Putative anoikis-resistant subpopulations in colorectal carcinoma: a marker of adverse prognosis

MADHURA PATANKAR,1,2,* TANELI MATTILA,1,2* JUHA P. VÄYRYNEN,1,2,3 KAI KLINTRUP,4,5 JYRKI MÄKELÄ,4,5 ANNE TUOMISTO,1,2 PENTTI NIEMINEN,6 MARKUS J. MÄKINEN1,2 and TUOMO J. KARTTUNEN1,2

1Department of Pathology, Cancer and Translational Medicine Research Unit, University of Oulu; 2Department of Pathology, Oulu University Hospital and Medical Research Center Oulu, Oulu, Finland; 3Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, MA, USA; 4Department of Surgery, Oulu University Hospital and Medical Research Center Oulu; 5Department of Surgery, Research Unit of Surgery, Anesthesia and Intensive Care; and 6Medical Informatics and Data Analysis Research Group, University of Oulu, Oulu, Finland

Putative anoikis-resistant subpopulations in colorectal carcinoma: a marker of adverse prognosis. APMIS 2020; 128: 390–400.

Anoikis is a form of apoptosis induced when a cell loses contact with the extracellular matrix (ECM). Anoikis resistance is essential for cancer cell survival during the formation of metastases (4,5). There is only indirect evidence suggesting that anoikis resistance is a prognostic factor (7-10). So far, the assessment of anoikis resistance has been limited to in vitro cell culture-based tests (4) as there are no methods to evaluate the presence and level of anoikis resistance using routine pathological specimens. However, based on the essential role of anoikis resistance in metastasis, the adoption of this concept to histopathological analysis might provide useful information.

We have shown that in carcinomas, anoikis resistance allows cells to form multicellular clusters where the inner cells lose their contact with the...
ECM, but still survive (11,12). We have previously shown that in CRC, in micropapillary structures (MIPs), defined as luminal extensions formed by piled-up carcinoma cells (11), suprabasal cells form a tumor cell population without ECM contact and still with low apoptosis rate, suggesting that these cells represent one putative anoikis-resistant (AR) tumor cell population (11). In the present study, we have used a similar approach to identify other types of multicellular carcinoma cell clusters without ECM contact and without evidence of increased apoptosis rate in CRC. We quantitated the structures composed of such subpopulations of carcinoma cells in a series of CRC and evaluated their prognostic significance.

MATERIAL AND METHODS

Patients

We studied a prospective series of 149 CRC patients (13) operated in Oulu University Hospital between 2006 and 2010 (Table 1). From the series, 12 (8%) cases were excluded due to insufficiency of sample material, leaving a total of 137 cases. Clinical and follow-up data were obtained from the clinical records and Statistics Finland. Cancer-specific survival (CSS) was defined as time from operation to cancer-related death. The Ethics Committee of Oulu University Hospital approved this research project (58/2005, 184/2009).

Histology and immunohistochemistry

Basic histological assessments including grading and stage determination were based on conventional histopathological sections (13). For other analyses, we used previously (13) constructed tissue microarrays (TMAs). For the TMAs, we used H&E stained slides to select optimal sample locations, and depending on the size of the tumor, manually sampled 1–4 (median 3) cores of 3.0 mm diameter. Of the cores, 1–3 were taken from the invasive front and 1–2 from random locations in the tumor bulk, only avoiding necrotic areas. The current analysis for putative AR populations was only focused on the cores representing the bulk.

For determination of cell proliferation rate and apoptosis rate, we used immunohistochemistry for Ki67 and M30, respectively. The antigen retrieval was performed with Tris-EDTA buffer, pH 9, in a microwave (800 W for 2 min, 150 W for 15 min). Antibodies for Ki67 (code NCL-Ki67-MM1, dilution 1/50; Leica Biosystems, Newcastle-upon-Tyne, UK) and M30 (CytoDEATH cat. no. 12140322001, dilution 1/1000; Enzo Life Sciences, Lausen, Switzerland) were incubated for 30 min at room temperature, and bound antibodies detected with Envision Detection kit (Dako, Copenhagen, Denmark). Laminin gamma 1 immunohistochemistry was conducted using Bond RX stainer (Leica Biosystems, Nussloch, Germany) using monoclonal mouse antibodies (Anti-LAMC1, clone CL3199, dilution 1/150; Atlas Antibodies, Stockholm, Sweden) with a pretreatment with Leica Bond Epitope Retrieval Solution 1, and Leica Bond Polymer Refine

