

## ORIGINAL PAPER

**PREPARATION OF FROZEN CELL-BLOCK SECTIONS AMENABLE TO DIAGNOSTIC IMMUNOCYTOCHEMISTRY: A TECHNICAL REPORT ON THE CRYO-EMBEDDED CELL-BLOCK METHOD**JOO SUNG LEE<sup>1</sup>, SUK JIN CHOI<sup>1,2</sup>, LUCIA KIM<sup>2</sup>, IN SUH PARK<sup>2</sup>, JEE YOUNG HAN<sup>2</sup>, JOON MEE KIM<sup>2</sup><sup>1</sup>Department of Pathology, Inha University Hospital, Incheon, Korea<sup>2</sup>Department of Pathology, Inha University College of Medicine, Incheon, Korea

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Modified agarose cell-block (CB) technique can be effectively used to have the CB embedded in the OCT compound for the preparation of frozen CB (F-CB) sections in the same way as in the preparation of frozen sections from cryoembedded fresh tissue samples for the intraoperative consultation. In this report, we demonstrate the amenability of F-CB sections to the diagnostic immunocytochemistry. The pelleted cytologic material was at first compactly embedded in ultralow-gelling temperature agarose gel and then re-embedded in conventional agarose gel. The resulting agarose-embedded cell-pellet was cut in halves so that one half is embedded in OCT compound for cryosectioning on a cryostat, while the other half is reserved for the preparation of paraffin-embedded CB (P-CB). The F-CB sections were comparable to sections cut from the paraffin-embedded CB in terms of the quality of H&E-staining and immunocytochemistry. We suppose this method can also facilitate a rapid quantitative and qualitative assessment of the future P-CB.

We have extended this technique to the cryo-embedded cell-block method, in which the compact agarose cell pellet is directly embedded in the OCT compound so that the frozen sections can be cut from the cryo-embedded cell block in the same way as in intraoperative frozen section analysis. In this technical report, we illustrate how the frozen cell blocks (F-CBs) can be effectively prepared not only from liquid-based cytology samples but also from conventional fine-needle aspiration cytology slides.

**Key words:** cytology, fine needle aspiration, cell-block, frozen section, immunocytochemistry.

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**Introduction**

Diagnostically useful information can be obtained from immunocytochemistry (ICC) on cytology samples. Cell-block (CB) is the most commonly used cytology sample preparation for diagnostic ICC because an optimally prepared paraffin-embedded CB (P-CB) can provide multiple sections that can be processed in the same manner as formalin-fixed paraffin-

embedded tissue sections. However, the result of ICC on P-CB sections is not always satisfactory because the cellular yield of a CB can be inconsistent and the cellularity can vary widely through the levels of the CB sections [1]. This is because the conventional sedimentation CB technique is prone to significant losses of diagnostic cells and minute tissue fragments during the CB preparation, especially when the CB

needs to be prepared from a sample with low cellularity [2, 3].

The cellularity of a CB can be improved by preventing the loss of cytologic material during the CB preparation and by minimising the volume of the cell pellets embedded in paraffin [2]. The loss of cytologic material during the CB preparation can be significantly decreased by using a cell-gel method [4]. We have modified the cell-gel method, which enabled us to dramatically improve the quality of CB in terms not just of cellularity at the viewing spots of CB sections but also of distribution of cytologic material through the different levels of the CB. This technique can be effectively used in an attempt to ameliorate diagnostic ICC on the P-CB sections [5, 6]. However, the CB quality cannot be ensured at the time of CB preparation, especially when the CB needs to be prepared from a clinical cytology sample with very limited cellularity, although rapid on-site evaluation (ROSE) of the direct smears from the aspirate can improve the CB outcomes [7].

We speculated that a rapid, advance assessment of the CB quality can be feasible if a CB is cryo-embedded and processed in the same way as a fresh tissue sample for intraoperative frozen section diagnosis [8]. Haematoxylin and eosin (HE)-stained sections cut from the frozen CB (F-CB) could be amenable to ROSE of the quality of the future P-CB in advance

before the sections cut from the P-CB are available. In this technical report, we outline in detail the scheme of this cryo-embedded CB technique, which can be effectively used to have the cells or minute tissue fragments optimally represented on the viewing spot of the F-CB section, which can be not only for diagnostic ICC but also for ROSE of the adequacy of the future P-CB.

## Material and methods

In order to demonstrate the diagnostic applicability of the cryo-embedded CB technique, we present illustrative cases including diagnostically challenging ones that were easily and correctly diagnosed with the aids of ICC on the F-CB sections (Table I). The schematic flow chart of this technique is represented in Fig. 1. The study protocol was reviewed by the Institutional Review Board of Inha University Hospital (IUH IRB 2020-03-034) and complied with the tenets of the Declaration of Helsinki. Immunocytochemistry on cell-block sections is a routine diagnostic test carried out in the department of pathology and as such is not a part of any research study on patients; hence, the Institutional Review Board of Inha University Hospital granted a waiver of informed consent.

