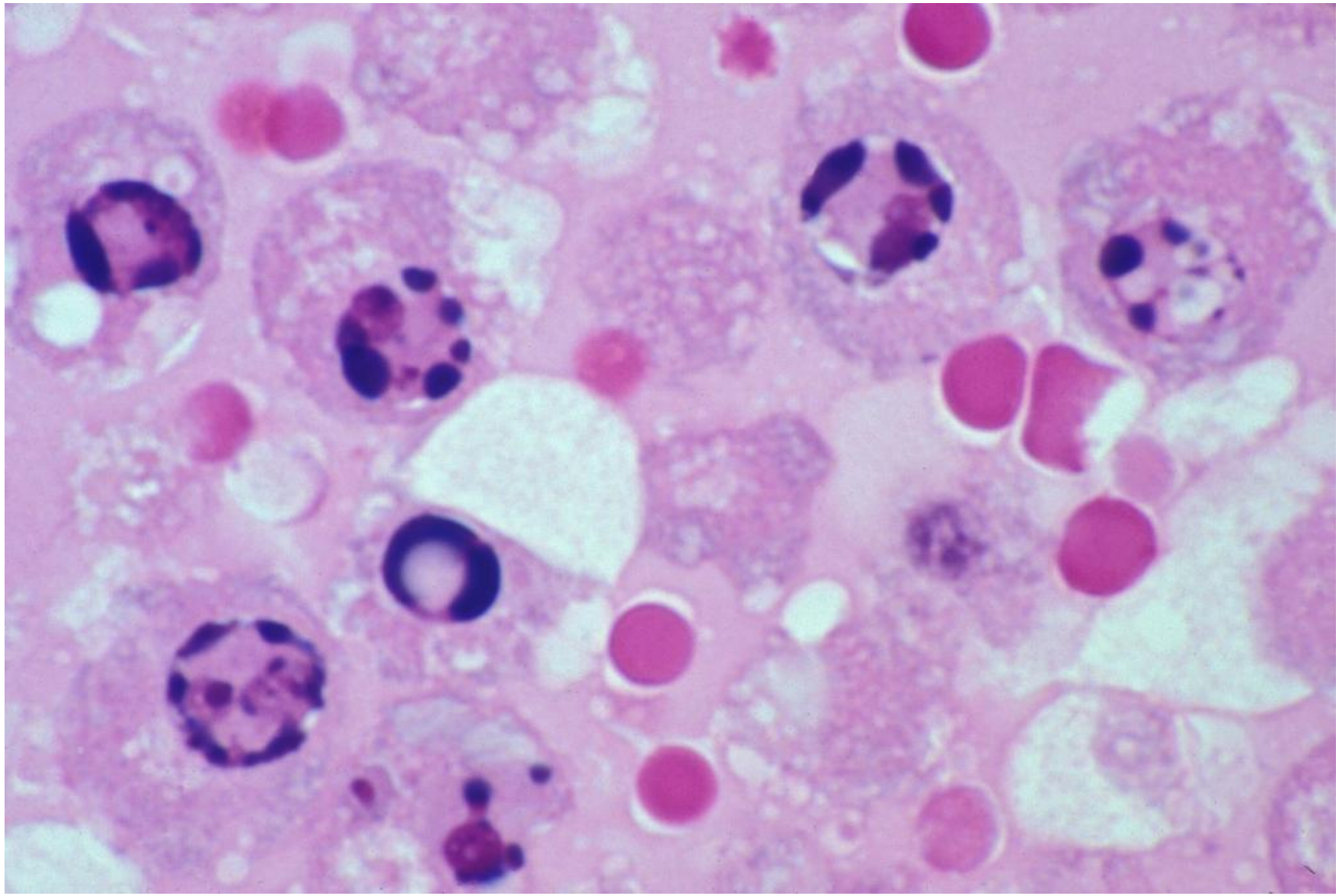
Histochemistry of apoptosis

For investigating the biological significance of apoptosis, the exact and sensitive histochemical identification of apoptosis and apoptotic cells is essential. However, we need to recognize both the pitfalls and caveats in performing histochemical staining in formalin-fixed, paraffin-embedded sections, and in interpreting the findings obtained. DNA fragmentation-based approaches, such as TdT-mediated dUTP-biotin nick end-labeling (TUNEL), *in situ* nick translation (ISNT) and immunostaining for single-stranded DNA, represent DNA alterations in the apoptotic cell, but they are technically unstable and occasionally give false positive and false negative findings. In contrast, immunostaining for intracellular proteins cleaved and activated by caspases, including cleaved caspase 3, cleaved poly(ADP-ribose) polymerase, cleaved cytokeratin 18 and cleaved actin (fractin), is technically reproducible, but the intracellular accumulation of the activated proteins is not necessarily synchronized. We should know the appropriate pretreatments for enhancing the sensitivity of these techniques, as well as their limitations and comparisons in histochemically demonstrating apoptosis and apoptotic cells.

Ref.: Tsutsumi Y, Kamoshida S. Pitfalls and caveats in histochemically demonstrating apoptosis. Acta Histochem Cytochem 2003; 36(4): 271-280. doi: 10.1267/ahc.36.271



Typical microscopic features of apoptosis: pleural effusion in multiple myeloma (cell block, H&E)

Histochemical identification of apoptosis

A) Identification of DNA changes

1) Terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)

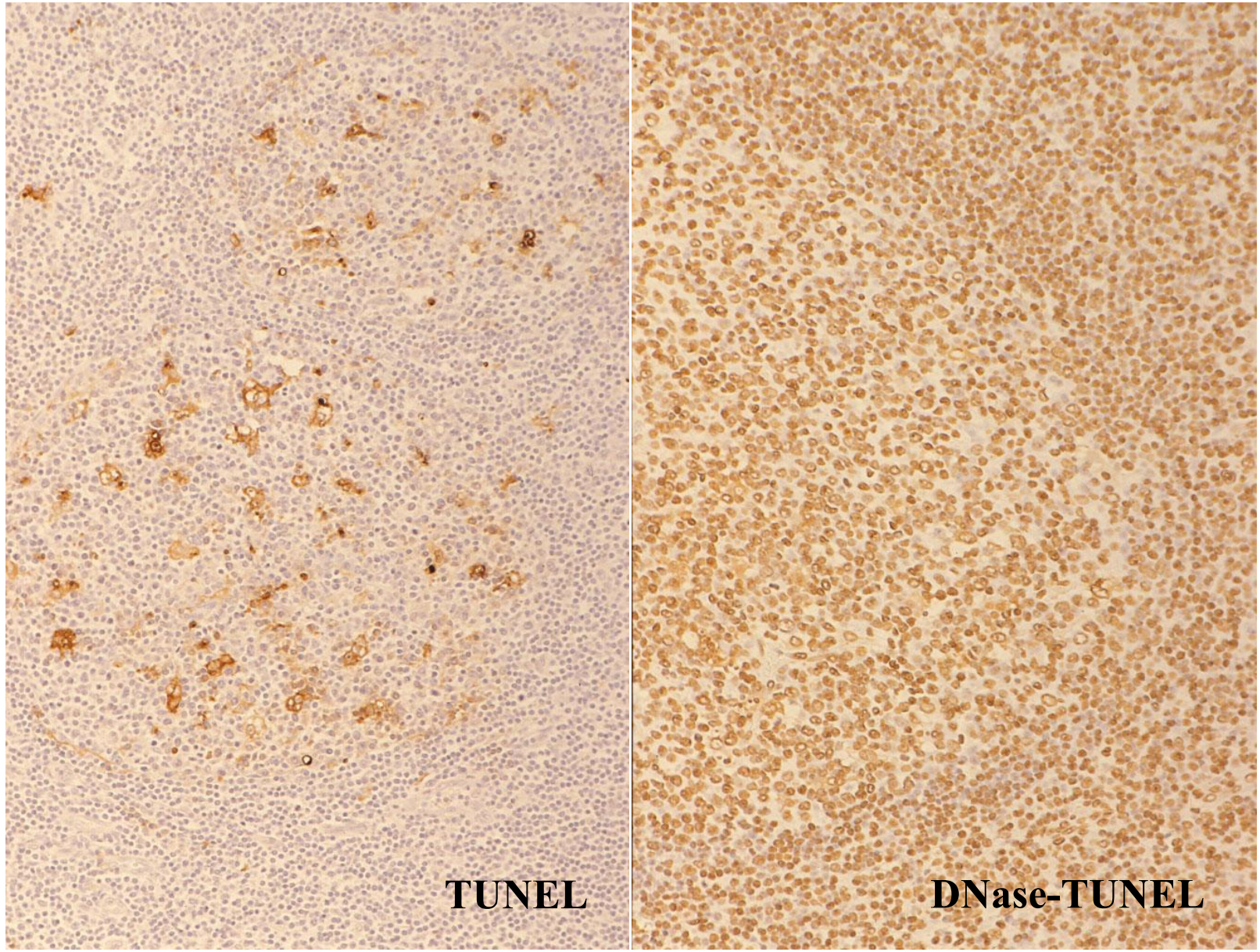
2) *in situ* nick translation (ISNT)

3) immunostaining for single-stranded DNA (ssDNA)

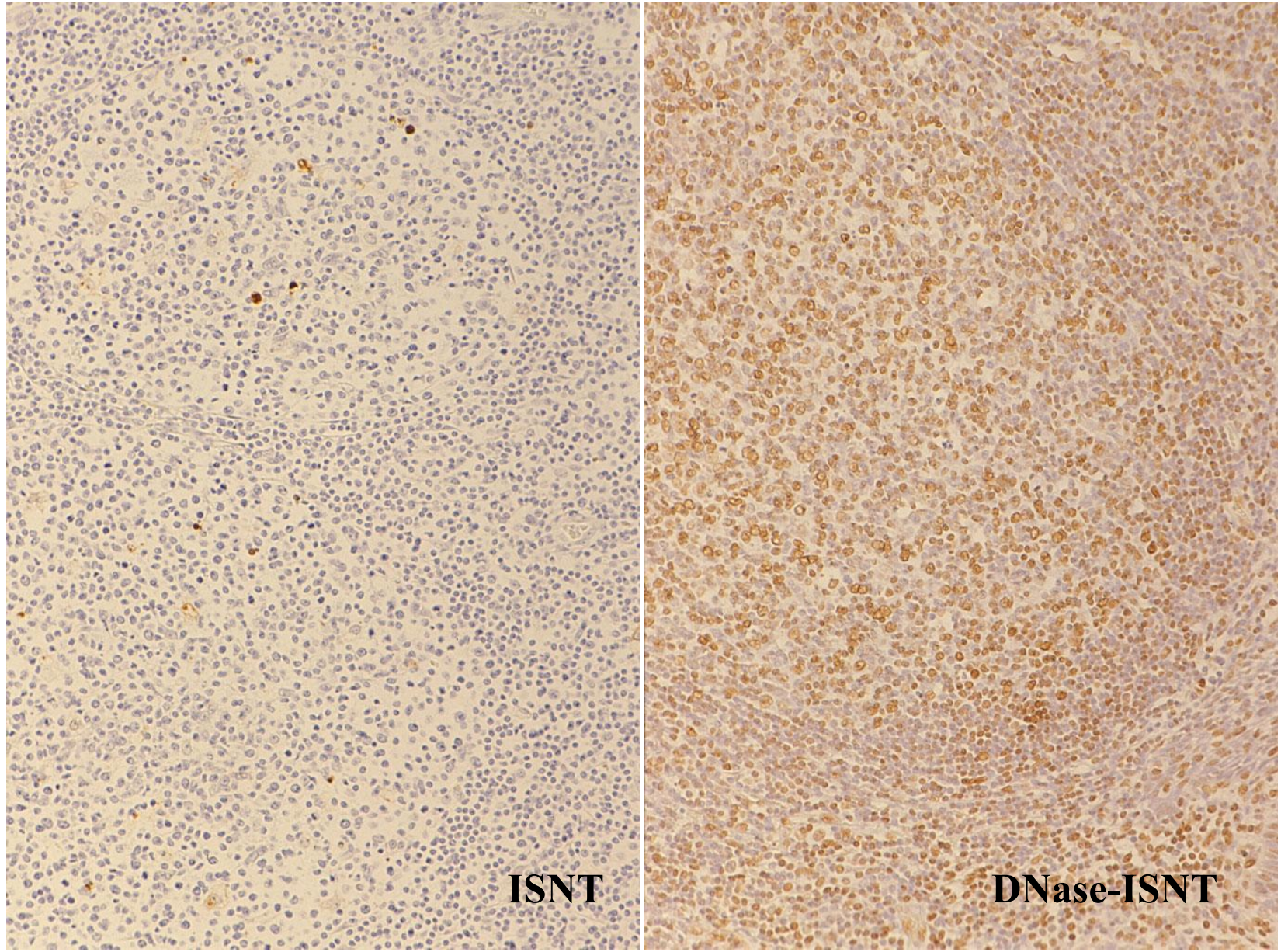
B) Immunohistochemical identification of apoptosis-associated events

