Cell-cycle and apoptosis regulators (p16$^{INK4A}$, p21$^{CIP1}$, β-catenin, survivin, and hTERT) and morphometry-defined MPECs predict metachronous cancer development in colorectal adenoma patients

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Abstract. Background and aims: Although adenomas may be precursors to colorectal cancers (CRC), knowledge concerning the development of metachronous CRC is scarce. We assessed whether differential expression of cell-cycle and apoptosis-regulating proteins and a monotonous population of elongated cells (MPECs) in colorectal adenomas could predict metachronous CRC.

Methods: Application of immunohistochemistry on tissue microarrays in consecutive, population-based colorectal adenomas. Influence of classic features (e.g., intraepithelial neoplasia grade, histological type, size) was examined.

Results: Of 171 patients with colorectal adenoma 86% (n = 147) were eligible for study; 10 (7%) developed metachronous CRC. Median time to cancer was 69 months (range, 25–256). Median follow-up was equal for the non-cancer and cancer groups. Elevated expression of cell-cycle regulators p16$^{INK4A}$, p21$^{CIP1}$, and cytoplasmic/nuclear β-catenin correlated with increased CRC risk (all $P<0.0001$), as did elevated expression of the anti-apoptosis protein survivin ($P<0.0001$) and human telomerase reverse transcriptase (hTERT; $P<0.001$). Survivin, hTERT, and nuclear β-catenin were the most predictive molecular markers (hazard ratios [HRs]: 6.3, 9.4, and 5.8, respectively). In a combined multivariate model, MPECs had the best overall prognostic ability (HR 28.2, 95% CI: 3.6–223.0), together with survivin, and hTERT. Within adenomas containing MPECs, several molecular markers further defined high-risk patients.

Conclusions: Among several markers predictive for metachronous CRC development in colorectal adenomas, MPECs, survivin and hTERT may, when validated, provide information superior to conventional histology, with relevance for the clinical management of patients with colorectal adenoma.

Keywords: Colorectal, adenoma, immunohistochemistry, cell cycle, apoptosis, metachronous cancer, molecular marker, diagnosis

1. Introduction

Colorectal cancer (CRC) is the second most frequent cause of cancer-related deaths in the Western world [60]. Although there have been advances, developments in diagnosis, surgical technique, systemic therapy, and post-operative follow-up have only moderately increased survival over the past decades [1,30, 31,36], and still almost 50% of patients are expected to die from CRC within five years of diagnosis. Thus, early detection and treatment of pre-invasive neoplastic lesions is essential to reduce mortality [61,62].

Colorectal adenoma (CRA) is a risk factor and a surrogate endpoint biomarker for CRC as carcinoma de-
velops from adenomas [26,27,53]. CRAs can be identified and removed endoscopically, and polypectomy with subsequent surveillance is the cornerstone of CRC prevention [2,24,61,62]. Yet despite their relatively common occurrence, the majority of adenomas are not associated with later occurrence of invasive cancer. Post-polypectomy surveillance yields only one cancer per 300–350 colonoscopies [45]; thus, identifying patients with high-risk adenoma features is a priority and would obviously contribute to a more efficient and focused surveillance for metachronous CRC development, even with potential for chemoprevention [27,41].

Clinicopathologic research on CRA has mainly focused on features associated with risk for synchronous CRC (cancer present in the index adenoma, or detected <12 months after detection/polypectomy) [40], whereas development of metachronous CRC (⩾24 months after index adenoma; ⩾5 cm from index location) has received considerably less attention. A major goal then, rather than detecting an already developed CRC, is to detect patients whose adenomas are associated with a high risk of developing long-term, metachronous CRC. Given increased surveillance of these patients, this may prevent cancer from developing in the first place. Clinicopathological adenoma characteristics such as histological type, size, and number of adenomas are associated with subsequent synchronous cancer development and its detection, with intraepithelial neoplasia (IEN) grade among the strongest prognosticators [2,5,9,35,42]. Studies suggest that CRC is typically diagnosed 10–15 years after adenoma detection [2,32]. This temporal lag represents a compelling opportunity and rationale for intervention. However, very few studies have addressed the adenoma features associated with the long-term risk of developing CRC, in particular with respect to associated molecular risk factors.