**Table I.** Cases diagnosed with the aid of immunocytochemistry on cell-block sections prepared by cryo-embedding and paraffin-embedding method

| CASE | SITE (AGE/SEX)                    | CYTOLOGY SAMPLES/ SLID PREPARATION                   | CYTOLOGIC FINDINGS  | CELL BUTTONS PREPARED FROM              | ICC ON FROZEN CELL-BLOCK SECTIONS  | DIAGNOSIS AIDED BY ICC                          |
|------|-----------------------------------|--|---|---|--|---|
| 1    | Thyroid (70/F)                    | US-FNA, SurePath™ preparation                        | High grade malignancy   | Residual SurePath™                      | CK <sup>+</sup> , patchy, PAX8 <sup>+</sup> , LCA  | Anaplastic thyroid                              |
| 2    | Pancreas tail (59/M)              | EUS-FNA, conventional smear                          | Malignant round cell tumour   | Scraping of conventional cytology slide | PAX8 <sup>+</sup> , TTF1 <sup>+</sup> , CD56 <sup>+</sup> , Ki67 80-90%, Chromogranin <sup>Weakly+</sup> , Synatophysin <sup>Weakly+</sup> | Metastatic small cell carcinoma of lung primary |
| 3    | Cervical lymph node (4/M)         | Direct FNA, conventional smear                       | Lymphoid malignancy   | Scraping of conventional cytology slide | CK(AE1/AE3) <sup>-</sup> , CD3 <sup>-</sup> , CD20 <sup>-</sup> , TdT <sup>+</sup> , CD2 <sup>-</sup> , CD5 <sup>-</sup> , CD1a            | T-lymphoblastic leukemia/ lymphoma              |
| 4    | Neck (parotid region) mass (39/F) | Direct FNA, conventional smear                       | High grade carcinoma suggestive of salivary duct carcinoma                | Scraping of conventional cytology slide | CK7 <sup>-</sup> , P63 <sup>+</sup> , P40 <sup>+</sup> , S100 <sup>-</sup> , Her2/neu <sup>-</sup>   | Metastatic Squamous cell carcinoma              |
| 5    | Ascitic fluid (56/F)              | Mucinosanguinous fluid (40 ml) SurePath™ preparation | A few clusters of atypical epitheloid cells suspicious for adenocarcinoma | Residual SurePath™ sample               | WT1 <sup>-</sup> , CK7 <sup>+</sup> , CK20 <sup>+</sup> , CDX2 <sup>+</sup>  | Metastatic mucinous carcinoma                   |

ICC – immunocytochemistry; EUS FNA – endoscopic ultrasound-guided fine needle aspiration; LCA – leukocyte common antigen; CK – cytokeratin; US-FNA – ultrasound-guided fine needle aspiration