1) **cleaved caspase 3**, cleaved caspase 6 and cleaved poly(ADP-ribose) polymerase (PARP)

2) cytoskeletal proteins such as cleaved cytokeratin 18, cleaved vimentin and cleaved actin (fractin)

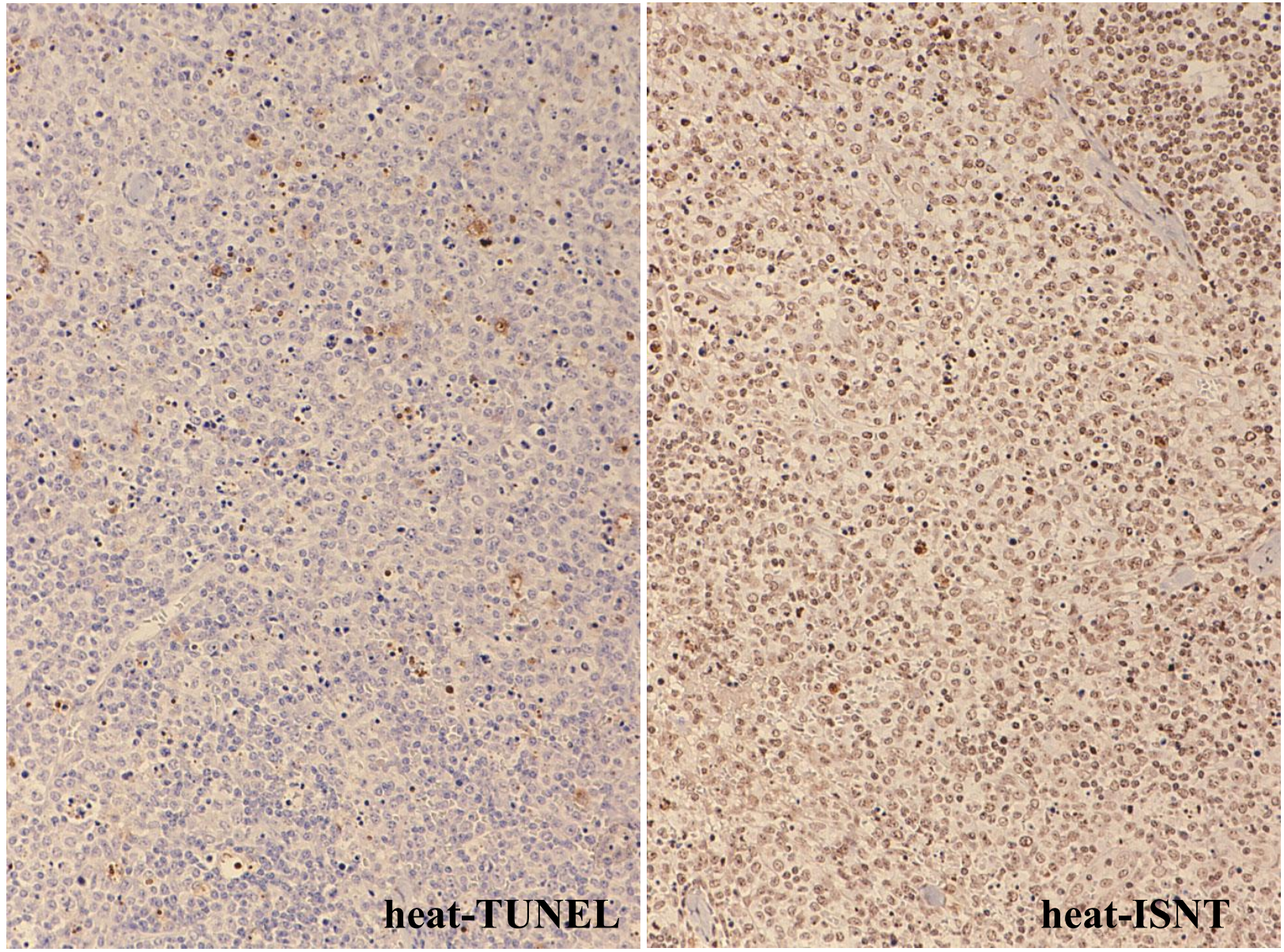


TUNEL for visualizing apoptosis in the germinal center of the palatine tonsil. All the nuclei turn to be positive with TUNEL after DNase treatment.

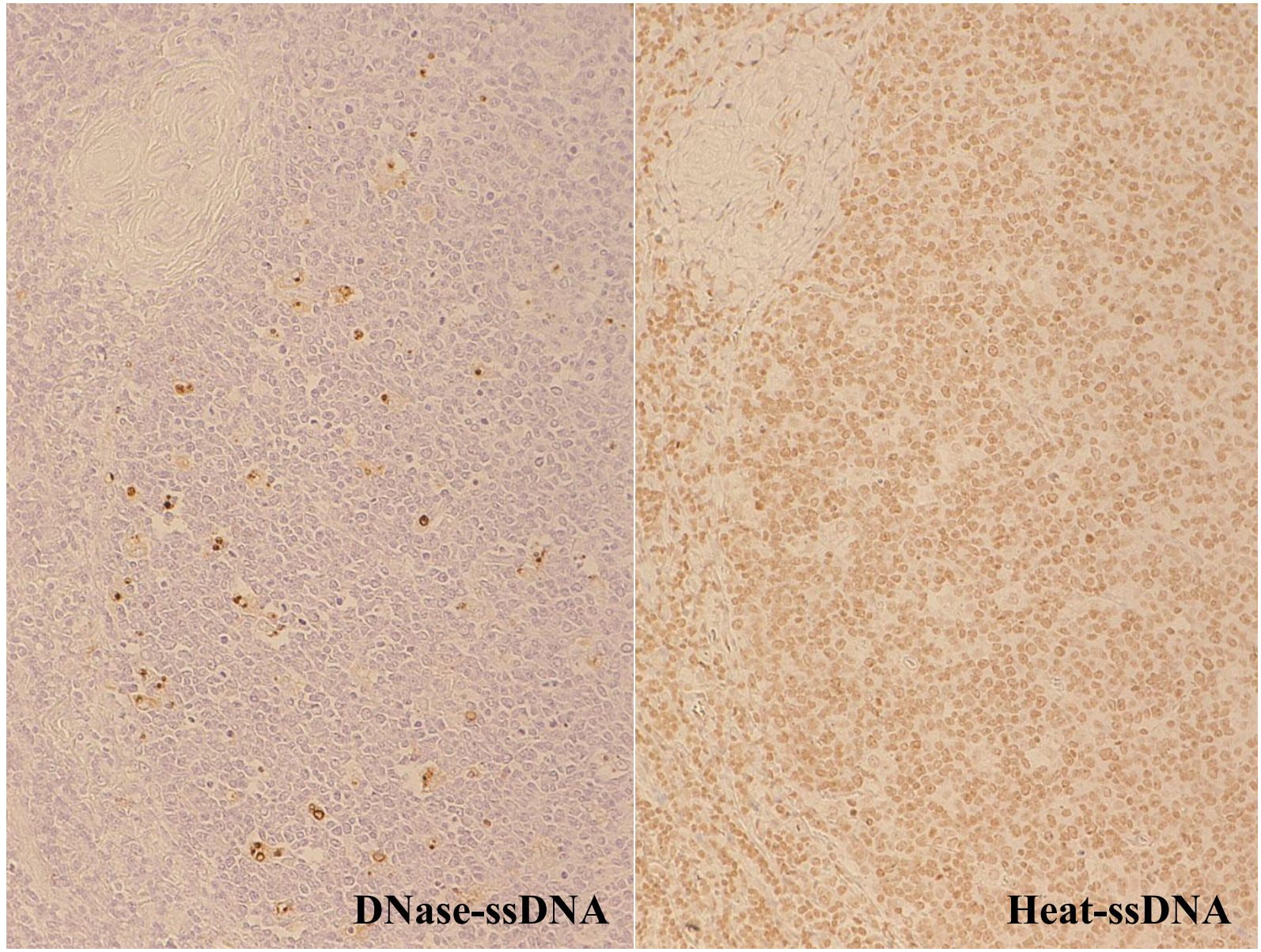


ISNT for visualizing apoptosis in the germinal center of the palatine tonsil. All the nuclei turn to be positive with ISNT after DNase treatment.