Recently, in a population-based series of CRAs with long-term follow-up of up to 20 years, we found that a quantitatively defined monotonous population of elongated cells (MPECs) within the adenomas overshadowed the diagnostic abilities of conventional features such as histological type, IEN grade, adenoma size, and number of polyps [50]. This is in agreement with earlier studies [37–39]. In the present study, we further explored the predictive ability of molecular biomarkers in CRAs. Specifically, markers of cell cycle and apoptosis with established or potential role in CRC development and progression were included. Thus, the combination of biomarkers and morphologically-defined MPECs were investigated for the ability to predict metachronous CRC development in patients with CRA.

2. Materials and methods

2.1. Study population

The patients under study has been described in detail elsewhere [50]. Briefly, patients were accrued from a retrospective search of the electronic files (using relevant SNOMED codes) at the Department of Pathology, Stavanger University Hospital, which serve as the only primary center for a stable population of about 285,000. The study population consists of 171 consecutive, population-based patients with no prior history of hereditary, previous, or synchronous colorectal cancer. Synchronous CRC was defined as cancer present in the index adenoma or detected <12 months after detection or polypectomy [37], whereas metachronous CRC was defined as cancer found ⩾24 months after index adenoma and ⩾5 cm from the index location. All colorectal adenoma fulfilled the WHO 2002 criteria [17] and were diagnosed between 1978 and 1990, allowing for a potential long-term follow-up of 12 years (and up to 20 years), and no less than 24 months. Patients with known inflammatory bowel disease (Crohn’s disease or ulcerative colitis) or polyposis were excluded. Of the 171 patients previously described [50], 147 (86%) had enough material for further analysis with molecular immunohistochemistry markers.

Follow-up and therapeutic interventions that occurred between the index diagnosis and the end of the follow-up interval were not formally standardized, but representative of the practice at the time of diagnosis and follow-up period. Briefly, all patients having adenoma with high-grade IEN or polyps >2 cm received a second colonoscopy after 3 to 6 months; patients with small adenomas with low-grade IEN received a colonoscopy after 12 months. Thereafter, patients received a follow-up colonoscopy every two to three years for up to 10 years, with shorter intervals employed if new or more advanced adenomas were detected. In a few select cases, colon barium enema radiography was performed if colonoscopy alone could not establish a “healthy/clean colon” (i.e., no polyps or high-grade IEN). No other interventions or systemic treatment were given. Thus, there were no systematic differences in the follow-up and only a potential, but negligible difference in cancer risk with regard to the “missed adenoma” rate among the included patients. Further, the rather conservative criterion of at least a 24-month interval from detection of adenoma to metachronous CRC reduces the risk of erroneously including a missed synchronous cancer. All aspects of
this study were approved by the Regional Ethics Committee, the Norwegian Social Science Data Service, and the Norwegian Data Inspectorate.

2.2. Adenoma selection

The adenoma found at first colonoscopy was reviewed concerning type, grade, and quality for immunohistochemical assessment. If more than one adenoma was found during the colonoscopy session, the one with the highest degree of IEN and/or the most abnormal histological type (i.e. villous) at review was used. If two or more adenomas were present with the same grade and/or type, the largest adenoma was used. If the adenoma was too small or the biopsies were superficial, fragmented, or contained fixation artefacts unreliable for immunohistochemical determination, a second adenoma (if available) was used. Tissue was suitable or available for immunohistochemistry analysis in 147 patients (86%).

2.3. Quantitative digitalized image analysis

The methods of measurement have been described in detail previously [50]. Briefly, measurements were performed in the area of the adenoma with the highest degree of IEN (with a surface of at least 5 µm²) using a quantitative image analysis measurement system (QPRODIT, Leica™, Cambridge, UK) with a 63× microscope objective. The motorized scanning system allows selection and systematic random sampling of the full number of fields of vision (FoVs) required (from 250 up to 500) and point-weighted sampling and morphometric assessments of epithelial cell nuclei in each FoV. Measurements were performed without knowledge of the clinical outcome. Nuclear size and shape, stratification, and arrangement/sociology features were analyzed. Within the measurement area, selection was restricted to strips of dysplastic epithelium in longitudinally cut glandular epithelial crypts (excluding crypt bottoms; Fig. 1A), also referred to as the “proliferative zone” [44]. The MPECs are characterized by a monotonous population of closely packed, elongated cells with very little cytoplasm. Morphometrically, many cell and nuclear characteristics can be used to describe MPECs. A simple identification method is the longest axis of the nuclei, which was expressed as the variance in the connectivity between two nuclei, which is typically low in adenomas with MPECs (for details see [50]).