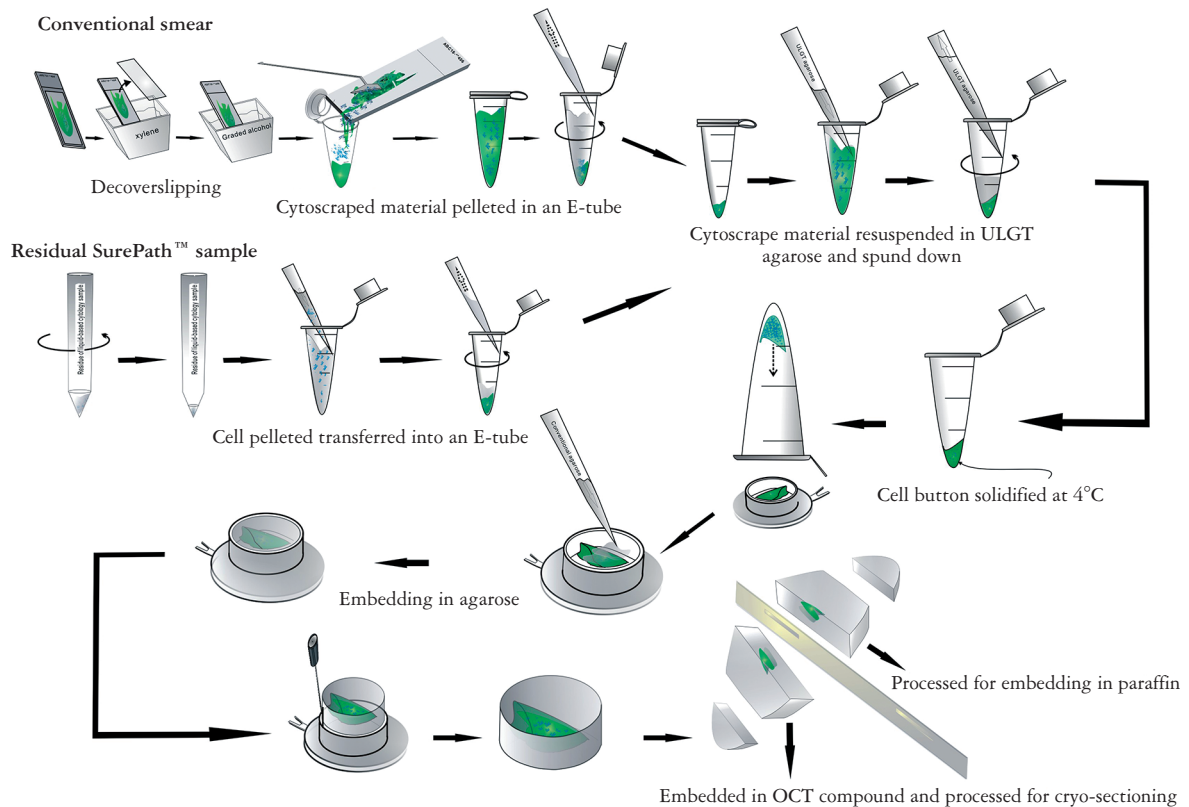


Fig. 1. Schematic flow of the work process involved in the preparation of agarose-cell pellet block

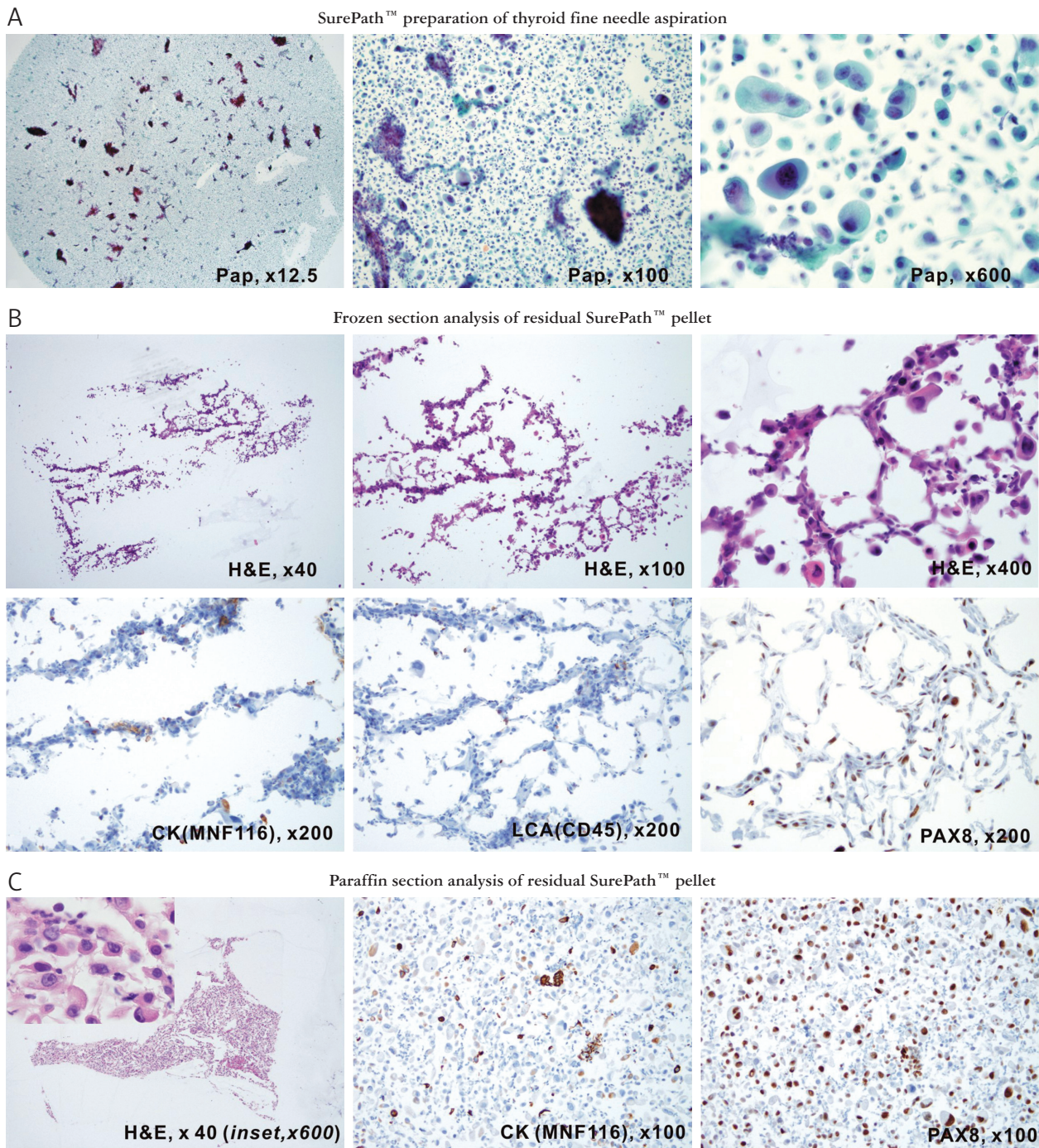
### Preparation of two types of agarose embedding media

Two types of agarose solutions were used in order to pre-embed the cell pellets. Three percent (w/v) ultra-low gelling temperature (ULGT) agarose (Agarose Type IX-A, Sigma-Aldrich, St. Louis, MO, USA) that has a gelling temperature of  $< 17^{\circ}\text{C}$  was used as a resuspending medium. Three percent standard agarose (Agarose Type I-A, Sigma-Aldrich) with a gelling temperature of  $34\text{--}38^{\circ}\text{C}$  was used as a re-embedding medium. In order to preserve the gelation quality of the agarose solutions, they were kept at  $4^{\circ}\text{C}$  with the cap fully tightened. When ready to be used, they were re-melted using a microwave oven. The re-melted ULGT agarose solution was then kept at room temperature while the re-melted standard agarose solution was kept in an oven set at  $60^{\circ}\text{C}$  to prevent premature solidification prior to use.

