Supplementary Materials and methods

Pathological staining processing. The specimen was fixed in 10% neutral buffered formalin at room temperature for a period ranging from 1 to 3 days. Subsequently, the DiaPath Donatello automated processor (Diapath S.P.A) was employed to progress through the following stages: i) The specimen was held in 10% neutral buffered formalin (with variable time, but an average of 20 min); ii) a 10-min bath in deionized water was used; iii) dehydration using alcohol was performed, commencing with a concentration of 70% (1 h), then proceeding to 95% (1 h), 99% (1 h and 30 min) and another station at 99% (1 h and 30 min); iv) clearing was achieved through three stations of xylene (each station lasting 1 h, totaling 3 h); and v) infiltration with paraffin wax in three stations (each station for 1 h, totaling 3 h).

Following this, the specimen blocks were embedded in paraffin wax using the Sakura Tissue-Tek embedding center (Sakura Finetek Europe B.V.). Subsequently, the blocks were faced and tissue sections were obtained using the Sakura Accu-Cut SRM microtome (Sakura Finetek Europe B.V.). These sections were then floated in a Sakura 1451 water bath (Sakura Finetek Europe B.V.) maintained at a temperature between 40-50˚C and then placed onto regular glass slides. The glass slides were then incubated in an oven, typically at a temperature range of 60-70˚C, overnight.

Hematoxylin and eosin staining. On the following day, the slides underwent staining with hematoxylin and eosin using the DiaPath Giotto autostainer (Diapath S.P.A), which encompassed the following steps: i) The slides were subjected to xylene in three successive stations (7, 7 and 5 min); ii) slides were submersed in alcohol at a concentration of 100% for three stations (7, 6 and 5 min), followed by alcohol at 90% (4 min) and 70% (3 min), and then tap water (2 min); iii) hematoxylin Gill II staining was performed for 8 min, which was prepared from Sigma-Aldrich Hematoxylin Natural Black 1 (MilliporeSigma); iv) slides were rinsed in tap water (4 min), followed by ammonia water (1 min) and then rinsed again in tap water (1 min), and finally, immersed in 70% alcohol (2 min); v) slides were stained with eosin for 5 min, which was prepared from Sigma-Aldrich Eosin Y disodium salt (MilliporeSigma), followed by a final rinse in tap water (1 min); vi) gradual dehydration in alcohol was performed, starting at a concentration of 70% (15 sec), then 90% (2 min) and ending with 100% alcohol in three stations (3, 3 and 4 min); and vii) the slides were cleared in xylene using three stations (3, 5 and 4 min). Subsequently, the slides were allowed to air dry for 5 min, after which they were covered with the SurgiPath Sub-X mounting medium (Surgipath Medical Industries, Inc.) and a coverslip was applied.

Immunohistochemical (IHC) staining process. The IHC process involved the following steps: i) Paraffin-embedded tissue blocks were cut into sections 0.4 mm in width using a microtome and placed in a water bath, similar to routine processing. However, these sections were affixed onto glass slides with a positive charge; ii) the slides were placed in an oven overnight, maintaining a temperature of 60-65˚C; iii) the next day, the slides underwent antigen retrieval by boiling using the Dako PT Link (Agilent Technologies, Inc.). Each slide was immersed in a solution with a pH of either 6 or 9 (both Dako Envision FLEX target retrieval solutions; Agilent Technologies, Inc.), depending on the specific requirements of the target antibody. The device gradually heated the slides from 30˚C to boiling point at 100˚C for 45 min, then was cooled down to 65˚C, resulting in a total operation time of 2 h; iv) after antigen retrieval, the slides were washed twice for 3 min each, using Dako Wash Buffer solution (Agilent Technologies, Inc.), followed by welling using the Dako Pen (Agilent Technologies, Inc.); v) to block endogenous peroxidase, the slides were immersed in 5% hydrogen peroxide at room temperature for 7-10 min, then washed twice for 3 min each in the same buffer solution as before; vi) the slides were covered with an estrogen receptor primary antibody (clone EP1; cat. no. 11496833; read-to-use; Dako; Agilent Technologies, Inc.), and incubated at room temperature for 45 min. Subsequently, the slides were washed twice for 3 min each using the buffer solution; vii) next, the slides were covered with the secondary antibody [EnVision FLEX+, Mouse, High pH (Link); cat. no. 41355654; ready-to-use; Agilent Technologies, Inc.] for 45 min, followed by two 3-min washes with the buffer solution; viii) diaminobenzidine chromogen was applied for 5-10 min, and the slides were rinsed with running water for 2 min; ix) counterstaining was performed with hematoxylin Gill II for 2-5 min at room temperature, followed by immersion in alcohol (successive concentrations of 70, 90 and 100%) and xylene (two stations), with each station taking 5-10 min; and x) finally, the slides were covered with mounting medium and a coverslip, similar to the process used for hematoxylin and eosin staining.

The Allred system (1) was applied by adding the intensity of the stain to the proportion of the positive cells. Signal intensities were scored as follows: 0, no stain; 1, weak; 2, moderate; and 3, strong. The proportion of positive tumor cell nuclei was scored as: 0, none; 1, <1%; 2, 1-10%; 3, 11-33%; 4, 34-66%; 5, 67-100%. Scores of ≥3 were regarded as positive using light microscopy (Leica DM750; hematoxylin and eosin; x100 magnification).

Reference

1. Hammond ME, Hayes DF, Dowsett M, Allred DC, Hagerty KL, Badve S, Fitzgibbons PL, Francis G, Goldstein NS, Hayes M and Hicks DG: American Society of Clinical Oncology/College of American Pathologists guideline recommendations for immunohistochemical testing of estrogen and progesterone receptors in breast cancer (unabridged version). Archives of pathology & laboratory medicine. 2010 Jul 1;134(7):e48-72.