Table 1. Clinical and pathological features of colorectal carcinoma cases (n = 149) analyzed

| Feature                                      | Value          |
|----------------------------------------------|----------------|
| Age, mean (SD)                               | 66.81 (11.19)  |
| Sex                                          |                |
| Male                                         | 80 (53.69%)    |
| Female                                       | 69 (46.31%)    |
| Tumor location                               |                |
| Proximal                                     | 49 (32.89%)    |
| Distal                                       | 28 (18.79%)    |
| Rectum                                       | 72 (48.32%)    |
| WHO grade                                    |                |
| G1 (Well differentiated)                     | 21 (14.29%)    |
| G2 (Moderately differentiated)               | 106 (72.11%)   |
| G3 (Poorly differentiated)                   | 20 (13.61%)    |
| TNM stage                                    |                |
| Stage I                                      | 27 (18.24%)    |
| Stage II                                     | 55 (37.16%)    |
| Stage III                                    | 44 (29.73%)    |
| Stage IV                                     | 22 (14.86%)    |
| T class                                      |                |
| T 1                                          | 5 (3.38%)      |
| T 2                                          | 29 (19.59%)    |
| T 3                                          | 103 (69.59%)   |
| T 4                                          | 11 (7.43%)     |
| Metastasis                                   |                |
| Yes                                          | 19 (12.84%)    |
| No                                           | 129 (87.16%)   |
| Nodal metastasis                             |                |
| Yes                                          | 61 (41.50%)    |
| No                                           | 86 (58.50%)    |
| Lymphatic invasion                           |                |
| Yes                                          | 61 (42.07%)    |
| No                                           | 84 (57.93%)    |
| Blood vessel invasion                        |                |
| Yes                                          | 27 (18.62%)    |
| No                                           | 116 (81.38%)   |
| Infiltrating border                          |                |
| Yes                                          | 43 (29.05%)    |
| No                                           | 105 (70.95%)   |
| Cancer type                                  |                |
| Conventional adenocarcinoma                  | 115 (77.18%)   |
| Serrated adenocarcinoma                     | 34 (22.82%)    |
| Mismatch repair (MMR)                        |                |
| Proficient                                   | 137 (92.57%)   |
| Deficient                                    | 11 (7.43%)     |
| BRAF mutation                                |                |
| Yes                                          | 13 (9.56%)     |
| No                                           | 123 (90.44%)   |
| KRAS mutation                                |                |
| Yes                                          | 34 (25.00%)    |
| No                                           | 102 (75.00%)   |

Detection Kit DS 9800 for detection (Leica Biosystems). For collagen IV immunohistochemistry, we used mouse monoclonal anti-human collagen IV (clone CIV 22; code nr. M 0785; dilution 1/50; Dako) antibody, with pretreatment with 0.4% pepsin for 30 min at 37 °C and detection with Ultravision Large volume Detection system TP-125-HL (Thermo Scientific, Waltham, MA, USA). We used PBS instead of primary antibodies for negative controls. The stained sections were digitized (Leica-Aperio AT2; Leica Biosystems).

© 2020 The Authors. APMIS published by John Wiley & Sons Ltd on behalf of Scandinavian Societies for Medical Microbiology and Pathology
Detection of putative anoikis-resistant subpopulations

To identify putative AR subpopulations, we first identified carcinoma cells without contact with the ECM and then assessed apoptosis rate in cells with and without ECM contact. We identified subpopulations of tumor cells with and without contact with the ECM or stromal areas using H&E staining (Fig. 1) and immunohistochemical stainings for ECM proteins laminin and type IV collagen (Fig. 2). In these analyses, we assessed all tumor cells present in the whole area of each core.