### Preparation of compact agarose-cell pellet block

The residual sample remaining after the preparation of liquid-based cytology (LBC) slide using the SurePath™ (BD Diagnostics, Burlington, NC, USA) platform was sedimented by centrifugation at  $600\text{ g}$  for  $5\text{ min}$ . The pellet was resuspended

with  $10\%$  buffered formalin solution. Then, most of the supernatant fluid was pipetted off, leaving a small volume of residual fluid that could be used to resuspend the formalin-fixed sediment. A drop of a working solution of eosin was added to the resuspended sample so that the cells and tissue within the sample were eosin-stained. The resuspended sediment was then entirely transferred to a  $1.5\text{ ml}$  microcentrifuge tube, which was subjected to centrifugation for  $30\text{ seconds}$  at  $15,000\text{ rpm}$  on a table-top centrifuge (Eppendorf® microcentrifuge 5415C). The resulting eosin-stained sediment was resuspended in  $2\text{--}3\%$  (w/v) ULGT agarose solution, which was then re-pelleted by using the same microcentrifuge. After pipetting off the supernatant ULGT agarose solution, the microcentrifuge with a compact agarose-cell pellet at the bottom was put in the refrigerator at the temperature of  $-20^{\circ}\text{C}$  so that it would solidify rapidly. The solidified compact agarose-cell pellet was transferred into the cap of the microcentrifuge tube. Then, the cap was filled with a  $1\text{--}2\%$  standard agarose solution that gels at room temperature. It was then put in the refrigerator at  $-20^{\circ}\text{C}$  so that the compact agarose-cell pellet would re-embed rapidly in the standard agarose gel [5]. The cytologic material scraped from an HE- or Papanico-



**Fig. 2.** Anaplastic thyroid carcinoma assessed by liquid-based FNA cytology and immunocytochemistry on cell-block sections (Case 1). The residual sample remaining after SurePath™ preparation (A) was pre-embedded in an agarose-cell pellet block. One half of the block was embedded in OCT compound for the preparation of frozen sections (B). The other half was fixed in formalin and processed to be embedded in a paraffin block (C). ICC for cytokeratin (MNF116), leukocyte common antigen (LCA), and PAX8 on the frozen sections confirmed the diagnosis of anaplastic thyroid carcinoma, which was reaffirmed on ICC on paraffin-embedded cell-block sections. All magnifications are original microscope magnification

laou-stained conventional cytology slides was also processed in the same way as described previously [6]. The resulting compact agarose-cell pellet block was bivalved into halves using a clean microtome blade in such a way that each half carried half of the compact agarose-cell pellet.

#### Cryo-embedding and cryo-sectioning of the agarose cell pellet block

One half of the agarose cell pellet block was transversely mounted on a cryostat chuck using a cryo-embedding medium (Tissue-Tek® O.C.T.™) and frozen on the cryostat so that the resulting F-CB sections were

cut and mounted a glass slide. Once the optimal cellularity of the F-CB sections was secured with ROSE of the HE-stained slide, additional F-CB sections were cut and mounted on additional glass slides, which were reserved in 95% ethanol until they were ready to be used for the diagnostic ICC using a panel of antibodies.

#### Paraffin embedding of agarose-cell pellet block

The remaining half of the compact agarose-cell pellet block was processed in the same way as clinical biopsy samples and embedded in a paraffin-CB. Once the proper F-CB sections needed for HE- and ICC staining were ready, the remaining F-CB was defrosted and processed to be embedded in a paraffin block designated as frozen permanent CB (FP-CB).

#### Immunocytochemistry on the CB sections

F-CB sections cut from the cryo-embedded agarose-cell pellet blocks were routinely processed for ICC using the same antibodies and protocols optimized for formalin-fixed paraffin-embedded tissue sections. An automated slide stainer (Ventana BechMark XT, Ventana Medical System Inc., Tucson, AZ, USA) and the Ventana OptiView DAB detection kit were used to analyse the result of immunostaining. The corresponding P-CB and FP-CB were also cut and processed for diagnostic ICC using the same antibodies and protocols in order to examine the quantitative and qualitative differences in ICC staining according to the methods for obtaining the CB sections.

