The purity of epithelial plasma membrane preparations was assessed by probing for epithelia-specific plasma membrane...
markers, prostate-specific membrane antigen (1:150 dilution; Northwest Biotherapeutics, Bothell, WA) and epithelial-specific antigen (1:2,000 dilution; Neomarkers), plasma membrane markers for stromal cells [leukocyte marker CD45, 1:50 dilution (Neomarkers); fibroblast marker CD90, 1:100 dilution (Calbiochem, San Diego, CA)], as well as for major intracellular organelles [all antibodies were from BD PharMingen; nuclear marker P62 (1:3,000 dilution); endoplasmic reticulum marker, calnexin (1:2,000 dilution); and mitochondrial marker, Tom20 (1:1,000 dilution)].

**H&E and immunohistochemical staining of retrieved tissue banked sections.** Selected malignant tissues initially banked for research that were too small for macrodissection were retrieved, placed in a plastic bag, thawed by immersion in warm water for 2 minutes, and subsequently formalin fixed and paraffin embedded before staining with H&E for pathologic evaluation. Benign slices from these patients were also retrieved.

Three pathologists (K. Sircar, L. Gaboury, and C. Lussier) independently and blindly evaluated the histology of thawed tissue and its routinely processed, mirror image on 41 pairs of tissue slices from 17 patients after a period of freezing that varied between 1 and 20 weeks. Tissue staining quality was scored based on architectural and cytologic features using the following system: 0 (poor, diagnosis/Gleason grading severely limited by histology), 1 (fair, diagnosis/Gleason grading somewhat limited by histology), 2 (good, adequate for diagnosis/Gleason grading), and 3 (excellent, optimal tissue staining). The pathologic features of the paired thawed and routinely processed slices were also recorded.

Immunohistochemistry was done on thawed tissue banked sections using a standard avidin-biotin technique with diaminobenzine as the chromogen. An automated immunostainer (Benchmark, Ventana Medical Systems, Tucson, AZ) was used, as were the following antibodies: PSA (polyclonal, 1:1,500; Dako, Carpinteria, CA), prostatic acid phosphatase (polyclonal, 1:6,000; Dako), 34E12 (monoclonal, 1:200; Dako), and PIN-4 Cocktail or P504S (AMACR) + p63 (mouse and rabbit, prediluted; Biocare, Walnut Creek, CA).

**Results**

**Macrodissection and validation of banked tissues.** According to the pathology mirror image, macrodisssection of malignant foci was done on 52 cases in which there was a sufficiently large cancer focus (5 × 5 mm) for this technique to be practical. The remaining 32 cases were excluded based on insufficient cancer volume in our PSA-screened cohort.

Homogenates from benign and macrodisssected malignant tissues were then probed by Western blotting for the expression of AMACR, a marker that is significantly up-regulated in cancer (8, 9), and p63, a prostatic basal cell marker present in benign glands but absent in cancer (10), to assess the levels of tissue cross-contamination. In 62% of the macrodisssected cases (32 of 52), a low level of tissue cross-contamination was observed as benign tissues contained on average <6% AMACR+ (tumor) cells, whereas malignant tissues were contaminated, on average, by ~30% p63+ (benign) cells (Fig. 3). Therefore, our data indicates a higher cross-contamination rate for benign prostatic cells infiltrating into putatively malignant-labeled banked tissue. However, in 38% of the macrodisssected cases (20 of 52), a high degree (>50%) of tissue cross-contamination was assessed. These were not processed further as the ability to identify differentially expressed proteins is significantly reduced when the levels of tissue cross-contamination reached >50%. Hence, a final cohort of 32 cases showing minimal tissue cross-contamination and sufficient cancer volume was selected to undergo a plasma membrane isolation procedure.

