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Original contribution

SOX9 expression predicts relapse of stage II colon cancer patients☆,☆☆

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Summary The aim of this study was to investigate if the protein expression of sex-determining region y-box 9 (SOX9) in primary tumors could predict relapse of stage II colon cancer patients. One hundred forty-four patients with stage II primary colon cancer were retrospectively enrolled in the study. SOX9 expression was evaluated by immunohistochemistry, and mismatch repair status was assessed by both immunohistochemistry and promoter hypermethylation assay. High SOX9 expression at the invasive front was significantly associated with lower risk of relapse when including the SOX9 expression as a continuous variable (from low to high expression) in univariate (hazard ratio [HR], 0.73; 95% confidence interval [CI], 0.56-0.94; $P = .01$) and multivariate Cox proportional hazards analyses (HR, 0.75; 95% CI, 0.58-0.96; $P = .02$), adjusting for mismatch repair deficiency and histopathologic risk factors. Conversely, low SOX9 expression at the invasive front was significantly associated with high risk of relapse, when including SOX9 expression as a dichotomous variable, in univariate (HR, 2.32; 95% CI, 1.14-4.69; $P = .02$) and multivariate analyses (HR, 2.32; 95% CI, 1.14-4.69; $P = .02$), adjusting for histopathologic risk factors and mismatch repair deficiency. In conclusion, high levels of SOX9 of primary stage II colon tumors predict low risk of relapse, whereas low levels of SOX9 predict high risk of relapse. SOX9 may have an important value as a biomarker when evaluating risk of relapse for personalized treatment.

Abbreviations dMMR, mismatch repair deficiency; IHC, immunohistochemistry; MLH1, MutL homolog 1; MMR, mismatch repair; MSH2, MutS protein homolog 2; MSH6, MutS homolog 6; MSI, microsatellite instability; pMMR, mismatch repair proficient; PMS2, postmeiotic segregation increased 2; SOX9, sex-determining region y-box 9; TMA, tissue microarray

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1. Introduction

Colorectal cancer is one of the most frequent malignancies in the Western world [1]. Approximately one-fourth of patients with colon cancer are diagnosed as having stage II colon cancer [2]. Today, a minor group of patients with stage II colon cancer is offered adjuvant therapy based on high-risk histopathologic features (T4 stage, vein invasion, perineural invasion, margin involvement, number of sampled lymph nodes, perforation, and low differentiated histology) [2,3].

Despite proper surgical intervention, approximately 20% of all patients with stage II colon cancer have relapse of their cancer. Yet still no optimal biomarker has been established in the clinic to identify high-risk patients and predict relapse of stage II colon cancer. Thus, the incentive for novel prognostic and predictive markers to identify the patients, who most likely will benefit from additional treatment, is extensive.

One of the hallmarks of cancer is genomic instability [4]. MSI can occur as a consequence of dMMR [5]. Germline mutations in MMR genes are associated with the Lynch syndrome, whereas *MLH1* promoter hypermethylations are primarily found in sporadic colon cancer cases [6].

The cancer stem cell theory proposes that cancer stem cells are involved in initiation, progression, reoccurrence of cancer, and treatment response. SOX9 is a transcription factor involved in several developmental processes and is important for cell proliferation, senescence, and lineage commitment [7–10]. We hypothesized that there may be an association between the SOX9 expression in primary tumors of patients with stage II colon cancer and their risk of relapse. To test our hypothesis, we investigated the SOX9 expression by IHC in primary tumors of patients with stage II colon cancer.

2. Materials and methods

2.1. Patient cohort

The study was performed as a retrospective cohort study. Formalin-fixed, paraffin-embedded primary tumors from 144 patients diagnosed as having and treated for stage II colon cancer at Glostrup University Hospital, Gentofte University Hospital, and Herlev University Hospital in Denmark, were included consecutively from January 2005 to August 2008 using the national pathology registry of Denmark and patient medical records. The patients had undergone complete surgical resection as primary treatment. The inclusion period was based on the desire of a follow-up period of at least 5 years. Registered data and inclusion and exclusion criteria can be found in the Appendix. Enrollment and exclusion of patients is shown in Fig. 1. The study was approved by the Scientific Ethics Committee of the Capital Region of Denmark (H-1-2013-028) and by the Data Protection Agency of the Capital Region of Denmark (2007-58-0015).

2.2. Tumor tissue

The tumor tissue had been processed as part of the diagnostic routine after curative surgery. The tumor tissue was fixated in 10% neutral-buffered formalin for at least 48 hours prior to paraffin embedding. From each patient, 2 tissue blocks of the primary tumor were obtained. Full slides of the tumor were used for IHC against SOX9. TMAs were constructed with four 1-mm cores from each secondary tissue block as previously described [11]. A fifth 1-mm core in a tumor cell-enriched area (≥50% tumor cells) was punched for DNA extraction.

2.3. IHC analysis

All analyses and assays were conducted blinded to patient outcome.

2.3.1. SOX9 IHC and evaluation

Three-micrometer slides were cut and incubated for 45 minutes at 60°C. IHC was performed using the EnVision FLEX, High pH detection system (Dako, Glostrup, Denmark) and the automated Autostainer Link 48 (Dako Glostrup, Denmark) according to the manufacturer's instructions. Anti-SOX9 antibody (1:10 000; Merck Millipore, Darmstadt, Germany) was used for SOX9 detection. Specification of the antibody can be found in the Supplementary Table. The tissue slides were counterstained with Mayer hematoxylin using the automated slide stainer Tissue-Tek Prisma/Film (Sakura, Copenhagen, Denmark). Finally, the slides were scanned using the Nanozoomer 2.0-HT (Hamamatsu, Herrschin, Germany).

The stability of the SOX9 antigen was evaluated by staining normal colon tissue that had been subjected to formalin fixation for 3, 27, 51, and 123 hours. A control slide was included for each run and consisted of normal tissue from the colon, small intestine, testis, ventricle, and breast. The selection of the control tissues was based on previous reports of SOX9 staining [12–17].

SOX9 expression was evaluated at the invasive front of the tumor and at the luminal surface independently by a specialized pathologist and a trained molecular biologist supervised by a specialized pathologist. We defined the invasive front as the area where the tumor periphery invades deepest into the colonic tissue. The luminal surface refers to the luminal surface of the neoplastic glands.

Five random areas were selected for each region, using the image analysis software Visiopharm Integrator System (version 4.5.6.516; Visiopharm, Hørsholm, Denmark). Percent positive and negative tumor nuclei were counted and given a score: score 0 (0-5% positive nuclei), score 1 (>5%-25% positive nuclei), score 2 (>25%-50% positive nuclei), score 3 (>50%-75% positive nuclei), and score 4 (>75% positive