## Results

### Preparation of frozen cell-block sections from agarose cell pellet block

By pre-embedding compact cell pellets in ULGT agarose and re-embedding them in standard agarose, it was possible for us to prepare compact agarose-cell pellet blocks within 20 minutes. By cryo-embedding one half of the compact agarose-cell pellet block in the same way as fresh tissue samples for intraoperative frozen section diagnosis, it was not difficult for a histotechnician to obtain serial frozen sections that were optimal for HE- and ICC-staining. The quality of the frozen sections was satisfactory not only for the evaluation of cytomorphology but also for the purpose of ICC. The result of ICC on sections cut from an F-CB was comparable to that on sections cut from corresponding P-CB or FP-CB.

### Case description

#### Case 1

A 70-year-old previously healthy woman was found to have a 20-day history of painful swelling in her

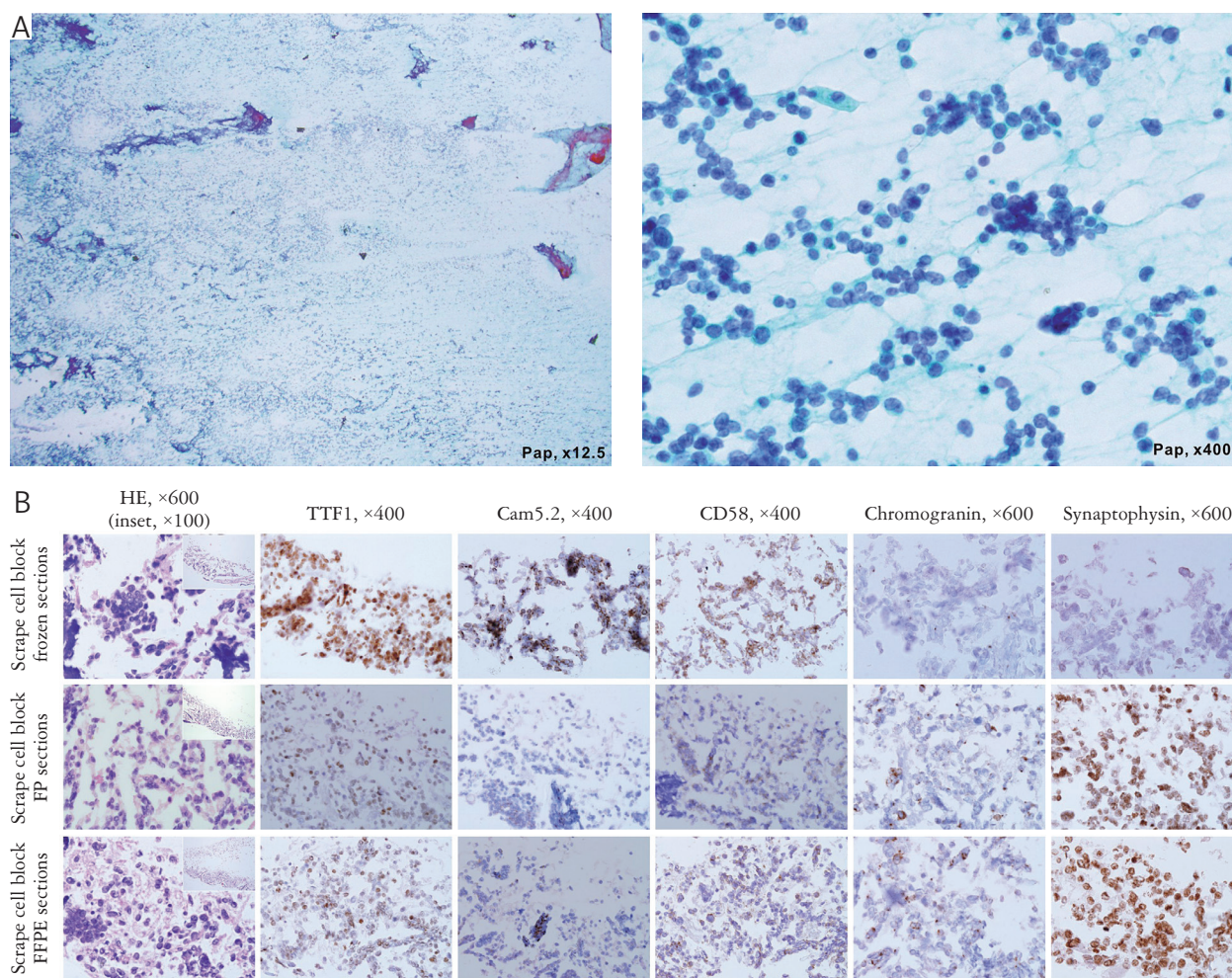
left neck. Ultrasound examination disclosed a conglomerated mass in the left thyroid along with multiple cervical lymphadenopathies. The SurePath™ preparation of the thyroid FNA represented a well-preserved population of malignant tumour cells characterized by pleomorphic nuclei and abundant cytoplasm (Fig. 2A). The conventional smears of the FNA from the cervical lymph node disclosed the same cell population. An agarose-cell pellet block was prepared from the SurePath™ residue. The block was processed for the preparation of F-CB and P-CB. The diagnosis of anaplastic thyroid carcinoma was rendered with much more confidence based on immunocytochemical features assessed on F-CB sections (Fig. 2B). The diagnosis was reaffirmed with immunocytochemical analysis on FP-CB sections (Fig. 2C).