Carcinoma cells without contact with the ECM or with mesenchymal areas of the carcinomas were found to be within the following three types of structures: (i) MIPs; (ii) cribriform structures; (iii) solid structures (Fig. 1). We defined MIPs as cells piled up at the luminal side of the glandular structures, the minimum thickness of this pile being two cells, and the lateral extent at minimum two cells. Cribriform structures were comprised of groups of cells at least four cells in diameter, and containing scattered, empty spaces without cells. Solid structures consisted of groups of cells at least four cells in diameter forming solid sheets. Suprabasal cells of MIPs and inner cells in cribriform or solid structures were devoid of ECM contact (Fig. 1). The cells in contact with ECM comprised cells of a single cell layer thick columnar epithelium and the outermost cell layer of multilayered epithelium, including the basal layer of MIPs (11) and the outermost layer of cribriform and solid structures (Fig. 1).

The proportions of cells positive for M30 and Ki-67 were separately determined in carcinoma cell subpopulations with and without ECM or stromal contact as defined above (Fig. 2). For the determination of positivity rates in subpopulations without ECM or stromal contact, a representative series of each type of putative AR structures was identified in each TMA tissue core representing tumor bulk (Fig. 1). In 5–20 of each type were assessed. For the determination of positivity rates in cells with ECM contact, a minimum 10 separate groups of about 10–50 adjacent cells were assessed around cells without ECM contact and in the cells representing unilayered columnar epithelium. In cases with more than one core, mean indexes were calculated and used for the case. One investigator (MP) manually assessed M30 stainings and two investigators (TM and MP) assessed Ki-67, both by using a digital image analysis platform (QuPath, version 0.1.2) (14). For all stainings, cases with inconclusive staining patterns were additionally analyzed by an experienced pathologist (TJK). For assessment of interobserver agreement of the recognition of different subpopulations and cell counts, 10 cases were independently studied by three observers (TM, MP, TJK).

Quantitation of putative anoikis-resistant structures

For each case, the area (mm$^2$) occupied by tumor was determined in all TMA cores representing tumor bulk (Fig. 3) by using virtual images of H&E stained sections (Aperio ImageScope; Leica Biosystems). All occurrences of each putative AR subpopulation type within the core (Fig. 3) were recorded to determine their areal density (structures/mm$^2$ tumor tissue). In 38 (42%) cases, more than one core representing tumor bulk was available. In these cases, mean areal densities were calculated.

Statistical analysis

IBM SPSS Statistics, version 22 (IBM Corp., Armonk, NY, USA), was used for statistical analyses. Reproducibility of the assessments was evaluated by using interclass correlation coefficient (ICC) based on a mean rating ($k = 2$), absolute-agreement, 2-way mixed-effects model. As areal densities of different subpopulations showed skewed distributions, we assessed their association with categorical clinicopathological features with Mann–Whitney or Kruskal–Wallis tests, and correlations with Spearman rank correlation. Similarly, we used Wilcoxon matched-pair test for comparing apoptosis and proliferation rates in different tumor cell subpopulations. For

Fig. 1. Microphotographs of colorectal carcinomas showing examples of putative anoikis-resistant tumor cell subpopulations. Annotations indicate putative anoikis-resistant subpopulations without contact to mesenchymal areas of cancers within different structures, including micropapillary structures (A; red annotation), a cribriform structure (B, yellow), and a solid structure (C; blue). Annotations in orange mark examples of cells with a contact with the mesenchymal areas of the tumors, including basal cells in MIPs (A) and the outermost cells of cribriform or solid structures (B, C). Hematoxylin and eosin staining. Scale bar = 50 µm.
survival analyses, we used receiver operating characteristics (ROC) analysis to determine optimal cutoff values for areal densities of subpopulations by using the Youden index. For univariate survival analysis, we generated Kaplan-Meier curves for cancer-specific survival (CSS) and disease-free survival (DFS) and Log-rank test estimated differences. Cox regression models verified the independent prognostic effects of the subpopulation counts on CSS when adjusted for covariates. Due to the low number of cases in multivariate analyses, we estimated the models for one covariate at a time, as described previously (15,16). We considered two-tailed, exact p-value < 0.05 as statistically significant for all the tests.