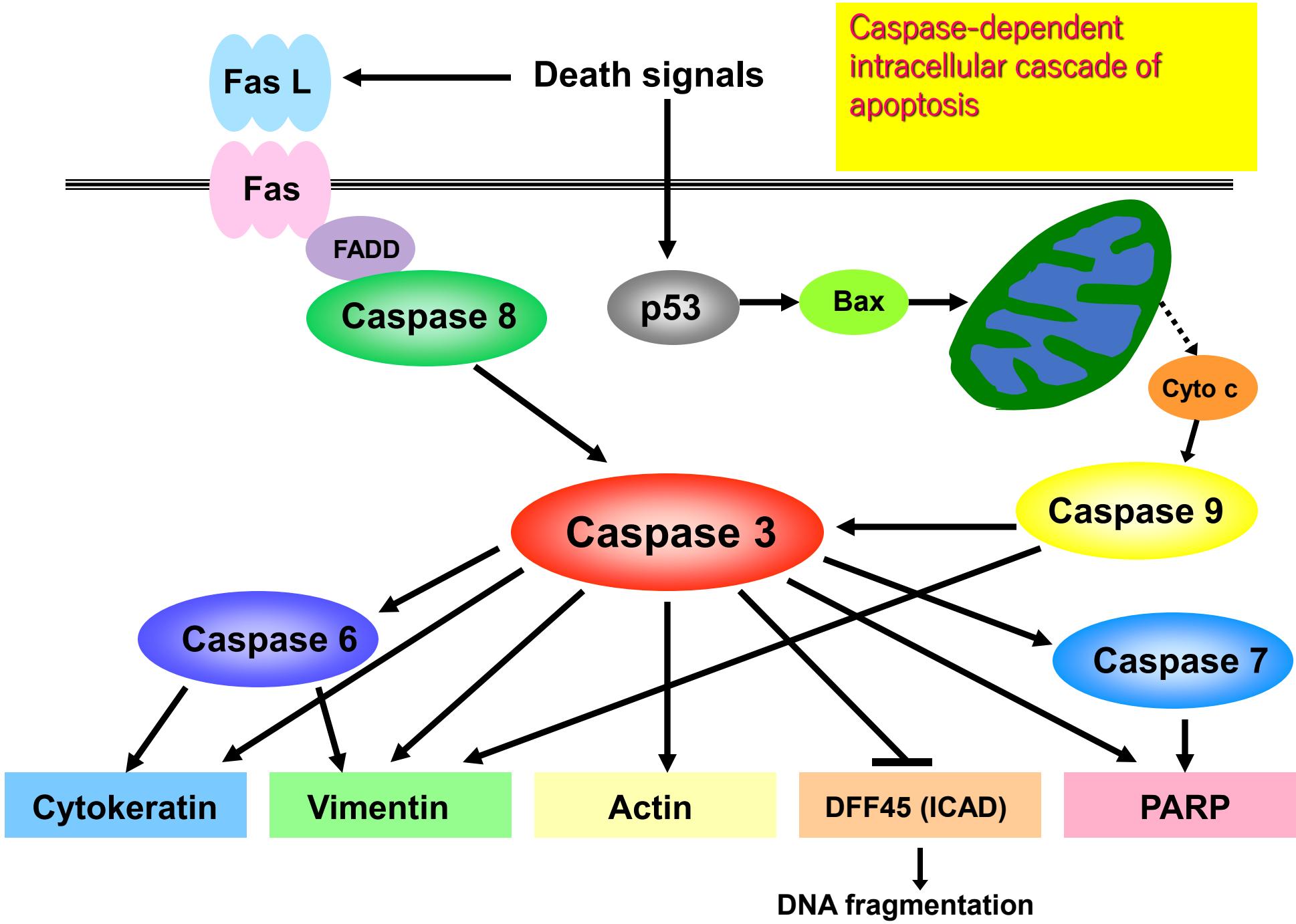
Necrotizing lymphadenitis



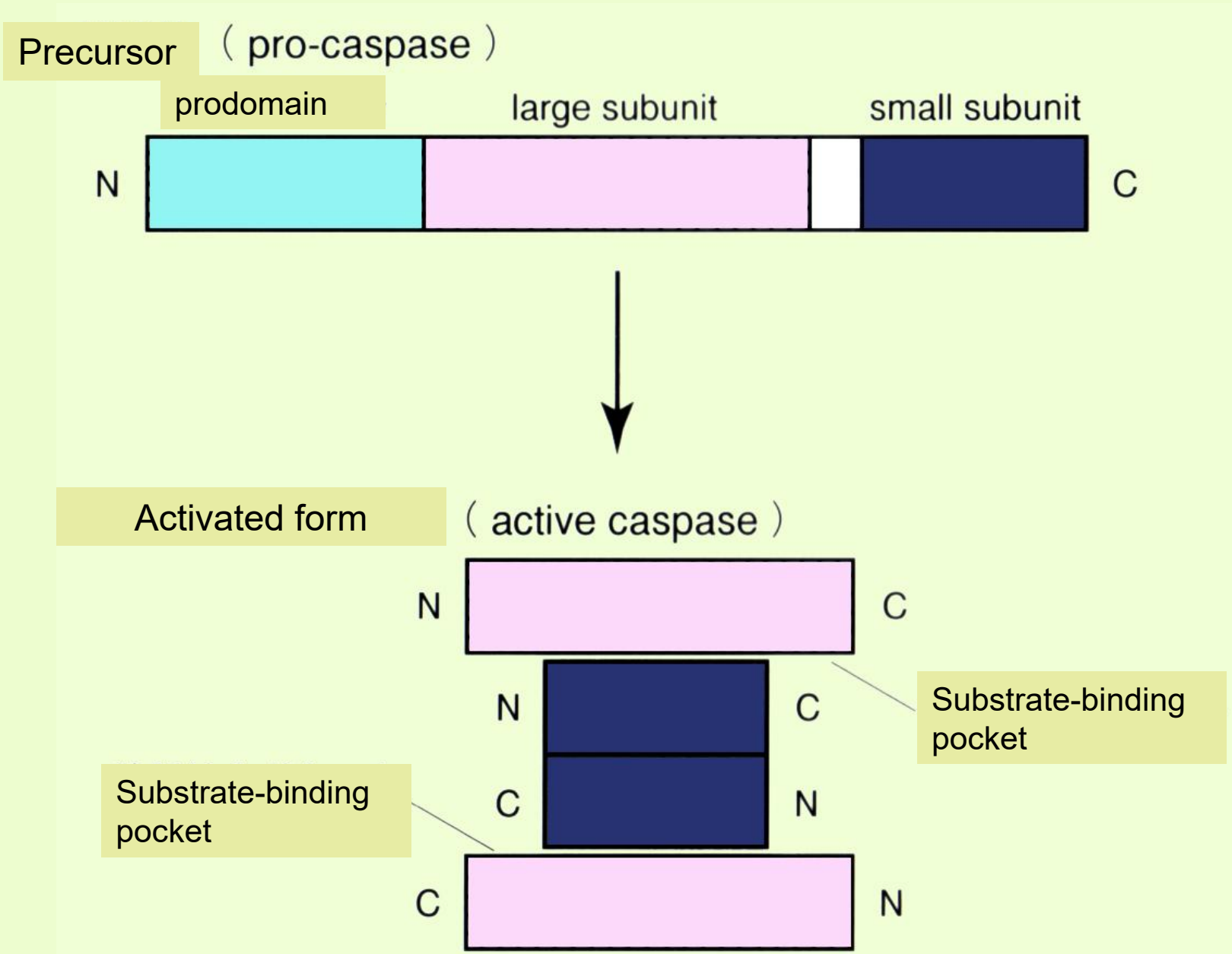
Visualization of apoptosis in necrotizing lymphadenitis (Kikuchi) with TUNEL and ISNT after heating treatment.

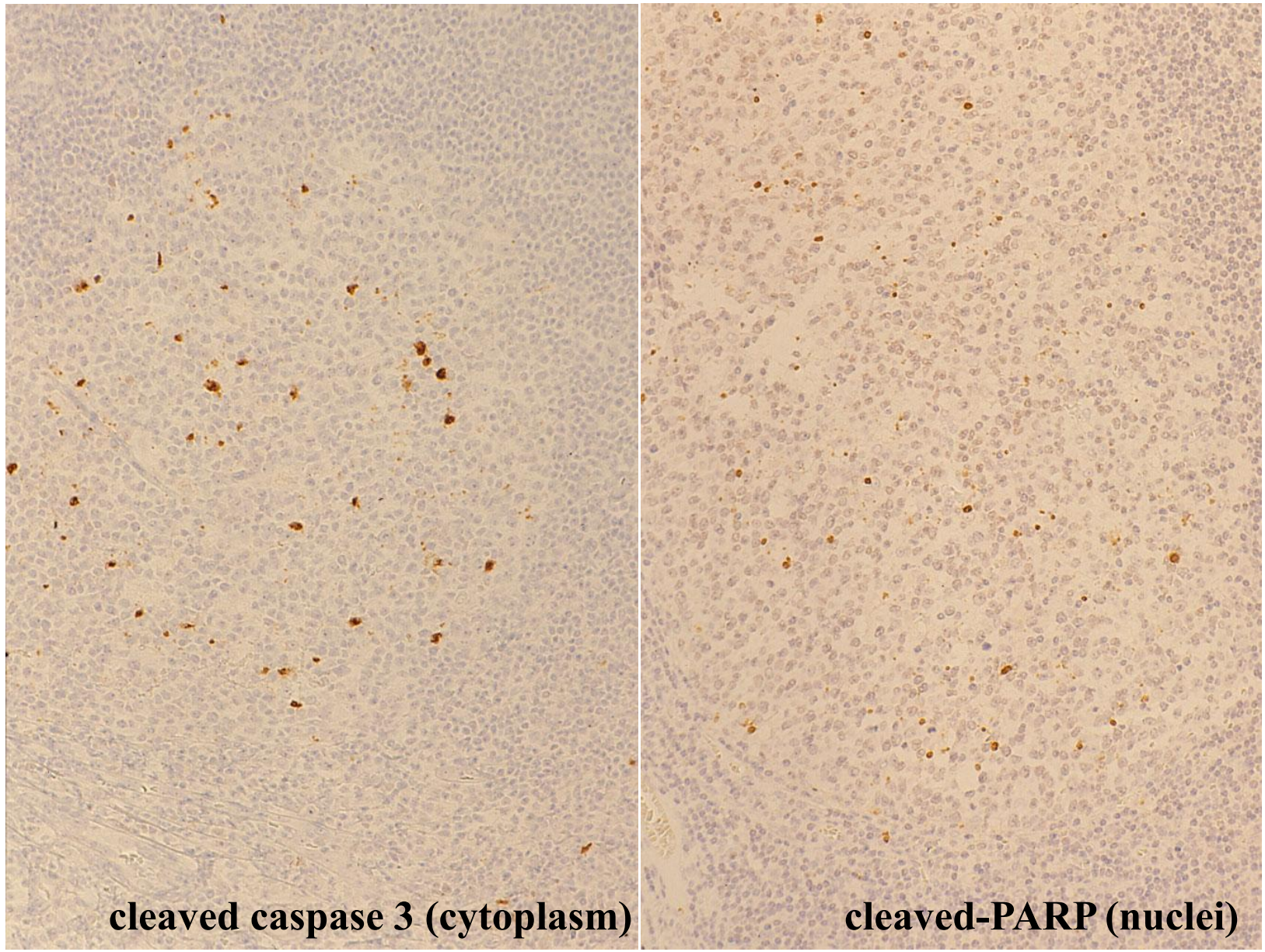


Visualization of apoptosis with immunostaining for ssDNA in the germinal center of the palatine tonsil. DNase treatment is effective, while all the nuclei turn to be positive after heating treatment.

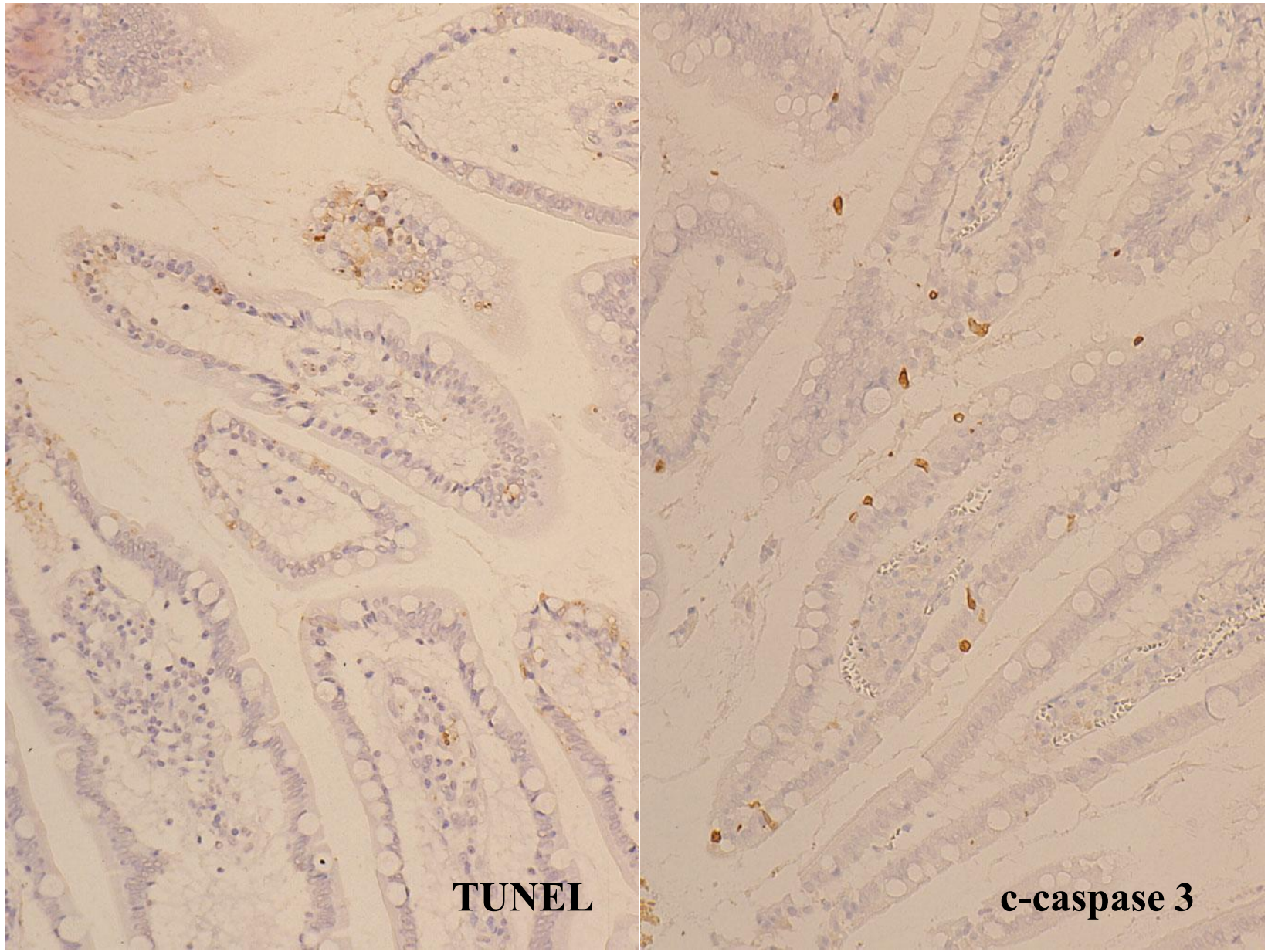

Caspase-dependent intracellular cascade of apoptosis
Death signals
Fas L
Fas
FADD
Caspase 8
p53
Bax
Cyto c
Caspase 9
Caspase 3
Caspase 6
Caspase 7
Cytokeratin
Vimentin
Actin
DFF45 (ICAD)
PARP
DNA fragmentation