**Immunomagnetic purification of epithelial plasma membranes from primary prostate tissues.** A novel purification approach was applied to isolate epithelial plasma membranes from primary human prostate tissues. In the first step, a crude plasma membrane fraction containing plasma membranes from all cell types present in prostate tissue, as well as contaminant organelles, was obtained after performing various velocity/isopycnic centrifugation steps. In the second step, antibodies specific to epithelial cell surface antigens were incubated in the presence of the crude plasma membrane fraction. After washing the excess of unbound antibodies, the crude plasma membrane was then incubated with magnetic beads coupled to secondary antibodies. The magnetic beads/epithelial plasma membrane immunocomplex was then retrieved and separated away from the other organelles and stromal plasma membranes by using a magnet. The end product is shown to be a highly purified epithelial plasma membrane fraction (Fig. 4). Indeed, probing of immunopurified epithelial plasma membrane preparations from both benign and malignant tissues with epithelial plasma membrane markers (prostate-specific antigen and epithelial-specific antigen) showed an 8 to 16 fold enrichment over homogenates. Expression of stromal plasma membrane markers (leukocyte marker CD45, fibroblast marker CD90) as well as contaminating intracellular organelle markers (mitochondrial marker Tom 20, endoplasmic reticulum marker Calnexin and nuclear marker p62) was barely detectable (Fig. 4). We found that a minimum of 0.3 g of lesional tissue was required to generate an amount of plasma membrane (20 μg) required to carry out the downstream proteomic analysis.

**H&E and immunohistochemical staining of retrieved tissue-banked sections.** Retrieval and thawing of frozen tissues, banked between 1 and 20 weeks, using our protocol had a minor effect on the quality of the H&E-stained sections of benign or malignant prostate (Fig. 5). Evaluation of tissue architecture and cytology by three pathologists showed that the banked/thawed sections all scored in the good to excellent range, confirming the clinical utility of these tissues (Table 1).

Banked/thawed tissues also showed the expected immunoprofile of benign and malignant prostate in samples that were...
banked between 1 and 20 weeks. Again, immunoreactivity was retained irrespective of the period of tissue banking (Fig. 5).

Pathologic comparison of routinely processed and retrieved tissue-banked sections. Of 41 slices that were retrieved and thawed, their routinely processed mirror image showed the following histology: 29 slices were entirely benign with the dotted focus showing a mean diameter of 14.8 mm, 12 slices had cancer with a mean cancer diameter of 3.5 mm.

In 27 of 29 benign slices, the histology of the thawed tissues matched their corresponding routinely processed mirror image sections, i.e., areas labeled benign on the formalin-fixed tissues were benign in the thawed tissues without evidence of high-grade prostatic intraepithelial neoplasia or cancer. In two benign routinely processed slices, the thawed mirror image showed a small focus of cancer (Gleason score 6). Thus, the pathologic cross-contamination rate by cancer into putatively “benign” banked slices was 6.9%.

In 8 of 12 routinely processed malignant slices, the histology of the thawed tissue matched in that it also showed cancer. In four malignant routinely processed slices, the thawed mirror image did not show cancer, i.e., the small cancer focus was lost in the mirror image slice. Hence, the cross-contamination rate by benign tissue into putatively “malignant” banked slices was 33% in a sample with small diameter cancer (median, 3.25 mm).

Discussion

Therapeutic targeting of plasma membrane proteins as a form of anticancer treatment first requires the procurement of purified epithelial plasma membranes from populations of benign and malignant human prostatic epithelial cells. Obtaining high-quality tissue suitable for plasma membrane isolation and downstream proteomic analysis also requires access to fresh tissue within a relatively short period of time postoperatively. Because prostate cancer cannot be reliably identified by gross examination (1, 6, 11), a mirror image banking protocol was applied for banking prostate tissue that enabled clinical diagnosis while providing fresh tissues suitable for plasma membrane purification. The validity of this protocol to obtain enriched populations of benign and malignant cells was confirmed via up-regulation of the AMACR/p63 ratio in tissue-banked slices labeled malignant and AMACR/p63 down-regulation in the slices labeled benign (Fig. 3). This standardized technique was used for all prostates in this study and could be completed within 25 minutes by pathology assistants and junior residents. Beyond the specific needs of proteomics, mirror image banking has wider applications in terms of generic prostate tissue banking. For example, access to fresh, frozen prostate tissues is superior to formalin fixed, paraffin embedded tissues for the recovery of mRNA and DNA from clinical samples (12, 13).

To our knowledge, the present series is the first to evaluate epithelial plasma membrane preparations from matched human benign and malignant prostatic tissue. A key variable for the generation of such plasma membrane preparations was the use of highly specific antibodies that bound their cognate antigens at the cell surface with sufficient specificity to purify epithelial plasma membranes of benign and tumor cells away from plasma membranes of stromal cells and other contaminating organelles. This was clearly achieved as the epithelial plasma membrane preparations displayed little contamination from stromal plasma membranes or from other intracellular organelles (Fig. 4).