RESULTS

Recognition of putative anoikis-resistant subpopulations

Three types of multicellular carcinoma cell islands were identified, including MIPs, cribriform, and solid structures (Fig. 1). Laminin and type IV collagen stainings showed discontinuous staining on the stromal interface of carcinoma cell islands of all types. However, no extracellular positivity was present within the MIPs, cribriform (Fig. 2), or solid structures, indicating absence of organized ECM within these structures. Apoptosis rate as shown by the proportion of M30 positive cells was higher in the cells with ECM contact than in those without ECM contact (p < 0.001; Figs 2 and 4). This finding supported the concept that the latter subpopulations, including suprabasal cells in MIPs and inner cells in cribriform and solid structures, are resistant to anoikis and can be classified as putative AR populations. Proliferation rates were mostly similar in different subpopulations, and only MIPs showed lower rates than the other subpopulations (Fig. 4).

Quantitation of putative anoikis-resistant subpopulations

We determined areal densities (structures/mm² tumor tissue) for structures containing putative AR cell populations (Fig. 3) and calculated the sum of areal densities of all three subtypes. Areal densities of each subpopulation type correlated with the total areal density sum (MIP, p = 0.337; cribriform, p = 0.560; solid, p = 0.558; all p < 0.001). Among the putative AR subpopulations, the areal densities of cribriform and solid structures correlated (p = 0.204; p = 0.017) whereas MIP areal density showed an inverse correlation to that of solid structures (p = 0.234; p = 0.006).

There were 58 cases with more than one core available for determination of putative AR areal density. The areal densities correlated moderately between the cores (Pearson c. 0.338, p = 0.010; ICC 0.412, p = 0.025). To assess interrater reproducibility, three investigators (MP, TM, TJK) annotated subpopulations and determined Ki67 indexes in a

---

Fig. 2. Laminin, type IV collagen, M30, and Ki-67 immunohistochemical stainings in colorectal carcinomas. As an example of a structure containing putative anoikis-resistant subpopulation, cribriform structures are shown. There is occasional presence of laminin (A) and type IV collagen (B) reactivity at the outer margin of cribriform carcinoma cell islands (arrows; inserts), but no extracellular expression of these proteins is seen among the inner cells of the structure representing a putative anoikis-resistant subpopulation. In M30 staining (C), the number of positive cells among the inner cells of a cribriform structure (arrowheads) is lower as compared with outer cells of the cribriform structure (arrows), suggesting a lower apoptosis rate in the putative anoikis-resistant subpopulation. In Ki-67 staining (D), positive cells are evenly distributed among both the inner cells (arrowheads) and the outer cells (arrows) of a cribriform structure. Scale bar = 200 μm.
series of 10 unselected cases. In this series, ICC for Ki-67 based on all subpopulation types was 0.814 for average measures ($p < 0.001$) and 0.686 for single measurements ($p < 0.001$), indicating moderate to good reliability applicable for both the identification of subpopulation type and Ki-67 measurement.

Amount of putative anoikis-resistant subpopulations and clinicopathological features of carcinomas

We then analyzed relationships between areal densities of putative AR subpopulations and clinicopathological features of CRC. We found a significant association between low tumor grade and high areal density of MIPs ($p = 0.005$) and between high tumor grade and high areal density of solid islands ($p = 0.029$; Table 2). Although BRAF and KRAS mutations did not show any association with the overall amount of AR structures, KRAS mutation was associated with low abundance of solid subpopulation ($p = 0.048$; Table 2), but no association was found with the tumor stage or carcinoma type (serrated or conventional; Table 2).