2.4. Construction of tissue microarray (TMA)

For TMA construction, we used H&E-stained slides from each block to define representative adenoma regions. One tissue cylinder with a diameter of 0.6 mm was punched from each adenoma from the area with the highest degree of IEN of each block and brought into a recipient paraffin block using a precision instrument (Beecher Instruments, Sun Prairie, WI) [29]. The punch biopsies thus reflect both the area with the highest degree of intraepithelial neoplasia and the area used to identify the MPECs (Fig. 1A–F). Each TMA block could hold up to 48 cylinders of different adenomas.

Orientation was secured via two 1.5-mm punches with lymph node material along the x and y axes in the lower right corner. Serial sections for each TMA block were used for immunohistochemical staining. To guarantee that all serial sections contained the original dysplastic area, the last serial section was checked for the original dysplastic lesion (“sandwich technique”).

2.5. Immunohistochemistry

The adenomas were routinely fixed in 4% buffered formaldehyde and embedded in paraffin. Paraffin sections 4 µm thick were mounted onto sialinized slides (Dako, Glostrup, Denmark, S3002) and dried overnight at 37°C followed by 1 hour at 60°C. The sections were deparaffinized in xylene and rehydrated in a graded series of alcohol solutions. Of the adenomas with metachronous cancer and from nine randomly selected non-cancer adenomas, 21 serial paraffin sections of the selected adenoma were mounted onto sialinized slides and stained for immunohistochemistry according to the protocol described below. Stains were assessed for intensity, distribution, and heterogeneity within the adenomas, before evaluation of the TMA slides.

Antigen retrieval and dilution of the antibodies was optimized before the study started. Antigen retrieval was performed by pressure cooking in 10 mM TRIS/1 mM EDTA (pH 9.0) for three minutes at full pressure and cooling for 15 minutes. Immunostaining was performed using an autostainer (DAKO, Glostrup, Denmark). TBS (S1968) was added at 0.05% and Tween 20 (pH 7.6) as the rinse buffer. Endogenous peroxidase activity was blocked by peroxidase blocking reagent S2001 (DAKO, Glostrup, Denmark) for 10 minutes, and the sections were incubated with the following monoclonal antibodies at the stated dilutions: p16 (clone 6H12), 1:25 (Novocastra, Newcastle upon Tyne, UK); p21 (clone 4D10),
Fig. 1. Examples of immunohistochemical analysis. A, example of a monotonous population of elongated cells (MPECs) (see [50]) (64× objective; MPECs, black arrows). B–F, representative cylinders from tissue microarrays (10× objective; hematoxylin counterstain) for (B) hTERT, (C) p21\(^{CIP1}\), (D) p16\(^{NK4A}\), (E) survivin and (F) β-catenin. The same markers are shown in G–K (40× objective) for β-catenin (G), p21\(^{CIP1}\) (H), p16\(^{NK4A}\) (I), survivin (J) and hTERT (K).
For β typically the 40 × tissue and identification of a sufficient number of ep-
crypts or parts of crypts (to allow orientation in the section had to show at least two transversely cut
cylinder sample was taken into account. A TMA cylin-
deric features, follow-up results, original histological
malignant tissue (due to fixation or cutting artifacts or IHC pre-
treatment), or too little or absent (epithelial) material. For each stain, an interobserver consensus score was
reached by taking the mean if the two different scores were less than 20 percentage points apart; oth-
ewise the slides were re-evaluated to reach a consensus. To assess the robustness and reproducibility of the
semi-quantitative assessments of the most important stains, blind re-evaluation of 10 re-cut and re-stained
randomly selected samples spread over the whole spectrum of positivity (i.e., between 0 and 100%) was per-
formed. The correlation was good for most features.