Structure of caspases





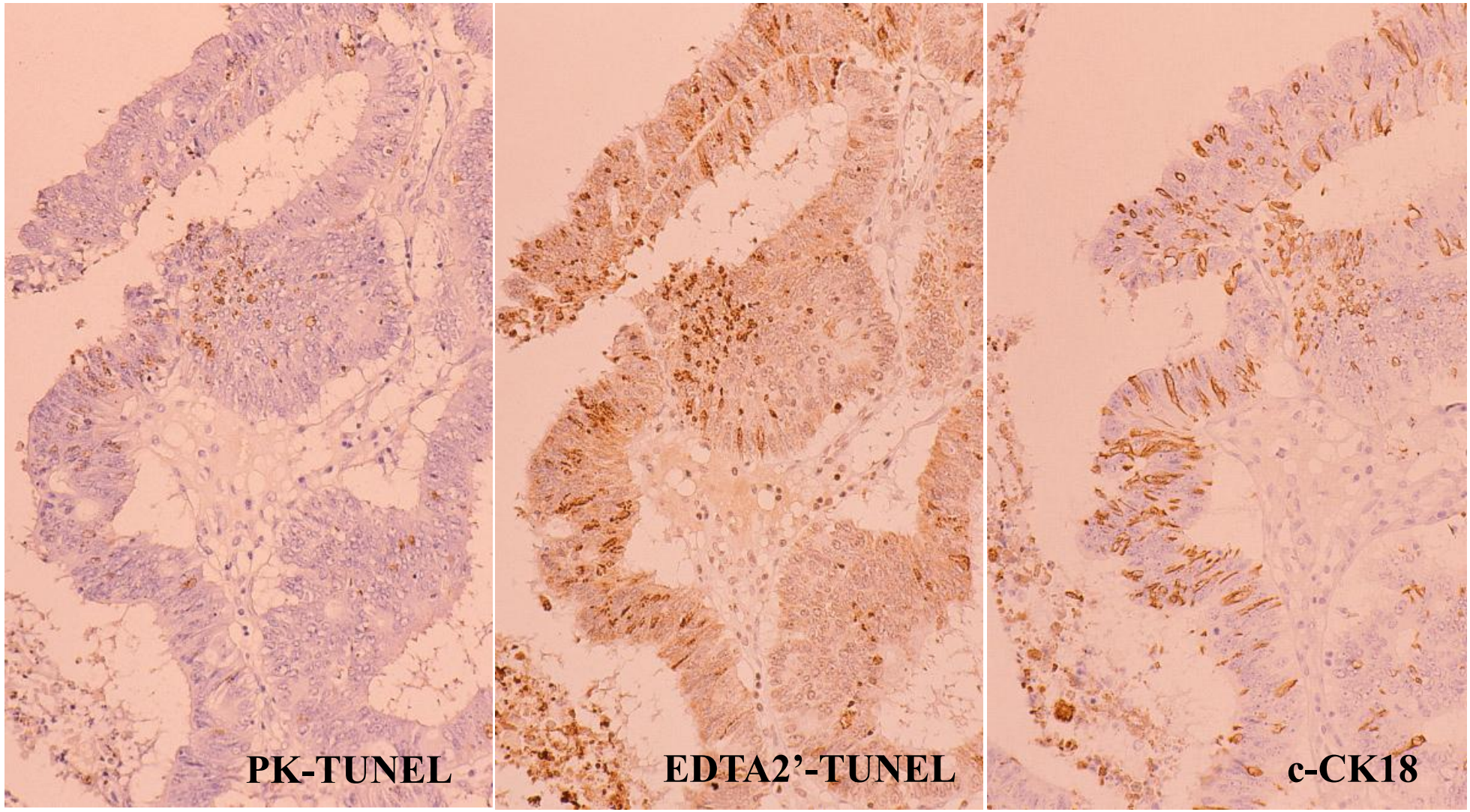
Visualization of apoptosis with immunostaining for cleaved caspase 3 and cleaved PARP in the germinal center of the palatine tonsil. Heating treatment is effective.



Visualization of apoptosis at the tip of villi of the normal ileal mucosa with TUNEL and immunostaining for c-caspase 3.

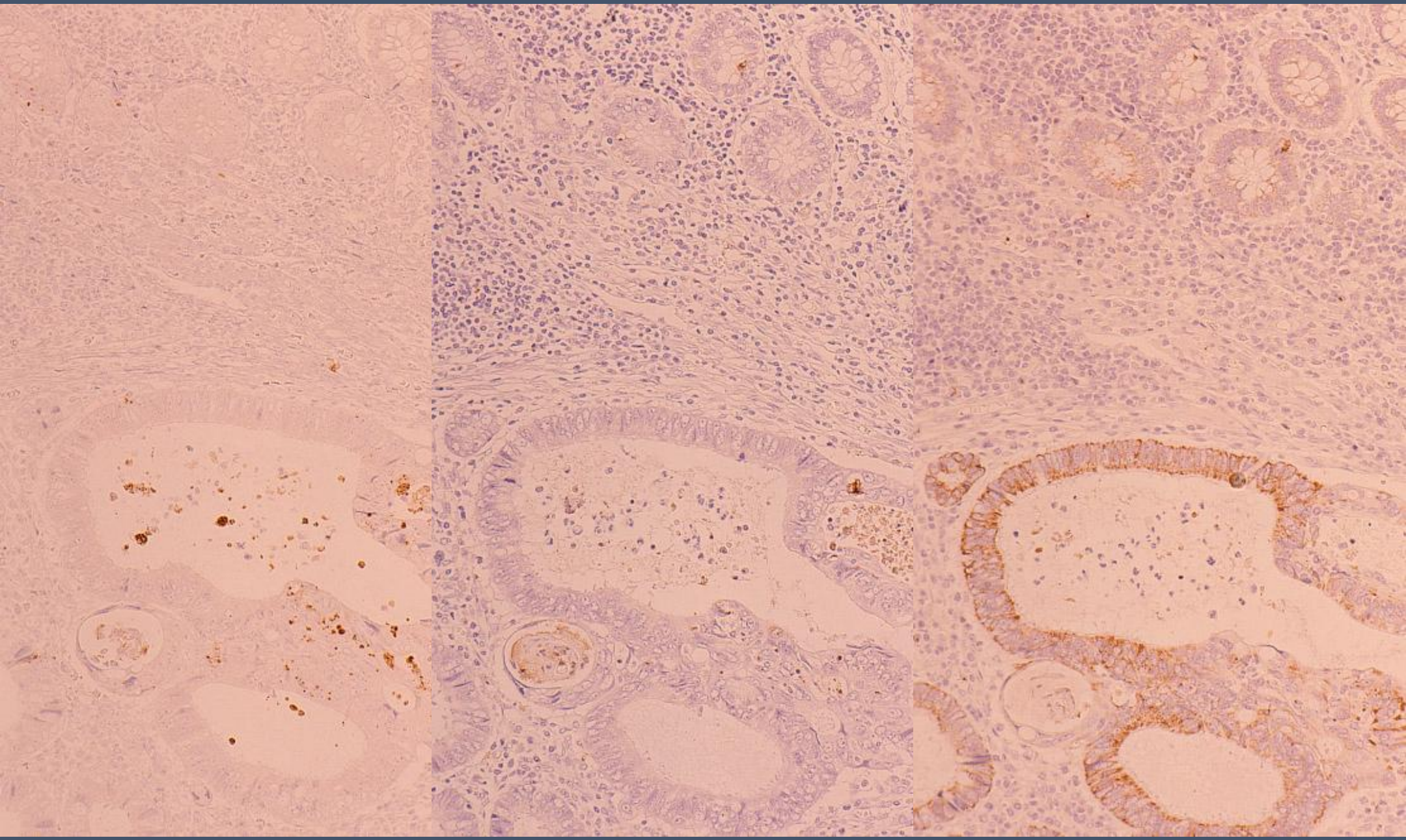
Treatments for increase of sensitivity in apoptosis histochemistry

Method of detection		Treatment for increased sensitivity
TUNEL method		1) Treatment of mung bean nuclease (single-stranded DNA-digesting enzyme) 2) Heating at 120℃ for 2-10 min in 10 mM citrate buffer (pH 6.0) or heating at 120℃ for 2 min in 1 mM EDTA (pH 8.0)
ISNT method		No report
Single-stranded DNA		Heating at 60℃ for 10 min in 10 mM citrate buffer (pH 7.0) Ficin treatment DNase treatment
Cleaved caspases 3&6		Heating at 120℃ for 2 min in 1 mM EDTA (pH 8.0)
Cleaved CK18, vimentin		
Cleaved actin (fractin)		
Cleaved PARP		



Demonstration of apoptosis in colon cancer with proteinase K-treated TUNEL, heat-treated TUNNEL in EDTA, pH 8 for 2 min, and immunostaining for cleaved CK18