Putative anoikis-resistant subpopulation areal density and carcinoma prognosis

To determine the prognostic value of putative anoikis-resistant subpopulations, we dichotomized areal density to high and low by using ROC curve analysis. Determination of cutoff by ROC curve was difficult for MIPs as the area under curve was low (0.527). However, at a closer look, MIPs were rare in grade 3 tumors (median 0.74/mm$^2$; range 0–3.9) compared to grade 1 and 2 tumors (1.4/mm$^2$; 0–16.1; $p = 0.005$) and correlated negatively with the number of solid structures (see above). Such associations are plausible based on the definition of MIPs, as these structures are extensions of the columnar epithelium and therefore rare in high-grade tumors, which, by definition (17), have an abundance of solid structures and only rare occurrence of gland-like structures with columnar epithelium (Fig. 1). Thus, for MIPs, we limited the ROC curve analysis to grade 1 and 2 cases with a resulting cutoff of 2.83/mm$^2$. The cutoffs for areal densities of cribriform and solid subpopulations were 4.1/mm$^2$ and 0.49/mm$^2$, respectively.
Fig. 4. Comparison of M30 (top image) and Ki-67 (bottom image) indexes (proportions of positive cells) in different tumor cell subpopulations in all studied CRCs, including cells with ECM contact (non-AR cells) and putative anoikis-resistant cells within structures such as micropapillary structures (MIPs), cribriform, and solid structures. Subpopulations were compared with the Wilcoxon matched-pair test. Range (whiskers), interquartile range (box), and median (horizontal line) are shown.
In univariate analysis, high areal density of MIPs (30/137; 22%) showed no association with prognosis (Fig. 5), but in grade 1 and 2 tumors, their high areal density (n = 27/117; 23%) tended to associate with worse CSS (p = 0.058). High areal density of the cribriform subpopulation (12/137; 9%) associated with worse survival (CSS: p = 0.004, Fig. 5), and a similar association was seen for the solid subpopulation (80/137; 58%; p = 0.017; Fig. 2). High areal densities associated with low DFS, but the association was significant only for the cribriform subpopulation (p = 0.015, data not shown). High total areal density based on the sum of areal densities of all three putative AR subpopulations (>6.86/mm²; 27/137; 20%) associated with worse CSS (p = 0.001; Fig. 5). In subgroup analyses, similar differences were found in stage III–IV tumors (p = 0.043), but not in stage I–II tumors (p = 0.645). Finally, high sum of areal densities of cribriform and solid subpopulations (>2.95/mm²; 60/137; 44%) associated with worse prognosis (p = 0.036). Although total areal density of putative AR subpopulations did not associate with DFS (p = 0.103), the areal density of cribriform...
structures showed a significant association ($p = 0.015$, data not shown).

The total putative AR areal density was an independent prognostic factor of CSS estimated with Cox regression model, when adjusted for features including the presence or absence of nodal metastases (N), tumor stage (T1-2 vs T3-4), presence or absence of distant metastasis, lymphatic invasion, blood vessel invasion, tumor grade, infiltrating border, tumor location, and age (Table 3). Independent association for CSS was similarly evident for the areal densities of cribriform and solid structures (data not shown).

**DISCUSSION**

We show here that subrabasal cells in MIPs (11) and the inner cells in cribriform and solid structures are devoid of contact with mesenchymal areas or basement membrane proteins, and yet show a decreased apoptosis rate compared to tumor cells that are in contact with mesenchymal areas or basement membrane proteins at the stromal-epithelial interface of the tumors. These findings suggest that these subpopulations, all comprised of inner cells of multicellular carcinoma cell groups, represent tumor cells with anoikis resistance in CRC. By definition, anoikis is a form of apoptosis induced by loss of ECM contact (4-5,11), and resistance to anoikis is considered essential for the survival of tumor cells during their travel to metastatic loci (5,6). Our analysis of 137 CRC patients indicated that high areal density of each of these putative AR subpopulation types associates with adverse prognosis, and for cribriform and solid subpopulations, the association is independent in multivariate analysis. However, the total areal density of all three AR subpopulation types was the most powerful prognostic factor, supporting generalizability of the concept of putative AR subpopulations and their prognostic value. Our observations suggest that the concept of anoikis resistance can be adopted to histopathological analysis of CRC and provide prognostic information.

Anoikis resistance has been mostly analyzed with *in vitro* cell cultures in anchorage-independent conditions (4). Such an approach allows mechanistic insight into anoikis resistance, but does not serve as a tool for routine analysis of anoikis resistance in clinical tumor specimens since fresh tumor specimens are needed. Although the prognostic and

@fig.5. Kaplan–Meier curves showing cancer-specific survival (60 months; log rank) in patients ($n = 137$) with high and low areal densities of the putative anoikis-resistant structures, including total putative anoikis-resistant structures areal density (A), areal densities of MIPs (B), cribriform structures (C), and solid structures (D).
predictive significance of anoikis resistance in human malignancies is unknown, reports of prognostic biomarkers functionally linked to anoikis resistance, such as tyrosine kinase receptor B and HCRP-1, support the importance of anoikis resistance as a prognostic factor (7,18). Our study is the first one to use the concept of histopathological recognition of anoikis resistance and to utilize it to get prognostic information. Confirmation of the biological equivalence of in vitro anoikis resistance and anoikis resistance as detected by the presence of putative AR subpopulations in histological analysis would require studies showing correlation between in vitro detection of anoikis resistance with that based on histopathology. So far, we lack such biological confirmation of the current concept of anoikis resistance recognition, but this does not depreciate the prognostic value of our concept. However, confirmation of the prognostic value with an independent case series is still warranted.

