RNA extraction and RT-PCR methodology

In total, 264 tissue samples were available for RNA extraction, which was performed on macrodissected tissue fragment lysates (overnight digestion with proteinase K) containing >50% tumor cells with Trizol-LS (Invitrogen/Life Technologies, Paisley, UK) according to the instructions of the manufacturer. RNA samples (1-3 µg) were immediately processed for reverse transcription with random primers and the Superscript III system (Life Technologies). cDNA samples were normalized at 25 ng/µl and stored at -20^{o}C until use. Relative mRNA expression was assessed with qPCR and hydrolysis probes by using premade single tube TaqMan® Gene Expression Assays (Applied Biosystems/Life Technologies) in an ABI7900HT system under default conditions for the following targets (in parentheses: assay ID; Genbank reference; amplicon location; size): *NOTCH1* (Hs00413187_m1, NM_017617.3, exons 4–5, 95 bp), *NOTCH2* (Hs00225747_m1, NM_024408.3, exons 23–24, 73 bp), *NOTCH3* (Hs00166432_m1, NM_000435.2, exons 16–17, 87 bp), *NOTCH4* (Hs00270200_m1, NM_004557.3, exons 18–19, 108 bp). Samples were tested in 10 µl reactions (50 ng template/reaction) with TaqMan® Universal PCR Master Mix and were run in duplicates in 384-well plates. A commercially available reference RNA (TaqMan® Control Total RNA, cat. no 4307281; Applied Biosystems/Life Technologies) was used as a positive control in each run. As an endogenous control and for the normalization of Cq (quantification cycle, which is synonymous to CT [cycle threshold]) values, an assay targeting GUSB mRNA (beta-glucuronidase [#4333767F]) was used. *GUSB* was preferred over usually applied endogenous controls because (a) no pseudogenes have as yet been reported for this gene, and (b) it has been identified as one among the best preserved mRNA targets in FFPE tissues (1). Relative quantification (RQ) was assessed in a linear mode as (40 – dCT) (2), whereby dCT = (avg CT target) – (avg CT *GUSB*). Assay inter-run variation (difference in dCTs for the reference RNA) was 0.94 (*NOTCH1*), 0.85 (*NOTCH2*), 1.5 (*NOTCH3*). *NOTCH4* was not expressed in the reference RNA; hence, only intra-duplicate stability was evaluated for this assay. Exclusion criteria for RQ analysis were *GUSB* CT values higher than 36 for each duplicate and

deltaCTs higher than 1 per duplicate pair. With the above criteria, 143 samples (54.2%) were found eligible for further analysis for *NOTCH1*, 148 (56.1%) for *NOTCH2*, 145 (54.9%) for *NOTCH3* and 144 (54.5%) for *NOTCH4*.

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