#### Case 2

A 59-year-old man who had been treated for small cell carcinoma of the lung was found to have a 5.4-cm sized mass in the pancreatic tail. Endoscopic ultrasound-guided FNA cytology was performed on the mass. The conventional smears of the FNA cytology presented loose clusters or minute syncytial fragments of malignant round cells characterised by fine chromatin and fragile cytoplasm, featuring small cell carcinoma or poorly differentiated neuroendocrine carcinoma (Fig. 3A). The cytological material scraped off from one of the Pap-stained direct smears was entirely incorporated into a compact agarose-cell pellet block. The agarose-cell pellet block was processed for the preparation of F-CB and P-CB. The diagnosis of metastatic small cell carcinoma of lung primary was rendered based on ICC F-CB sections using a panel of antibodies including TTF1, Cam5.2, CD56, chromogranin, and synaptophysin (Fig. 3B).

#### Case 3

A 4-year-old boy presented with multiple bilateral cervical lymphadenopathies. The conventional smears from the FNA of the lymph node (Fig. 4A) revealed cytological features consistent with an aggressive form of malignant lymphoid neoplasm characterised by high cellularity with individually dispersed atypical large mononuclear cells. The cytological material scraped off one of the Pap-stained direct smears was processed for the preparation of a compact agarose-cell pellet block, which was then processed for the preparation of F-CB and P-CB. The F-CB sections were used to demonstrate immunophenotypic features consistent with lymphoblastic leukaemia/lymphoma (Fig. 4B, upper), which was confirmed on ICC on P-CB sections (Fig. 4B, lower).



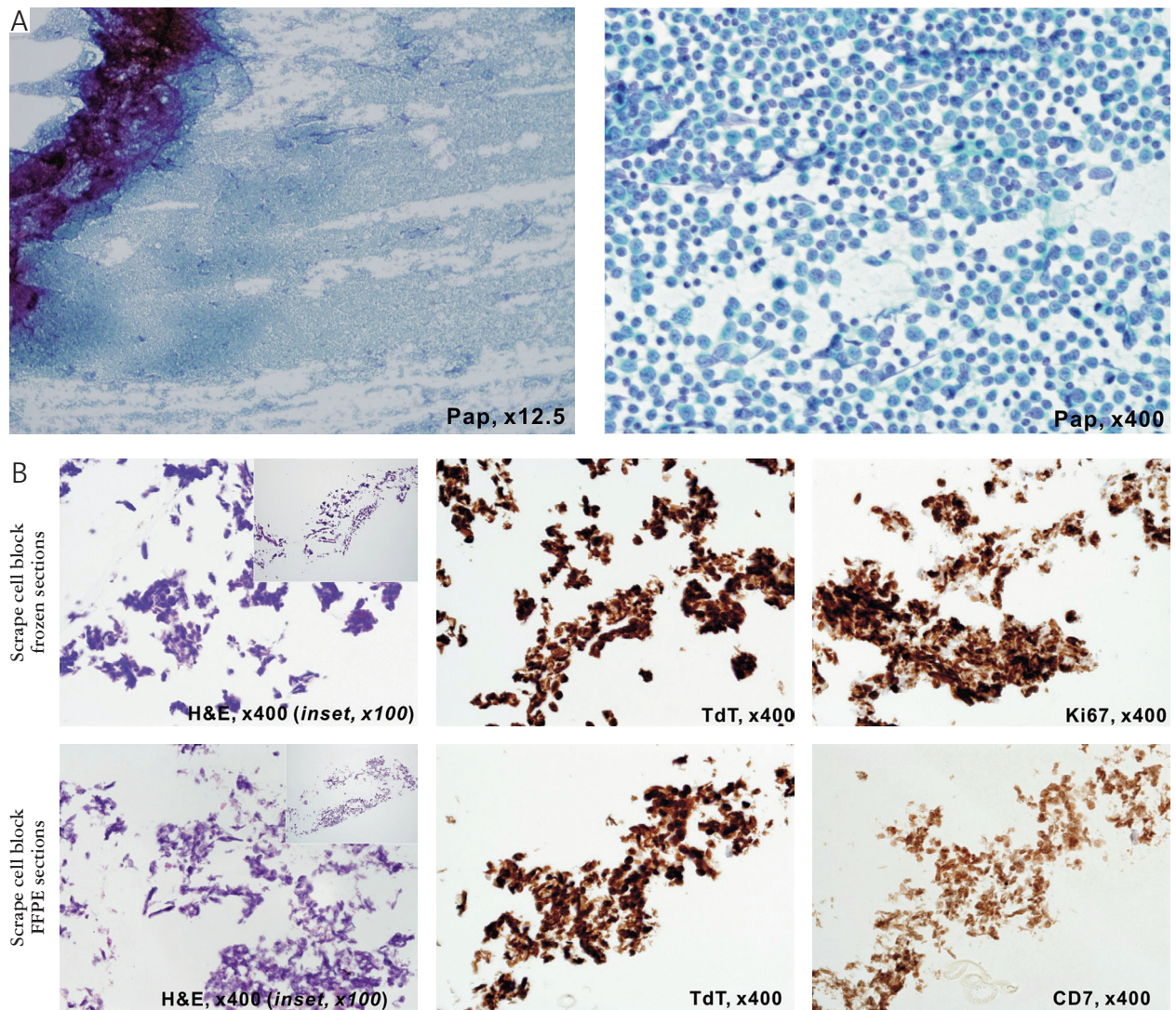
**Fig. 3.** Metastatic small cell lung carcinoma in the pancreas assessed by endoscopic ultrasound-guided FNA cytology and immunocytochemistry on agarose-cell block sections (Case 2). The cytologic material scraped off from a Pap-stained conventional smear (A) was pre-embedded in an agarose-cell pellet block. One half of the agarose-cell pellet block was embedded in OCT compound and processed for the preparation of frozen cell-block sections (B, upper) and frozen permanent cell-block sections (B, middle). The remaining half was processed to be embedded in paraffin (B, lower). ICC for TTF1, Cam5.2, and CD56 confirmed the diagnosis of metastatic small cell carcinoma of lung primary. All magnifications are original microscope magnification