The mechanisms causing the formation of the putative AR structures remain speculative. The ability of a cell to resist anoikis becomes evident when it loses its contact with the ECM. The mechanisms leading to loss of such contact include extrusion caused by cellular crowding (19), mechanical detachment, or regulatory aberration leading to loss of integrin function (20-22). To survive in such conditions, cells must respond either by inhibiting pro-apoptotic signaling or by activating anti-apoptotic mechanisms (5,6). It is likely that, in addition to anoikis resistance, other aberrations, such as suppression of apical polarity signaling (23), contribute to formation of the putative AR structures. Supporting the concept of multicellular groups without ECM contact indicating anoikis resistance, we have recently shown that transfection of Caco-2 cells with mutated KRAS or BRAF gene induces anoikis resistance (12) and, simultaneously, changes 3D growth from simple unilayered cysts to either focal piling of the cells reminiscent of MIP structures or solid growth reminiscent of solid structures, respectively (12).

The recognition of AR subpopulations was relatively reproducible. Tangentially sectioned glandular structures with a single-layered epithelium may mimic solid structures or MIPs. However, determination of areal densities of these putative AR subpopulations likely diminishes this bias, since high densities are an unlikely consequence of section

| Covariates        | Univariate HR (95% CI) | Model 1 HR (95% CI) | Model 2 HR (95% CI) | Model 3 HR (95% CI) | Model 4 HR (95% CI) | Model 5 HR (95% CI) | Model 6 HR (95% CI) | Model 7 HR (95% CI) | Model 8 HR (95% CI) | Model 9 HR (95% CI) |
|-------------------|------------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|---------------------|
|                   | p value                | p value             | p value             | p value             | p value             | p value             | p value             | p value             | p value             | p value             |
| Total AR > 6.86/mm² | 3.16 (1.50-6.66)       | 3.45 (1.61-7.40)   | 3.06 (1.44-6.53)   | 2.96 (1.40-6.34)   | 2.36 (1.1-5.06)    | 3.16 (1.49-6.71)   | 3.12 (1.46-6.67)   | 2.97 (1.37-6.44)   | 3.04 (1.42-6.51)   | 3.20 (1.52-6.75)   |
| N (yes/no)        | p = 0.002              | p = 0.001           | p = 0.004           | p = 0.005           | p = 0.027           | p = 0.003           | p = 0.003           | p = 0.006           | p = 0.064           | p = 0.002           |
| T1-2 vs T3-4      | 3.45 (1.05-11.3)       | 2.51 (0.75-8.36)   | p = 0.044           | p = 0.134           |                     |                     |                     |                     |                     |                     |
| Distant metastasis | 9.08 (4.53-18.2)       |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| (M)               | p < 0.001             |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| Lymphatic invasion (LI) | 7.15 (2.92-17.5)     | 5.48 (2.20-13.7)   |                     |                     |                     |                     |                     |                     |                     |                     |
| Bloodvessel invasion (BVI) | 4.87 (2.36-10.0) | 4.01 (1.91-8.42)  |                     |                     |                     |                     |                     |                     |                     |                     |
| Grade 1-2 vs 3    | 2.33 (1.01-5.39)       |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| p = 0.048         |                       |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| Infiltrating border (IB) | 2.62 (1.31-5.26)   | 1.73 (0.81-3.7)   |                     |                     |                     |                     |                     |                     |                     |                     |
| Location          | 1.59 (0.79-3.20)       |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| (rectum/colon)    | p = 0.102             |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| Age > 65          | 1.6 (0.76-3.36)        |                     |                     |                     |                     |                     |                     |                     |                     |                     |
| p = 0.215         |                       |                     |                     |                     |                     |                     |                     |                     |                     |                     |

Bold values indicate statistically significant p values. Univariate column presents the crude value.