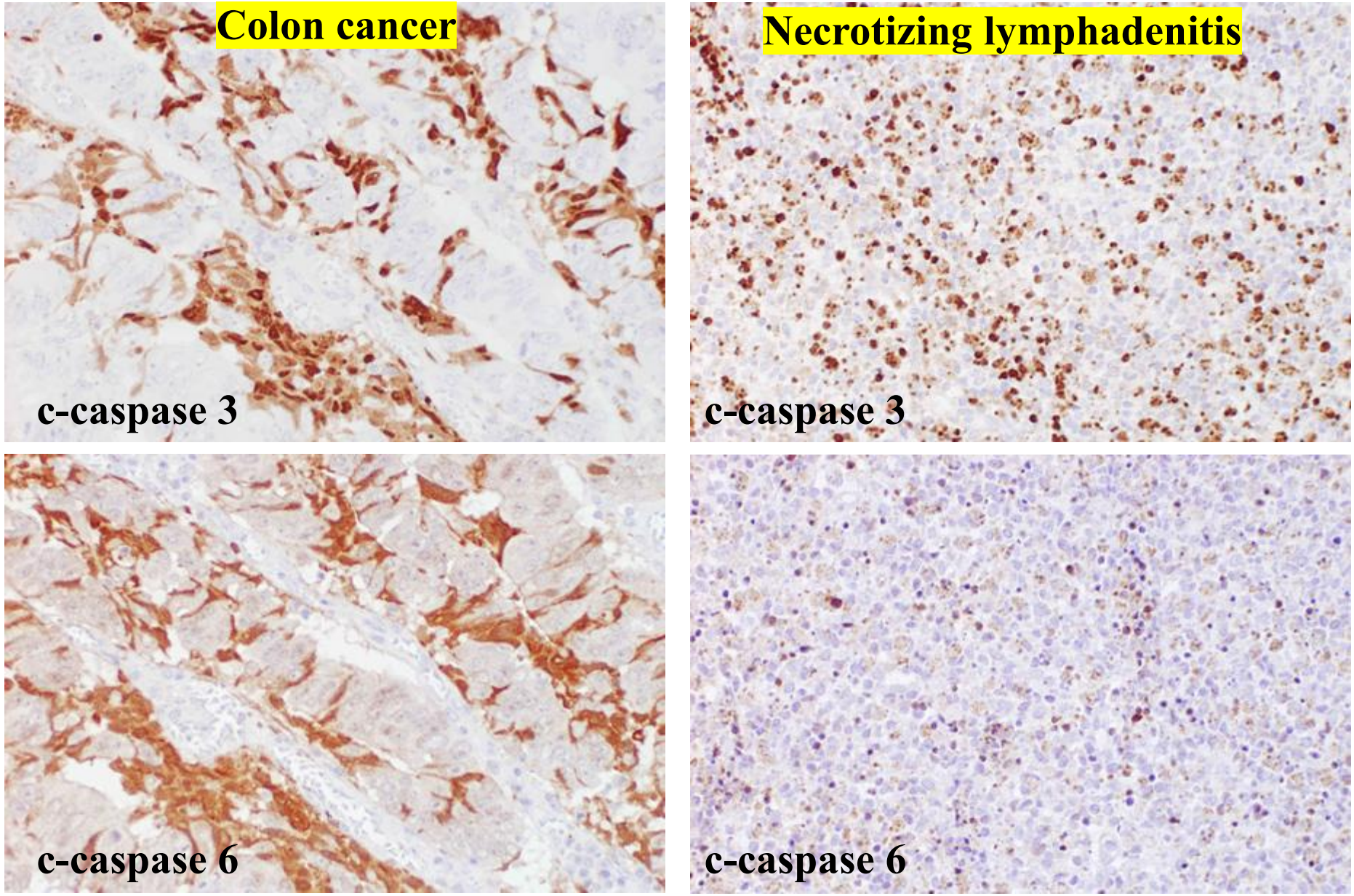
Colon cancer



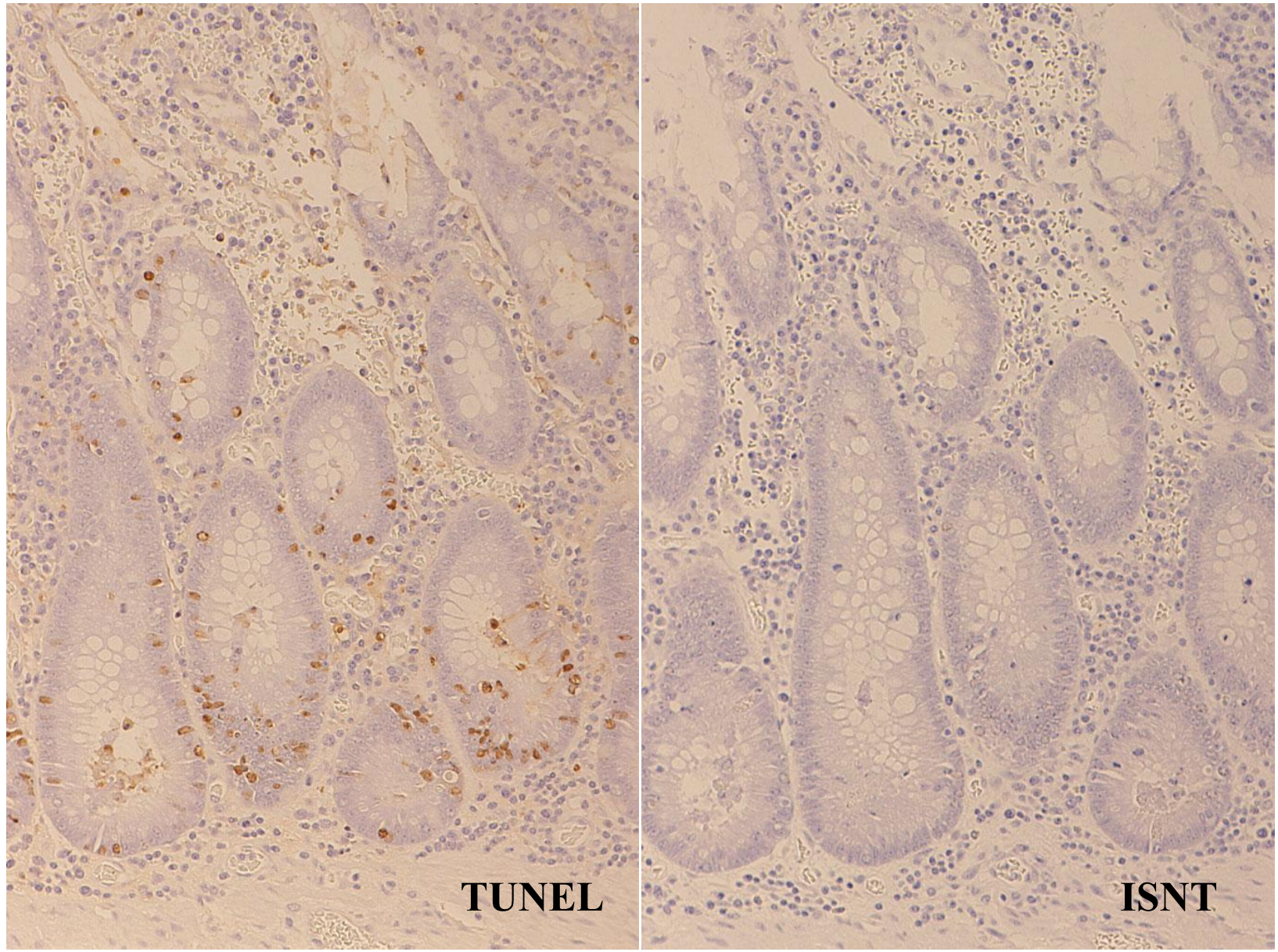
Cleaved caspase 3 | PK-TUNEL | EDTA2'-TUNEL

Effects of pretreatment in apoptosis histochemistry

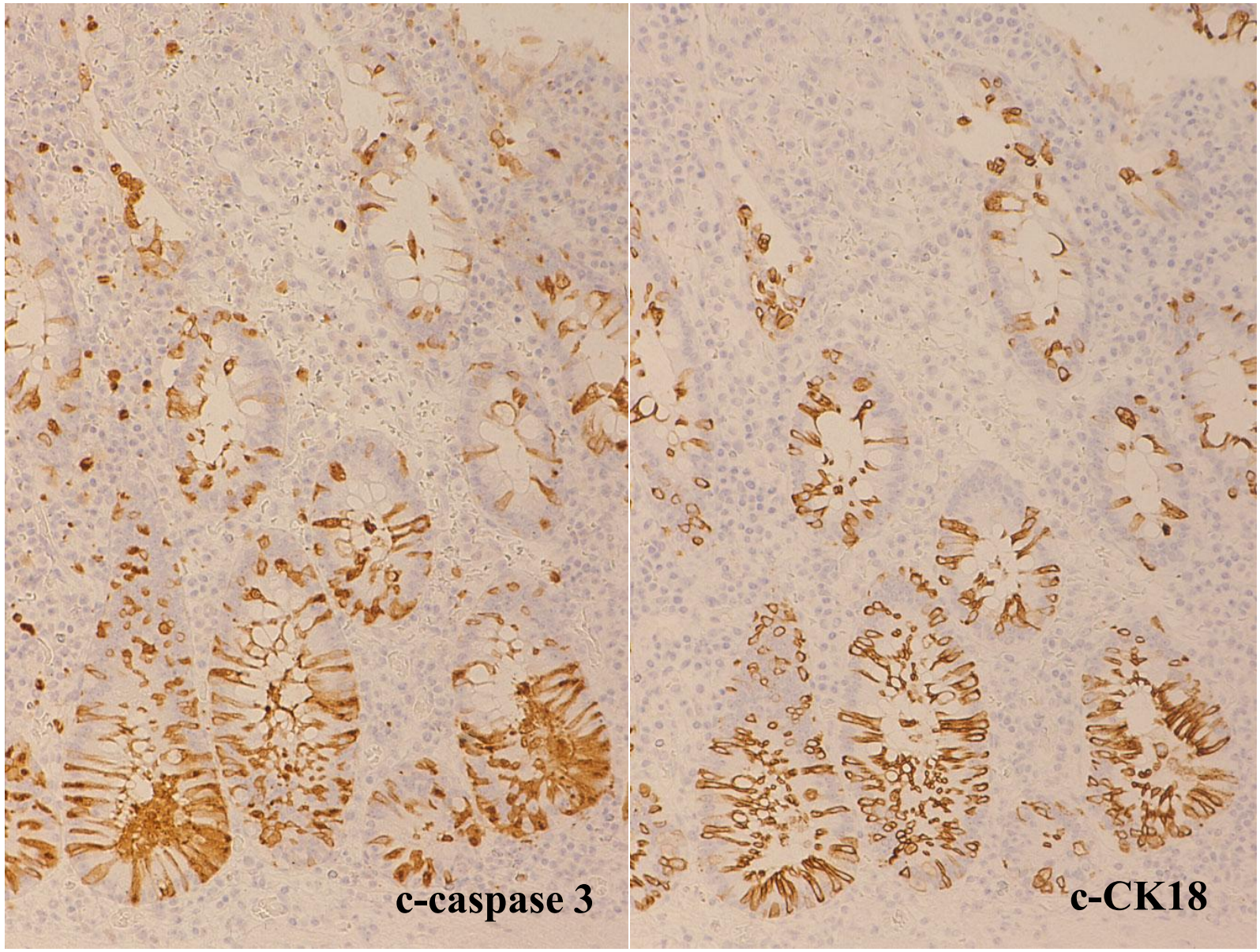
	120℃ heating	*60℃ heating*	*DNase*	*Proteinases*
TUNEL method	sensitized	～	all nuclei	PK needed *
ISNT method	all nuclei	sensitized@	all nuclei	PK needed
ssDNA	all nuclei	sensitized	sensitized	sensitized
cleaved proteins	mandatory	ineffective	～	antigenic loss
PK = protenase K、＊unnecessary when heated、@unstable (not reproducible)				



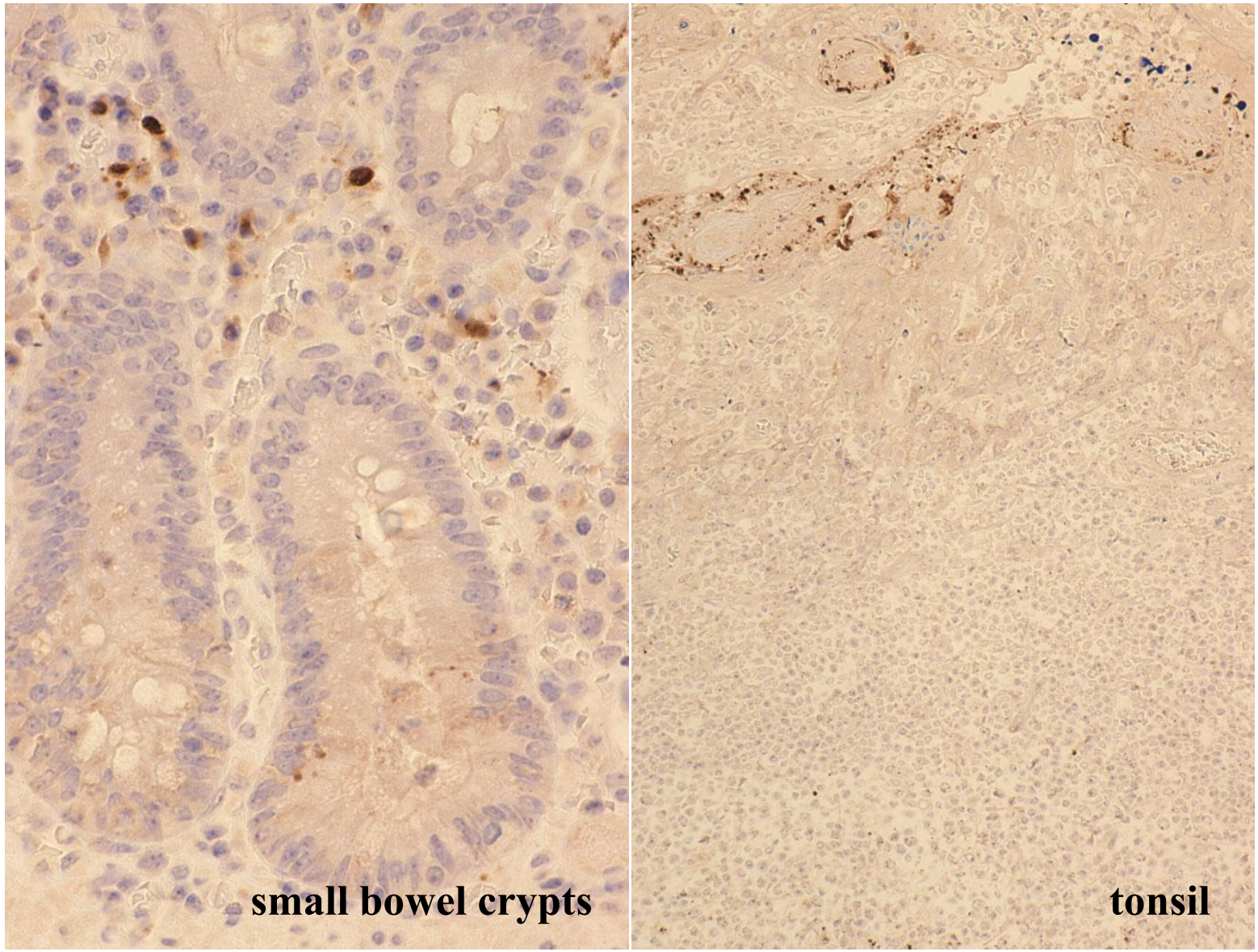
Comparison between cleaved caspase 3 (top) and cleaved caspase 6 (bottom) in colon cancer (left) and necrotizing lymphadenitis (right)