#### Case 4

A 39-year-old man presented with a 3-month history of a painless mass in his infraparotid neck region. The cytological findings of the conventional smears (Fig. 5A) of the mass were interpreted at first as high-grade carcinoma (Fig. 5B) suggestive of salivary duct carcinoma. However, clinical features were not compatible. Hence, one of the Pap-stained direct smears was scraped and the cytological material was incorporated into a compact agarose-cell pellet block, which was used for the preparation of an F-CB (Fig. 5C, D). ICC on F-CB sections using a panel of antibodies (Fig. 5E) showed that the tumour cells were negative for cytokeratin 7 (CK7) and GATA3 but positive for cytokeratin 5/6 (CK5/6), P40 and P63, which helped us to render a correct diagnosis of metastatic squamous cell carcinoma rather than salivary duct carcinoma [9, 10].

#### Case 5

A 56-year-old woman presented with massive ascites associated with a unilateral ovarian mass. About 40 ml of ascitic fluid was drawn for cytological examination. The Pap-stained slide prepared by the SurePath™ platform showed a few clusters of epithelioid cells of uncertain significance in a background consisting predominantly of normal mesothelial cells admixed with a minor component of inflammatory cells. In order to specify the nature of the atypical epithelioid cells, we pelleted the SurePath™ residue and incorporated it into an agarose-cell pellet block. The block was processed for the preparation of F-CB and P-CB. The HE-stained sections cut from the F-CB and P-CB revealed more vividly than the Pap-stained SurePath™ preparation the presence of clusters of mucinous type epithelial cells in the background of many reactive



**Fig. 4.** T-lymphoblastic lymphoma assessed by FNA cytology and immunocytochemistry on scrape cell-block sections (Case 3). The cytologic material scraped off from a Pap-stained conventional smear (A) was pre-embedded in an agarose-cell pellet block. One half of the block was embedded in OCT compound and processed for preparation of cell-block frozen sections (B, upper). The remaining half was paraffin-embedded (B, lower). Immunocytochemistry for TdT and Ki67 on the frozen sections of agarose-cell pellet block suggested lymphoblastic leukemia, which was reaffirmed using immunocytochemistry for TdT and CD7 on sections from a formalin-fixed paraffin-embedded agarose-cell pellet block. All magnifications are original microscope magnification

mesothelial cells and inflammatory cells. We used F-CB sections to demonstrate that the mucinous type epithelial cells express CK7, CK20, and CDX2. The result of ICC on the F-CB sections was comparable to the result of ICC on the FP-CB sections and P-CB sections, supporting the diagnosis of a metastatic intestinal-type mucinous tumour consistent with ovary primary.

## Discussion

The diagnosis of cytology samples often depends on the result of the ICC on cytology samples. The result of ICC is more reliable on CB sections compared to direct smears or liquid-based cytology prepara-

tions [3]. P-CB sections are the most common cell preparations recruited for ICC for the differential diagnosis of difficult cytology cases because cell pellets are processed and embedded in paraffin in the same manner as tissue samples [2].

The optimal interpretation of ICC depends on the quality of P-CBs in terms of adequate cellularity. For the preparation of an adequate CB from a cytology sample with limited cellularity, the cells in the sample should be entirely aggregated into a compact cell pellet [11]. Agarose has been effectively used to produce CBs [4, 12, 13]. However, the preparation of P-CB with optimal cellularity cannot always be successful due to the inevitable loss of cytologic material during the procedure of CB preparation [1].

Furthermore, the distribution of diagnostic materials on CB sections can be uneven through different levels of the CB, which can prevent adequate interpretation of diagnostic ICC, especially when a panel of antibodies against various markers needs to be applied for the differential diagnosis.