2.7. Statistical analysis

Statistical analysis was performed using SPSS version 11.0 (SPSS Inc., Chicago, USA). Development
of metachronous CRC was the endpoint. For patients without metachronous CRC, data were censored at the
time of death or at last contact. The continuous scores were discreted into positive/negative using previously
set thresholds (i.e., negative if <10% cells positive for Ki-67), or using the medians, tertiles, or quartiles when
needed. Where the survival results of neighboring sub-
groups were the same, these were grouped together
(i.e., the cases between the 25th–50th and 50th–75th percentiles) for survival analysis. Kaplan–Meier sur-
vival analysis was calculated for early and late differences by using the Breslow and log-rank methods, re-
spectively [43]. This was done in order to investigate the potential difference and minimize bias of early and
late clustering of events. Uni- and multivariate (Cox proportional hazards model) survival analyses were
used to evaluate classical and molecular features separ-
ately, with significance attributed when the P value
<0.05. A combined multivariate risk analysis was con-
ducted (Forward Wald) of all classical and molecular features with multivariate (Cox proportional hazards regres-
sion) analysis of markers with a P < 0.2 on univariate analysis and whose confidence interval (CI) could be calculated. Hazard ratios (HRs) are given with 95% CI.

3. Results

Ten (6.8%) of the 147 patients developed metachro-
nous CRC with a median follow-up of 144 months
(range, 25–256); median time to metachronous CRC
Clinicopathological demographics of 147 colorectal adenoma patients and Kaplan–Meier survival analysis of the early (Breslow) and late (log-rank) associated risk for metachronous colorectal cancer with hazard ratios (HR) and 95% confidence intervals (CI) for univariate and multivariate analyses (Cox proportional hazards model).

| No. patients | No. cancers | Mean follow-up, months (95% CI) | Breslow | Log-rank | P-value | Univariate | Multivariate |
|--------------|-------------|---------------------------------|---------|----------|---------|------------|-------------|
| Sex          |             |                                 |         |          |         |            |             |
| Female       | 72          | 4                               | 245 (233–256) | 0.6   | 0.55    | 0.7       | 0.2–2.4    |
| Male         | 75          | 6                               | 234 (219–250) |       |         |           |             |
| Age <60 yr   | 56          | 3                               | 242 (229–255) | 0.29  | 0.33    | 2.0       | 0.5–7.7    |
| ≥60 yr       | 91          | 7                               | 238 (225–251) |       |         |           |             |
| Location adenomas |        |                                 |         |          |         |            |             |
| Proximal     | 9           | 3                               | 160 (112–207) | 0.019 | <0.001  | 7.8       | 2.0–30.3   |
| Distal       | 138         | 7                               | 245 (237–253) |       |         |           |             |
| No. of adenomas |        |                                 |         |          |         |            |             |
| ≤2           | 134         | 7                               | 243 (233–254) | 0.002 | 0.006   | 5.5       | 1.4–21.4   |
| >2           | 13          | 3                               | 189 (139–239) |       |         | 7.1       | 1.7–28.8   |
| Size ≤2 cm   | 81          | 4                               | 243 (233–254) | 0.62  | 0.45    | 1.6       | 0.5–5.8    |
| >2 cm        | 66          | 6                               | 236 (220–252) |       |         |           |             |
| Histologic type |        |                                 |         |          |         |            |             |
| Tubular      | 112         | 6                               | 242 (229–254) | 0.16  | 0.42    | 1.8       | 0.7–4.6    |
| Tubulovillous | 30          | 3                               | 204 (184–224) |       |         |           |             |
| Villous      | 5           | 1                               | 182 (122–242) |       |         |           |             |
| IEN†         |             |                                 |         |          |         |            |             |
| Low grade    | 124         | 6                               | 243 (232–254) | 0.013 | 0.038   | 3.5       | 1.0–12.6   |
| High grade   | 23          | 4                               | 191 (163–219) |       |         |           |             |
| MPECs‡        |             |                                 |         |          |         |            |             |
| Absent       | 104         | 1                               | 254 (250–259) | 0.001 | <0.0001 | 22.1      | 2.8–174.8  |
| Present      | 43          | 9                               | 206 (178–234) |       |         | 24.5      | 2.8–174.8  |

* Result of multivariate analysis (Cox proportional hazards model) of variables with \( P < 0.05 \) on univariate analysis.
† IEN denotes intraepithelial neoplasia.
‡ MPECs denotes monotonous population of elongated cells.

was 69 months (range, 25–256), and median follow-up for patients without cancer was 146 (range, 25–256). The patient characteristics (Table 1) equaled the total population of 171 patients and were thus regarded as being representative of the whole population [50]. Of the classical adenoma features, proximal location, multiplicity of adenomas, IEN grade, and the MPECs feature were significant predictors of metachronous CRC development (Table 1). The MPEC feature was the strongest predictor compared to the other classical characteristics on multivariate analysis, only retaining MPECs and the number of adenomas in the model (HRs of 24.6 and 7.1, respectively). Metachronous CRC occurred disregarding the histological type (Table 1).