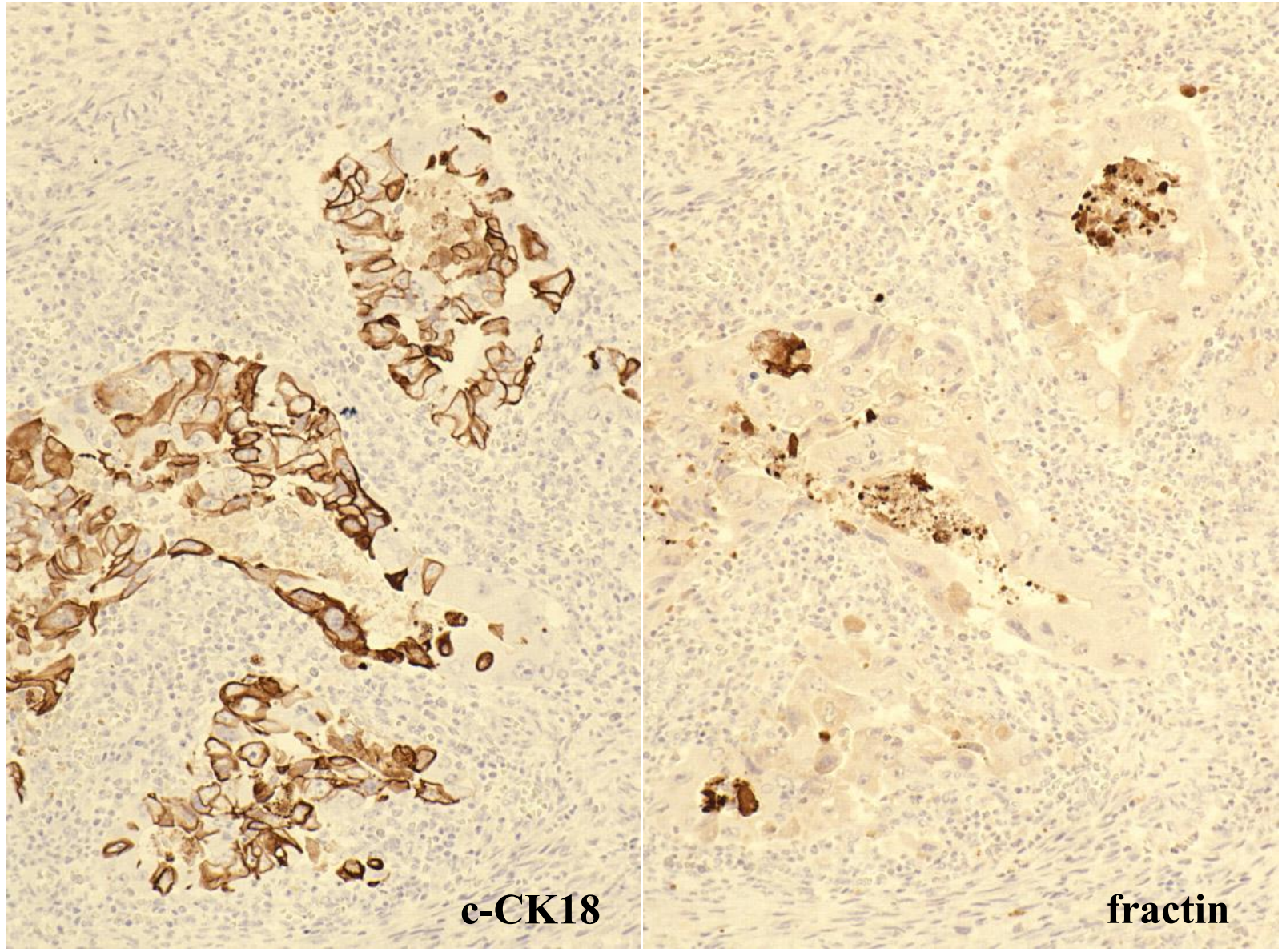
Paradoxical apoptosis in the small intestinal crypts (1). TUNEL-positive apoptotic cells are clustered in the crypt. ISNT is negative.



Paradoxical apoptosis in the small intestinal crypts (2). Apoptotic cells in the crypt are clearly visualized by immunostaining for c-caspase 3 and c-CK18.

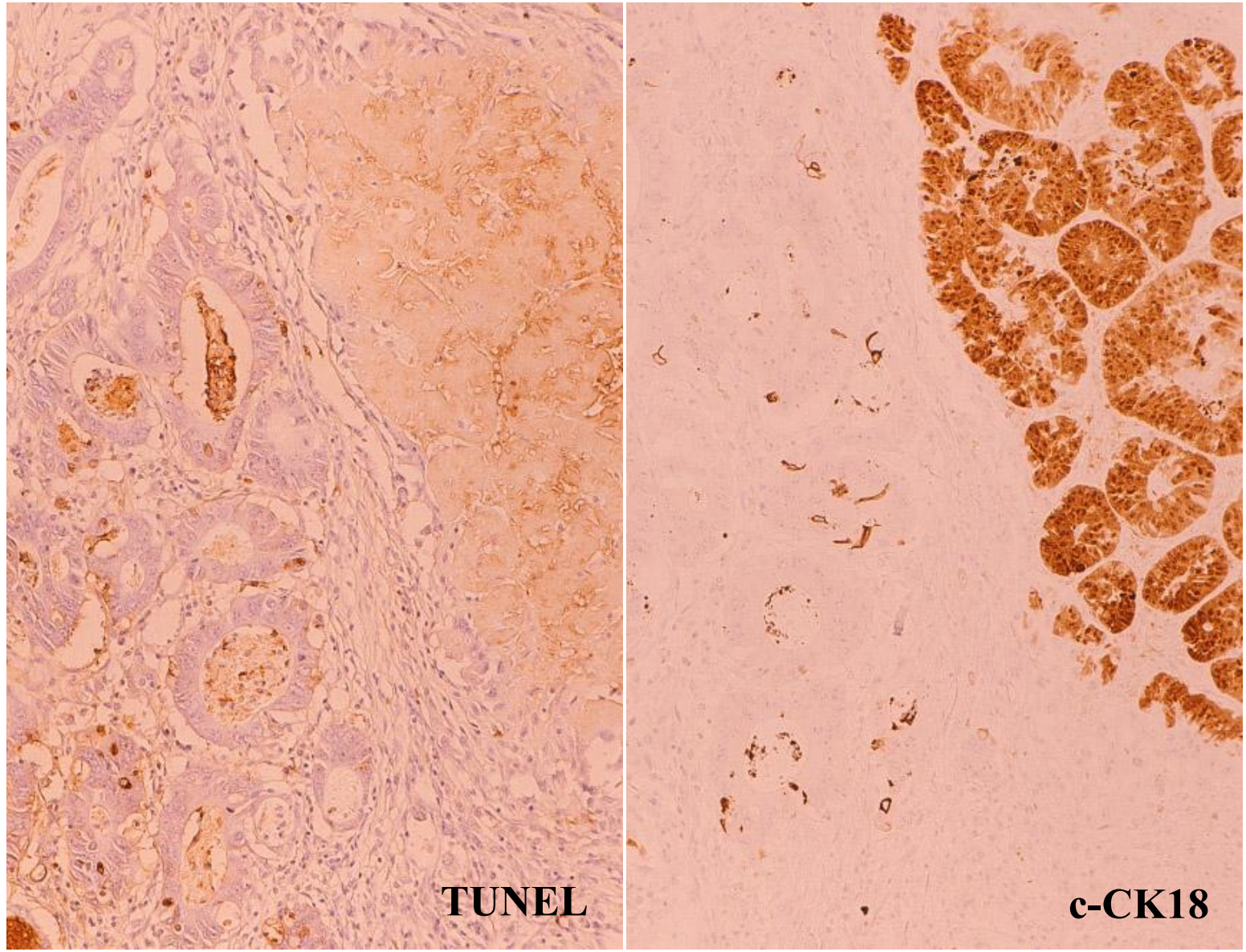


Immunostaining for cleaved actin (fractin) in the small intestinal crypts showing paradoxical apoptosis and palatine tonsil. Fractin is expressed in apoptotic cells of mesenchymal cell origin (mainly inflammatory cells). Epithelial apoptosis is not reactive.

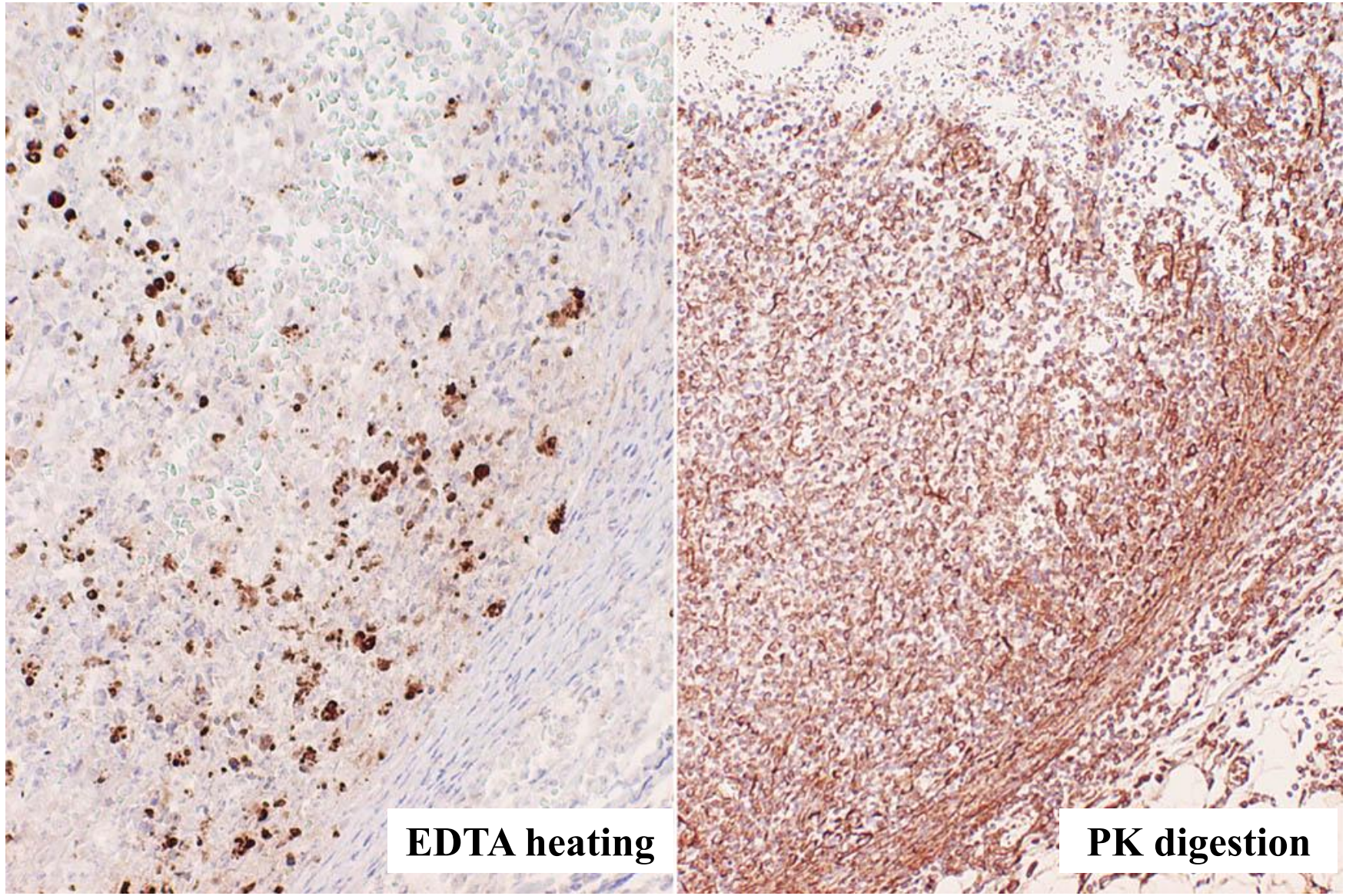


Detection of apoptosis in resected colon cancer after preoperative chemotherapy. Immunostaining for cleaved CK18 demonstrates apoptotic cancer cells (left), while cleaved actin (fractin) is reactive in apoptotic inflammatory cells in the cancerous gland (right).