In order to improve the quality of CB sections in terms of optimal-cellularity and even distribution of the diagnostic material through different levels, cell pellets should be as compact as possible so that the diagnostic material within the cell pellet can be concentrated on the viewing spot of the CB [14]. Recently we introduced a modified agarose-CB technique that is readily adaptable to any resource-limited labs: a technique that uses two types of agarose gels that are different in terms of temperature for gelation [5]. In this technique, the cell pellet is pre-embedded in ULGT agarose gel that does not gelate at room temperature and then re-embedded in standard agarose gel that gelates at room temperature. Finally, the cell pellet is processed to be embedded in paraffin in the same manner as biopsy specimens, resulting in a high-quality P-CB. In the same way, the cell scrapings obtained from an HE- or Papanicolaou (Pap)-stained direct smear(s) can also be incorporated into a compact cell pellet [6].

In this report, we have demonstrated that agarose-embedded compact cell pellet blocks can be embedded in a cryo-embedding medium that is routinely used for the preparation of cryo-embedded tissue sections for the purpose of intraoperative frozen section diagnosis. This technique enabled us to assess the quality of CBs prepared from cytology samples with limited cellularity in advance before the corresponding P-CB sections are available. The compact cell pellets embedded in the agarose gel were easy to cut in half using a microtome blade. Accordingly, we were able to have each half of an agarose-embedded cell pellet processed separately. In this split sample preparation, one was reserved in order to be processed for the preparation of high-quality P-CB, while the other was directly re-embedded in the OCT compound and directly cut on a cryostat for the preparation of F-CB sections.

The feasibility of a cryo-embedded CB method was investigated in our pilot study (abstract published in the proceedings of The Korean Society for Cytopathology 2018 Annual Spring Meeting [8]) which involved some cases of malignant serous effusions processed in pairs – i.e., the P-CB technique vs. F-CB technique, where it was evident that F-CB sections can be amenable not only to ICC but also to on-site adequacy assessment of the future P-CB. We also found that the result of the ICC on the F-CB sections was comparable to the result of ICC on P-CB sections in most cases. A critical step in this technique was to transfer the cell pellet pre-embedded in the ULGT agarose gel into the cap of the microcentrifuge tube.

Because ULGT agarose has lower gel strength compared to standard agarose, the cell pellet pre-embedded in the ULGT gel can be fragile and easily breaks up when handled. We found that, in order to prevent the cell pellet from crumbling into pieces during this procedure, the supernatant should be removed from the cell pellet as completely as possible and the pellet should be resuspended with an amount of ULGT agarose sufficient to rinse the residual supernatant from the pellet. This enabled us to procure a cell pellet compactly pre-embedded in the ULGT agarose gel, which is readily solidified at the bottom of the microcentrifuge in the refrigerator at a temperature of  $-20^{\circ}\text{C}$  just before the cell pellet block is transferred into the cap of the tube and re-embedded in the conventional agarose gel.

In cases where the cell pellet appeared to be too small to be seen on the CB sections, we added a small amount of eosin dye to the sedimented cytology sample so that the cells and minute tissue fragments within the cell pellet are prestained. This enabled the compact agarose-cell pellet block to be more easily visualised by the histotechnician, who determined the cutting level of the CB. We also experienced some cases in which it was not easy to remove the pellet from the bottom of the microcentrifuge tube into the cap of the tube. In these cases, we were able to float the pellet away from the bottom of the tube by filling the tube about half full of normal saline or distilled water. Then the pellet could be easily picked up using a needle or a tweezer. The water can be wiped off the surface of the pellet using tissue paper. This enabled us to remove the cell pellet out of the tube very easily without damaging the pellet.

Interestingly, in the case of small cell lung cancer in this study, the immunoreactivity for TTF1, Cam.52, and CD56 was much stronger on F-CB sections compared to that on sections cut from the FP-CB and P-CB, while the immunoreactivity for chromogranin and synaptophysin was stronger on sections cut from the FP-CB and P-CB. The decreased immunorecognition of for TTF1, Cam.52, and CD56 on FP-CB sections and P-CB sections might have been caused by the decreased immunorecognition or insufficient extent of antigen recovery that could be associated with routine tissue processing procedures needed for the preparation of P-CB [15].

The advance of rapid tissue processing technique and rapid immunohistochemistry can enable the pathologist to render a one-day diagnosis of difficult histopathology cases that should be diagnosed based on the result of immunohistochemistry for a panel of markers [16, 17]. We assume from our experience in this technical report that F-CB sections can also be amenable to rapid ICC for the rapid differential diagnosis of difficult cytology cases [18, 19]. We also speculate that the sections cut from the F-CB can



also be used for the purpose of on-site assessment of the adequacy of the future CB in the same way as ROSE of cytology material improves adequacy rates of FNA biopsy [20, 21, 22, 23].

In conclusion, we propose a cryo-embedded CB method enabling the preparation of high-quality F-CB sections that can be directly utilised for diagnostic ICC in advance before the P-CB sections are available. This method can also facilitate a rapid quantitative and qualitative assessment of the future P-CB, thereby avoiding the waste of financial resources, time, and effort that is inevitable when the diagnostic tests are performed using poor-quality P-CBs.

*The authors declare no conflict of interest.*

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