On univariate analysis, several of the molecular markers were also significantly associated with the development of metachronous CRC (Table 2), notably markers involved in cell cycle control (i.e., p16\(^{\text{INK4A}}\), p21\(^{\text{WAF1/CIP1}}\), nuclear and cytoplasmic \(\beta\)-catenin) and the anti-apoptosis regulators survivin and human telomerase reverse transcriptase (hTERT). Significant differences on univariate analysis remained stable for both early and late occurring events (Breslow vs. logrank on Kaplan–Meier analysis). The majority of adenomas showed some proliferative activity (>10% proliferating cells, as identified by Ki-67 positivity). The gatekeeper and apoptosis-related proteins p53 and bcl-2 were not significant, and they did not show an inverse relationship. COX-2 was positive in most adenomas but did not contribute as a predictor of cancer development; neither did the cell cycle mediators Rb and cyclin D, the minichromosomal maintenance pro-
Table 2
Kaplan–Meier survival analysis of molecular markers in colorectal adenoma associated with early (Breslow) and late (log-rank) metachronous cancer development, with hazard ratios (HR) and 95% confidence intervals (95% CI) on uni- and multivariate analyses

| Marker* | Threshold† | Events, n (%) | Kaplan–Meier survival | Univariate | Multivariate |
|---------|------------|---------------|-----------------------|------------|--------------|
|         |            |               | Breslow (P) | Log rank (P) | HR | 95% CI | HR | 95% CI |
| p16INK4A| ⩽5%        | 0/98 (0%)     | <0.0001    | <0.0001 | NA ‡ | NA | – | – |
|         | >5%        | 10/32 (31.3%) |            |          |      |      |    |     |
| Ki-67   | ⩽10%       | 0/8 (0%)      | 0.41       | 0.37    | –    | – | – | – |
|         | >10%       | 9/118 (7.6%)  |            |          |      |      |    |     |
| Cyclin D1| ⩽10%     | 2/46 (4.3%)   | 0.227      | 0.458   | –    | – | – | – |
|         | >10%       | 8/101 (7.9%)  |            |          |      |      |    |     |
| Rb      | <10%       | 5/56 (5.2%)   | 0.244      | 0.191   | –    | – | – | – |
|         | >10%       | 5/35 (15.2%)  |            |          |      |      |    |     |
| mcm-2   | ⩽50%       | 0/36 (0%)     | 0.056      | 0.055   | –    | – | – | – |
|         | >50%       | 10/96 (10.4%) |            |          |      |      |    |     |
| β-catenin, total| ⩽60% | 6/113 (5.3%) | 0.0006    | 0.002 | 5.7 | 1.6–20.3 | – | – |
|         | >60%       | 4/13 (30.8%)  |            |          |      |      |    |     |
| β-catenin, cytoplasm| ⩽60% | 0/100 (0%) | <0.0001   | <0.0001 | NA | NA | – | – |
|         | >60%       | 10/26 (38.5%) |            |          |      |      |    |     |
| β-catenin, nuclear| >1%      | 5/17 (29.4%) |            |          |      |      |    |     |
| β-catenin, membrane| ⩾0%  | 5/109 (4.6%) |            |          |      |      |    |     |
|         | 0%–99%     | 9/123 (7.3%)  | 0.016      | 0.056   | –    | – | – | – |
|         | 100%       | 1/3 (33.3%)   |            |          |      |      |    |     |
| Cox-2   | 0–99%      | 2/45 (4.4%)   | 0.718      | 0.479   | –    | – | – | – |
|         | 100%       | 8/95 (8.4%)   |            |          |      |      |    |     |
| Ets-1   | ⩾0%       | 4/25 (16%)    | 0.560      | 0.304   | –    | – | – | – |
|         | <1%       | 6/77 (7.8%)   |            |          |      |      |    |     |
| p53     | <80%       | 4/90 (4.4%)   | 0.054      | 0.067   | –    | – | – | – |
|         | ⩾80%       | 5/39 (12.8%)  |            |          |      |      |    |     |
| Bcl-2   | ⩾10%       | 8/85 (9.4%)   | 0.701      | 0.573   | –    | – | – | – |
|         | ⩽10%       | 2/36 (5.6%)   |            |          |      |      |    |     |
| Survivin| ⩾7.5%      | 2/94 (2.1%)   | 0.0002     | 0.0007  | 9.1  | 1.9–43.1 | 6.3 | 1.3–30.8 |
|         | >7.5%      | 8/41 (19.5%)  |            |          |      |      |    |     |
| hTERT   | <10%       | 1/59 (27.6%)  | 0.015      | 0.009   | 9.7  | 1.2–76.5 | 9.4 | 1.1–78.6 |
|         | ⩾100%      | 9/61 (2.5%)   |            |          |      |      |    |     |
| Tcf-4   | ⩾100%      | 2/12 (16.7%)  | 0.972      | 0.395   | –    | – | – | – |
|         | ⩾100%      | 7/107 (6.5%)  |            |          |      |      |    |     |