Moreover, our method enabled purification of the entire plasma membrane and not merely a portion of it. This is particularly relevant to epithelial cell–derived malignancies or carcinomas because epithelial cells are polarized, implying that their plasma membranes are not homogenous but rather separated into two distinct domains: the apical and basolateral domains. Figure 4 shows the presence of both the apical marker, prostate-specific membrane antigen (14, 15) and the basolateral marker, epithelial-specific antigen (16, 17). Hence, the purified membranes should allow for the identification by mass spectrometry of the entire complement of the plasma membrane. Such information will be critical for the optimal identification of novel immunotherapeutic targets.

The mirror image banking protocol was tested with respect to pathologic diagnosis and found to be highly satisfactory. Specifically, tissue-banked sections that were retrieved, thawed, and processed after various periods of time showed histologic features that were always diagnostically adequate, albeit qualitatively slightly inferior to routinely processed tissues (Table 1). This is important, as pathologists may need to recover selected tissue-banked slices to fully assess equivocal areas of extracapsular invasion or surgical margin positivity seen on the routinely processed mirror image sections. Moreover, cancer may only be present in the slices sent for tissue banking and thus may be required for cancer diagnosis and Gleason scoring. We only encountered one case in which cancer was not seen on routinely processed sections and all the banked slices were returned with minimal cancer identified on one such retrieved, banked section. However, our study sample was relatively small and this scenario of extremely low volume prostate cancer will inevitably, if rarely, occur. Thawed tissues also showed the retention of immunoreactivity with antibodies routinely used in diagnosing prostate cancer.

With respect to prostate cancer, certain limitations inherent to the disease and the procedure need to be addressed. First,
even the minimal amount of tumor (0.3 g) required for plasma membrane isolation and downstream proteomic applications are often unavailable due to low volume cancer. Thirty-eight percent of our patients were excluded on this basis. This is not surprising considering our PSA-screened patient cohort in which only 34% had clinically palpable disease. The second issue in a multifocal, highly infiltrative neoplasm such as prostate cancer is cross-contamination. Even after careful screening, our data suggests rates of cross-contamination of $\sim 30\%$ for benign into malignant-labeled banked tissue and $<6\%$ for tumor into benign-labeled banked tissue. When evaluating a 2-mm-thick tissue slice, mirror image histology can be used as a guide in selecting malignant areas, but infiltration between benign glands is intrinsic to prostate cancer and some degree of contamination cannot be entirely eliminated.

The pathologic discrepancies that we encountered between routinely processed and banked/thawed mirror image slices could be explained by the trimming effect: prostatic quadrant slices are relatively large—approximately the size of a standard cassette—and because three-dimensional tissues are never perfectly flat, $\sim 100$ to $150 \mu m$ needs to be trimmed off the pathology block before getting a full face section that the pathologist can read. Thus, when evaluating the pathology slide, we do not see the exact mirror image of the tissue banked slice, but rather, a section that is 100 to 150 $\mu m$ deeper. This creates space for cross-contamination, especially in a highly infiltrative, multifocal neoplasm such as prostate cancer. It was magnified by the trimming of both blocks when histologically comparing thawed and routinely processed mirror image pairs.

There is currently no standard, universally accepted method for processing radical prostatectomy specimens even for clinical diagnosis. Both complete and partial submission of the prostate are widely practiced and both are recognized as valid by the College of American Pathologists (18). Methods of procuring prostate tissue for research purposes are similarly variable. Common techniques include punch biopsies of fresh prostatic tissue with pathologic examination of the tissue surrounding this hole used to infer the histology of the procured area (11, 19). Although histologic correlation of the procured area with its surrounding tissue has been validated in a recent study (20), the basic drawback of this technique is the inaccuracy of knowing which area to punch: cancer was missed in roughly 50% of prostatectomy specimens. Where data is available correlating the size of prostate cancer with accuracy in identifying it grossly, the results are worse. The large series by

### Table 1. Histologic quality of routinely processed tissue versus retrieved, thawed mirror image banked slices

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<tr>
<th>Quality of slices (n = 41)</th>
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<th>Pathologist 2</th>
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References


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