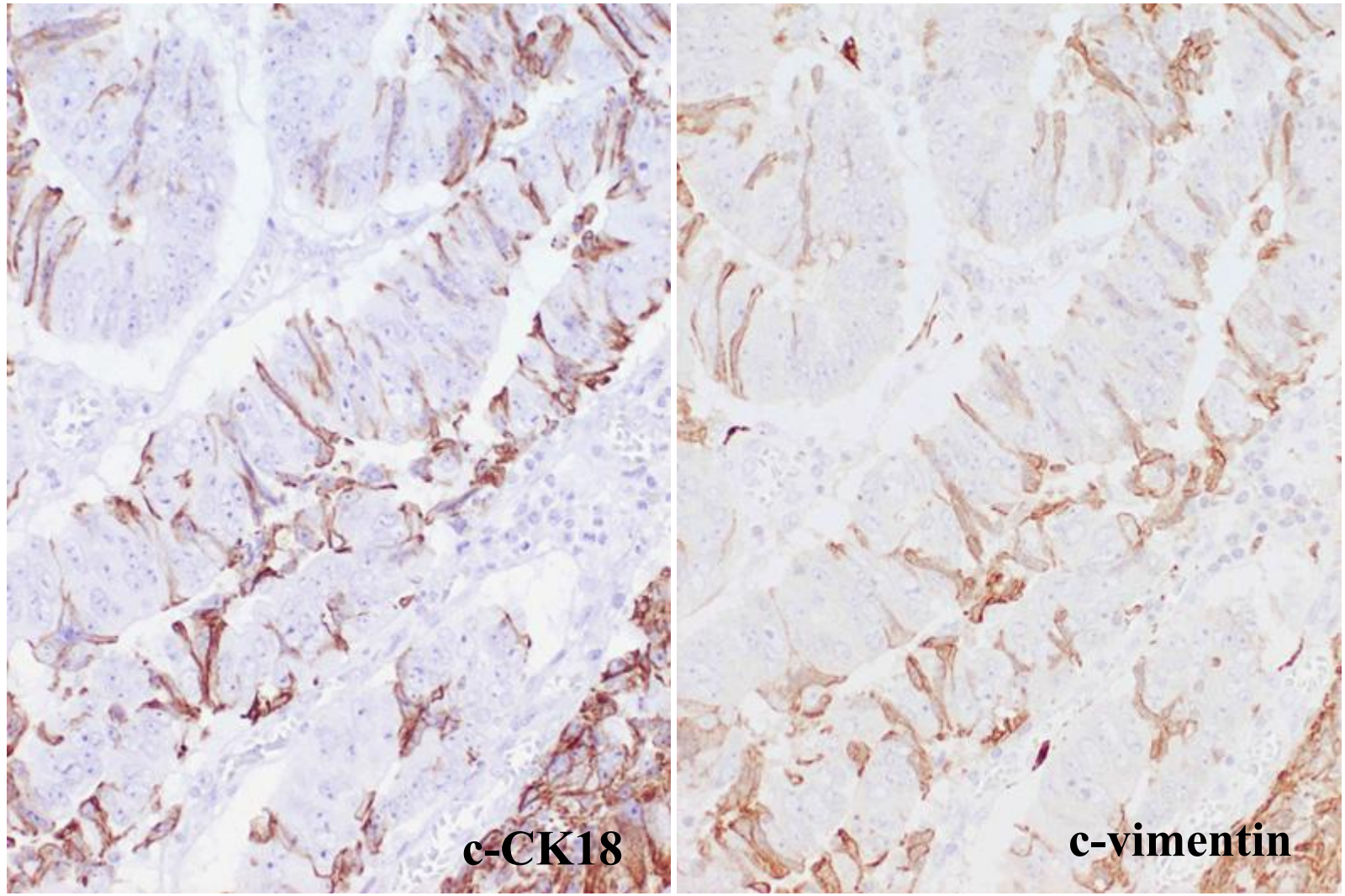
Colon cancer (coagulation necrosis)



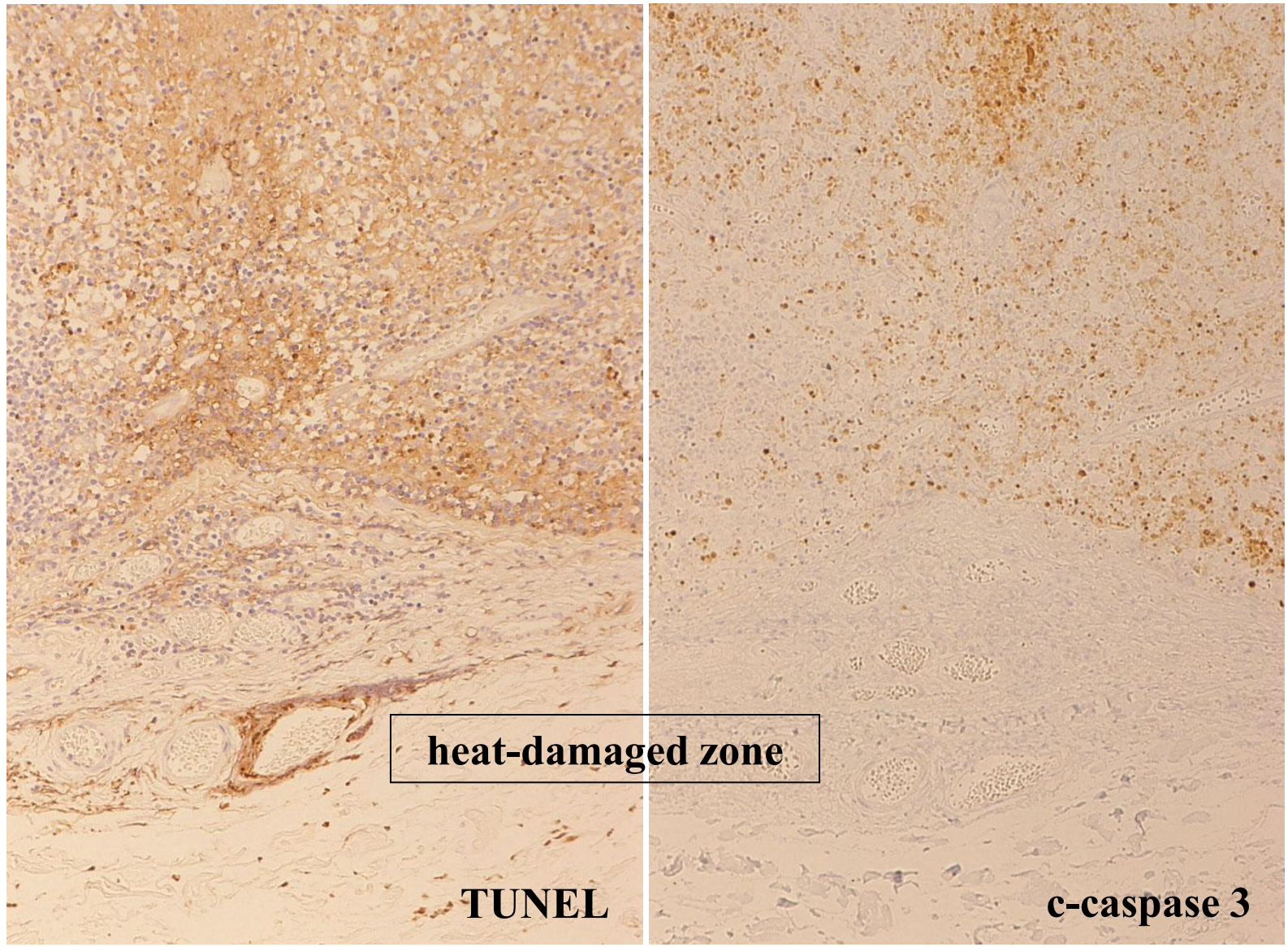
Detection of apoptosis in colon cancer with focal coagulation necrosis. TUNEL (left) and Immunostaining for cleaved CK18 (rigtht) demonstrate apoptotic cancer cells. Of note is that the necrotic cancer cells are difusely immunostained for c-CK18.



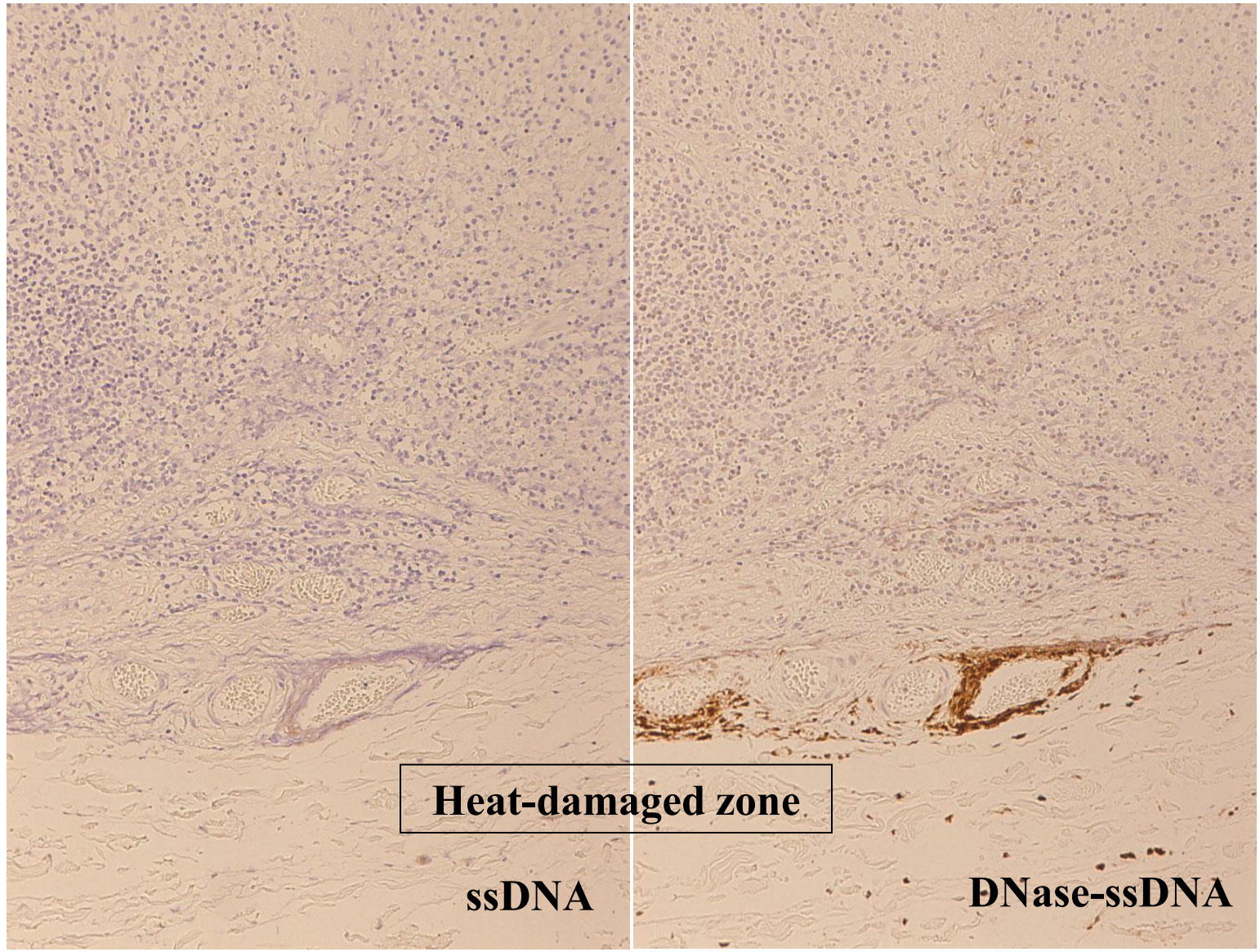
Immunostaining for cleaved vimentin in necrotizing lymphadenitis. Heating in EDTA, pH 8, is effective to detect apoptotic cells, but proteinase K (PK) digestion results in diffuse nonspecific staining in the cytoplasm.