* The cut-offs represents medians, quartiles, and tertiles.
† The most favorable group is mentioned first.
‡ NA, not applicable. No events in one group precluded appropriate evaluation, with extreme values for HR and infinite confidence interval.

The protein (mcm-2), nor the expression of transcription factor Ets-1 or tcf-4.

Multivariate analysis of the molecular markers retained survivin, hTERT, and nuclear β-catenin in the model (Table 2). Although highly significant, p16INK4A and cytoplasmic β-catenin were not included in the model because of infinite CI, which would thus violate the statistical model. In combined multivariate analysis of classical and molecular markers (Table 3), the independent predictors of metachronous CRC development were MPECs, survivin, and hTERT. The MPECs feature was the overall strongest feature (HR of 28.2; 95% CI 3.6–223.0) (Table 3).

When analyzing those adenomas with the presence of the MPEC feature only, the molecular markers further increased in their ability to predict the de-
velopment of metachronous CRC, as depicted in the Kaplan–Meier survival curves (Fig. 2A–F).

4. Discussion

This study identifies cell-specific and molecular features associated with the long-term development of metachronous CRC in a population-based cohort of consecutive patients with colorectal adenomas. Strongest among the features was the occurrence of MPECs within the colorectal adenoma. The prognostic value of this quantitatively defined feature is in agreement with previous studies and may serve as a better and more accurate identifier of high-risk adenomas than the classical use of IEN grade and histological type [37–39, 50].

The pathogenetic explanation for the elongated cell feature of MPECs remains uncertain at this stage. However, evidence has evolved over the past decades concerning the role of commensal bacteria in the large bowel and their possible contribution to carcinogenesis [22,34]. Of particular interest is the noted elongation of cells induced by cytotoxic distending toxins (CDTs) produced by bacteria in the intestine, which leads to alterations in the cell cycle and apoptotic machinery [21,34]. The first CDT was detected in 1987 in strains of Escherichia coli – a bacteria present throughout the large bowel in large numbers. Johnson et al. showed that intoxicated cells developed marked cell distension after treatment with culture supernatants, which later resulted in cell death [23]. More recently, experiments have shown that several bacterial toxins interfere with cellular signaling mechanisms in a way that is characteristic of tumor promoters [22,34,55]. One mechanism of cytotoxicity of CDTs is the breakage of double-stranded DNA in eukaryotic cells. This initiates the cells’ own DNA damage-response mechanisms, resulting in the arrest of the cell cycle at both the G1/S and the G2/M boundary [8,13,21]. Affected cells enlarge until they finally undergo apoptosis. Alternatively, depending on the cell type studied, CDTs lead to increased expression of p53 and the p53-regulated cyclin-dependent kinase inhibitor p21, and upregulates p27, which halts the cell in the cell cycle. Aberrantly expressed patterns of p53 and bcl-2 were also recently demonstrated in CRC patients coinfected with schistosomiasis [65]. We found upregulation of both p53 and p21 in the adenomas. Several of the markers upregulated in our material, namely p53, p21, p16, COX-2, survivin and hTERT, are indicative of a paralleled inhibition of apoptosis and halted cell cycle.

