TMA construction and immunohistochemistry

Three tissue cores of 0.6-mm from 285 clinically annotated laryngeal tumors were arrayed in five tissue microarrays (TMAs) with the use of a manual arrayer (Model I, Beecher Instruments, San Prairie, WI, USA). Each TMA block contained 63–342 tissue cores from the original tumour tissue blocks, while 12 cores in each TMA from various neoplastic, non-neoplastic and reactive tissues were also included, serving as assay controls. Serial 2.5-μm thick sections from the TMA blocks were cut at the Laboratory of Molecular Oncology of the Hellenic Foundation of Cancer Research, School of Medicine, Aristotle University of Thessaloniki, Greece, mounted on adhesive microscope slides and subjected to IHC labelling using Bond Max™, Bond III (Leica Microsystems, Germany) and i6000 (Biogenex, San Ramon, CA, USA) autostainers. The sections were stained with antibodies against: NOTCH1 [Anti-activated NOTCH1 (ab8925), Abcam, Cambridge, UK, at 1:200 dilution for 30 min], NOTCH2 [Anti-activated NOTCH2 (ab72803), Abcam, at 1:100 dilution for 30 min], NOTCH3 [Anti-NOTCH3 (ab23426), Abcam, at 1:500 dilution for 30 min], and JAGGED1 (Anti-JAG1, HPA021555, Sigma-Aldrich, St. Louis, MO, overnight at 1:125 dilution]. The NOTCH1 antibody recognizes the cleaved intracellular (activated) form, the NOTCH2 antibody detects the endogenous levels of fragment of activated NOTCH2 resulting from cleavage adjacent to Ala1734, while the NOTCH3 antibody spans an epitope from the residues 2300 to the C-terminus of the human NOTCH3 molecule. The antigen retrieval was performed using the Bond™ Epitope Retrieval Solution 1 at 98^0C for 20 minutes for all antibodies. Binding of antibodies was visualized using Envision (Dako, Glostrup, DK) for NOTCH1 and Bond Polymer Refine Detection (Leica Biosystems) for the remaining antibodies. DAB (3,3-diaminobenzidine) was used as a chromogen and hematoxylin as a counterstain. The quality of IHC staining was evaluated using the internal positive controls. The evaluation of immunostains was based on a semiquantitative immunoreactive score (IRS), which combines the staining intensity (SI) (ranging from 0: no staining to 3: strongly positive) and the percentage of immunoreactive tumour cells (PP) (0 = 0–5%, 1 = 6–25%, 2 = 26–50%, 3 = 51–100%). The

IRS was calculated as follows: IRS = SI x PP, for each sample, as previously described (1, 2), thus ranging from 0 to 9. The localization of the staining for each protein was also indicated as cytoplasmic, nuclear or membranous. Evaluation of the stained slides was performed by a pathologist (M.B.), who was blinded as to the patients' clinical characteristics and survival data.

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