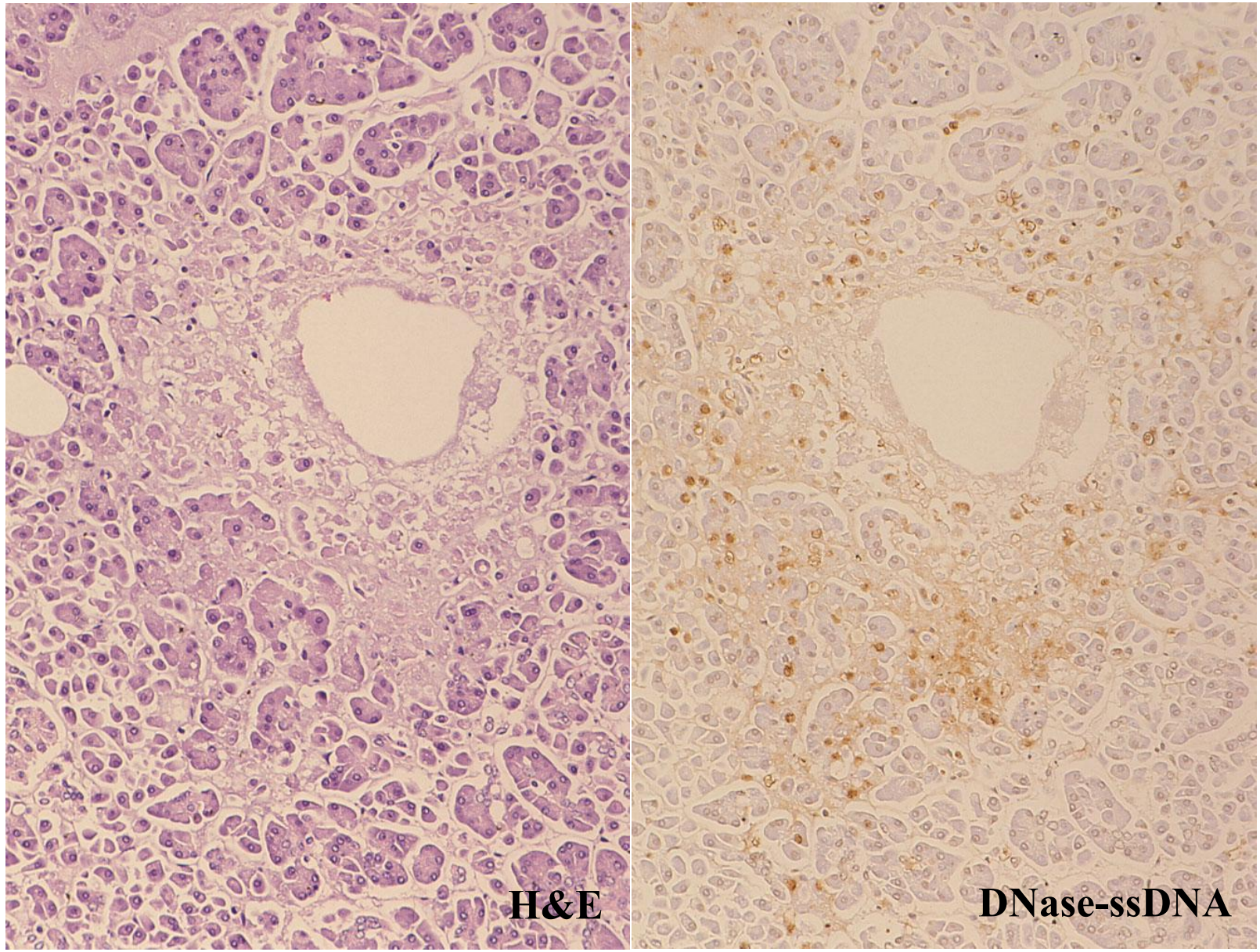
Nonspecific reaction of cleaved vimentin in colon cancer. Immunoreactivity of cleaved vimentin is expressed in vimentin-negative cancer cells (right), suggesting the cross-reactivity to cleaved cytokeratin 18 (left).



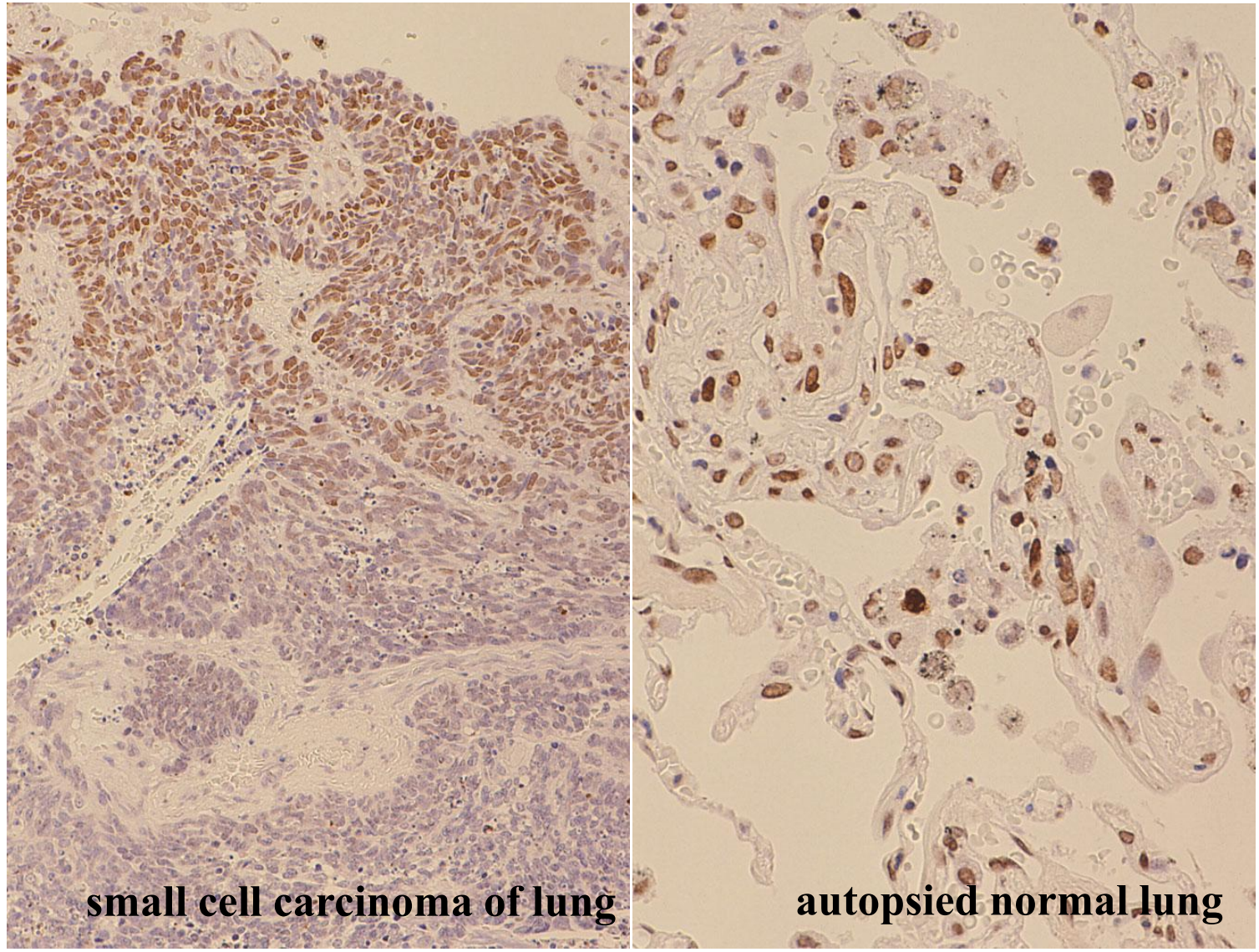
TUNEL and Immunostaining for cleaved caspase 3 in necrotizing lymphadenitis with a heat-damaged area at the periphery. Heat-damaged DNA shows nonspecific reactivity with TUNEL, while cleaved caspase 3 remains negative.



Immunostaining for ssDNA in necrotizing lymphadenitis with a heat-damaged area at the periphery. Heat-damaged DNA shows nonspecific immunoreactivity for ssDNA after DNase treatment, while untreated section remains unreactive.



Immunostaining for ssDNA in autopsied pancreas with autolytic change.
The autolytic cells reveal immunoreactivity for ssDNA after DNase treatment.



Immunostaining for ssDNA in autopsied small cell lung carcinoma and normal lung. Formalin fixation provokes fragmentation of DNA to yield ssDNA at the fragmented ends, causing “nonspecific or artificial” ssDNA immunoreactivity detected in certain cells, as shown here.

Representative artifacts in histochemistry for apoptosis

1) False negativity, probably due to overfixation

2) False positivity, particularly ssDNA, in overfixed areas at the periphery of tissues or in normal tissues

3) Heat-damaged tissue (Heat degeneration of DNA) showing false positivity with TUNEL and ISNT methods and ssDNA immunostaining after DNase treatment

4) ssDNA positivity in autolytic areas

5) Nonspecific (cross-reactive) immunostaining for cleaved vimentin.

Inconsistencies between methodologies

1) Germinal center of the palatine tonsil:
   Positive for TUNEL, ISNT, immunostaining for ssDNA and c-caspase 3, but negative for cleaved actin (fractin)
2) Paradoxical apoptosis in small bowel crypts:
   Positive with TUNEL and ssDNA, but negative with ISNT
   Positive for c-caspase 3, c-CK18、but negative for fractin
3) Coagulation necrosis in colon cancer:
   Positive for c-CK18 but negative with TUNEL and c-caspase 3
4) Caseous necrosis in tuberculosis:
   Positive with TUNEL and ssDNA, but negative for c-caspase 3
5) Cytoplasm of macrophages engulfing apoptotic bodies:
   Positive with TUNEL and c-caspase 3, but negative with ISNT and ssDNA

Comments

1) Apoptosis markers may also be positive in necrosis.

2) Reactivity patterns vary in different kinds of necrosis, such as comedo necrosis, coagulation necrosis and liquefaction necrosis.

3) More cells are labeled for cleaved caspase 3 and cleaved CK18 (in epithelial cells) than TUNEL、ISNT and ssDNA. Under ideal conditions, labeled cells by TUNEL method are comparable with those for cleaved caspase 3 and cleaved CK18.

4) Cleaved actin (fractin) is expressed in apoptotic non-epithelial cells.

5) Heat-retrieved cleaved vimentin immunoreactivity is cross-reactive with cytokeratin. Proteinase treatment destroys the specificity.

6) Heat-assisted immunostaining for cleaved proteins is convenient, stable and reproducible.

7) Distribution of stained markers is infrequently concordant.