Further, variable expression of minichromosomal maintenance protein (mcm-2) was found in the adenomas, indirectly suggesting alterations at the DNA level that induce repair mechanisms. However, if DNA damage is induced (e.g. by CDTs) the factors upregulated (i.e. survivin, p21, COX-2, p53) may prevent the cells from entering the cell death program, while striving at not letting them proceed in the cell cycle (by up-regulating p16, p21, and p53). Ki-67 staining identifies proliferative cells between the G1 and M phases of the cell cycle. In normal crypts, Ki-67 positivity is only seen in the lower one-third of the crypt. Dysplastic crypts show a reversed pattern in which the upper third of the crypt and mucosal surface are stained [47]. We noted previously that mitotic activity was not a prominent feature on subjective analysis of the MPEC feature [50]. In this study, Ki-67 did not allow for discriminating patients with, from those without, increased risk for metachronous CRC development. The presence of Ki-67 activity supports the finding of a deregulated cell cycle and altered apoptosis control in the adenomas, and could be an effect of an altered cell cycle by mechanisms described above.

CDTs could play a direct or additive role in cancer causation and progression, by as of yet only poorly investigated and understood mechanisms. However, the established link between Helicobacter pylori and gastrointestinal cancer rises the possibility of carcinogenic mechanisms induced by other bacteria in CRC development [54]. No other established familial, hereditary or known environmental risk-factors were present in the selected patients under study.

In this study, we noted cryptal distribution of the immunomarkers similar to that identified previously [6]. In the study by Boman et al. [6] on the formation of adenomatous crypts, the distribution of cells ex-
Fig. 2. Kaplan–Meier survival analysis (log-rank). Risk of developing metachronous colorectal cancer in 43 patients with adenomas containing a monotonous population of elongated cells (MPECs) according to molecular markers.
pressing crypt base cell markers (MSH2, Bcl-2, survivin) expanded toward the crypt surface. We found an equal distribution for survivin, bcl-2, and p53. However, survivin- and p16\(^{\text{INK4A}}\)-positive cells seemed to distribute randomly along the crypt wall, not restrictively to the crypt surface or bottom (Fig. 1). This noted distribution of survivin has been recognized previously [15]. When present, nuclear \(\beta\)-catenin tended to be more prominent in the lower parts of the crypt.

The distribution for hTERT was increasingly found towards the top, together with p21\(^{\text{CIP1}}\). As indicated by Boman et al. [6], during adenoma development, APC mutations may cause expansion of the crypt base cell population, including crypt stem cells, along with the developmental theory of the intestine [44]. Stem cell overpopulation can explain the shifts in patterns of proliferative crypt cell populations in early colon tumorigenesis, and mutant crypt stem cells clonally expand to form colonic adenomas and carcinomas [6].

The Vogelstein adenoma-to-carcinoma progression model has served as an educational basis for the understanding of colorectal carcinogenesis over the past decades [11,58]. Lately, its validity has been challenged as increased complexity and diversity in neoplasia development have emerged [7,16,19,52]. Rather than representing a linear model, the neoplastic process follows multiple pathways with distinct cytogenetic and molecular features [7,12,16,20,49,52]. This notion may provide difficulties in interpreting the exact role of any one biomarker, however, several findings in this study deserve attention.

For one, nuclear accumulation of \(\beta\)-catenin is indicative of deregulated \(\text{Wnt}\) signaling pathway, which is believed to occur as an early and crucial step in colorectal carcinogenesis [57,63]. Nuclear staining of \(\beta\)-catenin was notably upregulated in a large number of the adenomas in this study. The oncogenic properties of \(\text{Wnt}/\beta\)-catenin signaling stem from alteration in phosphorylation-dependent protein degradation and subcellular localization of \(\beta\)-catenin from cell membrane to the nucleus, where it binds to Tcf-4 to form a bipartite transcription factor. The \(\beta\)-catenin/Tcf-4 complex facilitates transcription of target genes that encode effectors for activation of cell proliferation and invasion and inhibition of apoptosis, leading to colorectal cancer development. Disruption of \(\beta\)-catenin/Tcf-4 complex activity causes decreased expression of c-MYC, which in turn upregulates p21\(^{\text{CIP1}}\)/\(\text{WAF1}\). This mediates G1 arrest and differentiation. Thus, the \(\beta\)-catenin/Tcf-4 complex constitutes the master switch that controls proliferation versus differentiation in normal and neoplastic colorectal epithelial cells [57]. The previous belief of \(\beta\)-catenin as a protein exclusively linked to the expression/mutational status of APC has now evolved into a molecular complex known to interact with a number of important pathways and mechanisms in carcinogenesis [63]. Thus, a detailed description is beyond the scope of this paper, and only major notions of importance to the current results are included.

