Supplementary Materials and Methods

Immunohistochemistry

Immunostaining was done with monoclonal antibodies directed against mucin 1 (clone MA695; dilution 1:100), mucin 2 (clone Ccp58; both Novocastra, Leica Microsystems GmbH, Wetzlar, Germany; 1:100), mucin 5 (clone 45M1; Thermo Scientific, Schwerte, Germany; 1:100), mucin 6 (clone CLH5; 1:100), CD10 (clone 56C6; both Novocastra; 1:10), E-cadherin (clone SPM471; ZYTOMED Systems GmbH, Berlin, Germany; 1:400), β-catenin (clone Cat-5H10; Life Technologies GmbH, Darmstadt, Germany; 1:300), and a polyclonal antibody directed against lysozyme (1:3000; DAKO, Glostrup, Denmark).

Antigen retrieval was done with ER1 (citrate buffer Bond pH 6.0; mucin 1, CD10) or ER2 (EDTA-buffer Bond pH 8.9; mucin 2, mucin 6, E-cadherin, β-catenin) according to the manufacturer`s instructions and using the autostainer Bond™ Max System (Leica Microsystems GmbH, Wetzlar, Germany). The immunoreaction was visualized with either the Bond™ Polymer Refine Detection Kit (DS 9800; brown labelling) or the Bond™ Polymer Refine Red Detection Kit (DS 9390; red labelling; both Novocastra). Epstein-Barr-Virus encoded RNA was detected using the EBER-probe (Novocastra) and the Bond Refine Detection system according to the manufacturer´s instructions (Leica Microsystems GmbH).

Evaluation of Immunostaining

Immunostaining of the TMAs was evaluated by applying an immunoreactivity scoring system (IRS). Briefly, category A documented the intensity of immunostaining as 0 (no immunostaining), 1 (weak), 2 (moderate), and 3 (strong). Category B documented the percentage of immunoreactive cells as 0 (no immunoreactive cells),

1 (few scattered immunoreactive cells, less than <1%), 2 (1-10%), 3 (11-50%), 4 (51-80%), and 5 (>80%). The addition of category A and B resulted in an IRS ranging from 0 to 8 for each individual case. For statistical analyses, the IRS was split at the median into negative and positive cases.

Mutational analysis

Mutational analyses of codons 12 and 13 of the *KRAS* and *NRAS* genes, codon 600 of the *BRAF* gene and mutational hotspots in exons 9 and 20 of the *PIK3CA* gene were performed by pyrosequencing on a PyroMark Q24 instrument (QIAGEN, Hilden, Germany). Fragments of the different genes were amplified by PCR with following primers: *KRAS* (codon 12/13), forward 5´-GGCCTGCTGAAAATGACTGAA-3´ and reverse 5´-TTAGCTGTATCGTCAAGGCACTCT-3´;[1] *NRAS* (codon 12/13), forward 5´-CTTGCTGGTGTGAAATGACTG-3´ and reverse 5´-TTCTGGATTAGCTGGATTGTCAGT-3´; *BRAF* (codon 600), forward 5´-TGAAGACCTCACAGTAAAAATAGG-3´ and reverse 5´-TCCAGACAACTGTTCAAACTGAT-3´; *PIK3CA* exon 9, forward 5´-AACAGCTCAAAGCAATTTCTACACG-3´ and reverse 5´-ACCTGTGACTCCATAGAAAATCTTT-3´; and *PIK3CA* exon 20, forward 5´-CAAGAGGCTTTGGAGTATTTCA-3´ and reverse 5´-CAATCCATTTTTGTTGTCCA-3´.[2] The resulting PCR products were analyzed by agarose electrophoresis and sequenced using following sequencing primers: *KRAS* (codon 12/13), 5´-TGTGGTAGTTGGAGCT-3´; *NRAS* (codon12/13), 5´-GTGGTGGTTGGAGCA-3`; *BRAF* (codon 600), 5´-GTAAAAATAGGTGATTTTGG-3´; *PIK3CA* exon 9, 5´-TAGAAAATCTTTCTCCTGCT-3´ and 5´-TTCTCCTGCTCAGTGATTT-3´; and *PIK3CA* exon 20, 5´-GTTGTCCAGCCACCA-3´.

Microsatellite Instability Assay

Microsatellite instability (MSI) was determined by comparison of the allelic profiles of the mononucleotide repeat markers BAT-25, BAT-26, NR-21, NR-24, and NR-27 in tumour and corresponding normal tissue.[3] All markers were coamplified in a pentaplex PCR assay with the QIAGEN Multiplex PCR Master Mix (QIAGEN, Hilden, Germany) following the manufacturer´s recommendations for amplification of microsatellite loci. The amplified loci were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems, Darmstadt, Germany). Samples were judged as microsatellite unstable (MSI-H) when the tumour showed instability in at least two of the five (40%) microsatellites analyzed.

External quality assurance

The *KRAS* mutational assay, the microsatellite instability assay and the immunohistochemical evaluation of DNA-mismatch repair proteins (MSH2, MSH6, MLH1 and PMS2) were certified successfully by the quality assurance program of the German Society of Pathology and the *Bundesverband Deutscher Pathologen e.V*.

Detection of *Helicobacter pylori* by Histology and Polymerase Chain Reaction

Infection with *H. pylori* was evaluated histologically using the modified Giemsa-staining and PCR.[4] *H. pylori*-specific DNA sequences were detected by a PCR-based assay targeting the 16S rRNA gene of *H. pylori*.[5] In short, a 109 bp fragment of the 16S rRNA gene was amplified with the primers Hp1 (5´-CTGGAGAGACTAAGCCCTCC-3´) and Hp2 (5´-ATTACTGACGCTGATTGTGC-3´) following the PCR parameters described by Chisholm et al.[5] Amplification products were assessed by agarose gel electrophoresis.

References

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3. Buhard O, Cattaneo F, Wong YF, *et al.* Multipopulation analysis of polymorphisms in five mononucleotide repeats used to determine the microsatellite instability status of human tumors. *J Clin Oncol* 2006;24:241-51.
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