The tumor suppressor protein p16\(^{\text{INK4A}}\) is typically inactivated in human malignancies, with a frequency second only to p53. Together, p16\(^{\text{INK4A}}\) and \(\beta\)-catenin play pivotal roles in the multistep progression towards carcinoma development [14,46,57,63,64]. Conflicting in this study though, is the apparent upregulation of p16\(^{\text{INK4A}}\) in adenomas that progress to CRC. For reasons explained above, we believe this to be an upregulation of a tumor suppressor secondary to other molecular changes that serve to drive the cell into division/proliferation. An upregulation of p16\(^{\text{INK4A}}\) may in fact reflect an attempt (albeit in despair) of the cell to stop such cell cycle progression and keeping cell cycle control.

In an equal manner, we believe, as one of the cyclin dependent kinase inhibitors involved in the G1/S checkpoint, p21\(^{\text{CIP1}}\) to be involved in colorectal neoplasia progression [48]. As p16\(^{\text{INK4A}}\) and p21\(^{\text{CIP1}}\) inhibit cell-cycle progression, we suggest the significance by overexpression of p16\(^{\text{INK4A}}\) and p21\(^{\text{CIP1}}\) found in this study be attributed to the attempt at controlling a deregulated cell-cycle activity. This is in line with the results of others [64], and has been noted in other premalignancies, i.e. in cervical intraepithelial neoplasia [3,4]. The additive effect of various, de-ranged cell-cycle mechanisms may thus represent a spectrum of continuous cell stimuli upon cells developing from colorectal crypts. Further accumulation of molecular alterations, such as the later loss of inhibitor functions from p16\(^{\text{INK4A}}\) and p21\(^{\text{CIP1}}\) may then drive the cells into cancer development, in line with the known, complex mechanisms of colorectal carcinogenesis [7,16,51,52]. This may call for a more dynamic interpretation of biomarkers assessed by immunohistochemistry [4].

Avoidance of apoptosis is a crucial step in carcinogenesis and may be early markers for CRC development [18,33,59]. The survivin protein, a member of the inhibitor-of-apoptosis family, blocks apoptotic signaling activated by various cellular stresses. Survivin is a bifunctional regulator of cell death and cell proliferation during embryonic development but un-
detectable in healthy adult tissues and re-expressed in many cancers, including colorectal cancer. Increased expression of survivin in CRC is associated with poor patient prognosis [28]. Stimulation of survivin expression by the β-catenin/Tcf-4 has been suggested to impose a coupling of enhanced cell proliferation with resistance to apoptosis in the colorectal crypts, and thus contribute to the development of CRC [28]. As evident in the current study, survivin was one of the most important markers predicting risk for cancer development. The co-expression with hTERT and β-catenin supports this link in neoplasia.

Immortalization of cells is a required hallmark of carcinogenesis [18]; telomere length maintenance by telomerase is required for cancer cells to proliferate indefinitely, yet how cancer cells activate telomerase remains unclear. Telomerase activation occurs during the progression from low-grade to high-grade dysplasia in adenomas and increases steadily with the progression of the degree of dysplasia and invasion during colorectal carcinogenesis [25]. Correlation between survivin and hTERT expression has been observed in CRC, and overexpression of survivin has been shown to enhance telomerase activity by up-regulation of hTERT expression [10], which is consistent with results found in our study. Apparently, survivin participates not only in inhibition of apoptosis but also in prolonging cellular lifespan. The current study points to an important role for survivin in metachronous CRC development. Together with the immortalizing effects of hTERT on the CRA cells [25], these factors facilitate survival. Over time further mechanisms may enable them to turn malignant [51].

In conclusion, deregulated cell cycle control (p16INK4A, p21CIP1, nuclear β-catenin), inhibition of apoptosis by survivin, and telomere-length stabilization and immortalization through hTERT appear to be factors important for the late development of cancer and may serve as potential molecular predictors for high-risk patients. The current results serve as new potential diagnostic markers for colorectal adenomas in patients with a high risk for developing metachronous CRC. Improved diagnostics, targeted and more cost-efficient follow-up strategies, potential molecular intervention, and eventual cancer prevention may result by adding high-risk markers to the current use of classical risk designators in patients with colorectal adenomas.

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