In Models 1–9, total AR was adjusted with one covariate at a time, as the number of cases was insufficient for conventional multivariate analysis (15,16).
ANOIKIS RESISTANCE IN COLORECTAL CANCER

Effects. We used tissue microarrays (TMAs) for the quantitation of putative AR subpopulations. Supporting the validity of this approach in terms of structural heterogeneity in carcinomas, we saw a moderate correlation between different cores. However, more studies are needed to address the within-tumor variation of the putative AR structures and the clinical significance of such variation.

Assessment of anoikis resistance in routine diagnostic work by the methods described in the present study does not require any special staining techniques as it can be performed by using conventional H&E stained sections. In addition, although we used digital images and image analysis programs for determination of areal densities of the putative AR populations, it would also be possible by using conventional microscopy; for example, by counting their occurrences in a certain number of visual fields with a known area. We did not measure the time consumption for the present analyses, but a crude estimate would be 3–30 min for each case, depending on the experience of the assessor and the quantity of putative AR structures. Other options for the quantitation of putative AR structures include classical morphometrical methods (24).

We observed an association of high areal density of cribriform structures with poor prognosis. This finding is in agreement with reports of poor prognosis in cribriform-comedo type adenocarcinoma, a rare CRC subtype (25-27). However, the prognostic significance of the overall extent of cribriform pattern, as shown in our study, has been unknown in CRC. High areal density of solid structures associated with high grade, as expected (17). Tumor grade is a rather weak prognostic factor (28), and the reproducibility of grading in CRC is suboptimal (29). The recently described grading system for CRC is based on the amount of so-called poorly differentiated clusters (30), composed of at minimum five cells without any gland formation. Based on this definition (30), larger poorly differentiated clusters may contain putative AR tumor cells, comparable to those in solid structures in our study. These analogies suggest that the current concept of morphological expression of anoikis resistance may present a plausible biological explanation for both the traditional grading criteria of CRC and the grading based on poorly differentiated clusters.

We used tumor bulk specimens for evaluation of anoikis resistance and hence, the quantitation of the putative AR structures at the front likely does not provide prognostic information. Bulk and front show other biological and structural differences (33). Taken together, we suggest that tumor bulk may be the part of the tumor where biological conditions in the carcinoma cells and the microenvironment allow the emergence of features that mark potential of the cells to resist anoikis. Hence, we quantitated the putative AR structures only in the tumor bulk.

In conclusion, it seems possible to recognize and quantify specific subpopulations of CRC tumor cells which likely indicate anoikis resistance, as shown by the absence of ECM contact and decreased apoptosis indexes in the cells. Importantly, abundance of such putative AR populations associated clearly with an adverse prognosis. Further studies showing correlation between histopathologically determined anoikis resistance with that based on conventional in vitro assays are necessary to confirm the current concept, and studies with larger case series are advised to confirm the prognostic value of our assay.

The authors are grateful to Medical Research Center Oulu, University of Oulu Scholarship Foundation for financial support. The authors would like to thank Riitta Vuento and Erja Tomperi for their expert technical help.

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

REFERENCES

1. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-Tieulent J, Jemal A. Global cancer statistics, 2012. CA Cancer J Clin 2015;65:87–108.
2. Siegel R, DeSantis C, Virgo K, Stein K, Mariotto A, Smith T, et al. Cancer treatment and survivorship statistics, 2012. CA Cancer J Clin 2012;62:220–41.
3. Puccini A, Berger MD, Zhang W, Lenz HJ. What we know about stage II and III colon cancer: it’s still not enough. Target Oncol 2017;12:265–75.
4. Frisch SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. J Cell Biol 1994;124:619–26.
5. Paoli P, Giannoni E, Chiarugi P. Anoikis molecular pathways and its role in cancer progression. Biochim Biophys Acta 2013;1833:3481–98.
6. Simpson CD, Anyiwe K, Schimmer AD. Anoikis resistance and tumor metastasis. Cancer Lett 2008;272:177–85.
7. Dawson H, Grundmann S, Koelzer VH, Galvan JA, Kirsch R, Karamitopoulou E, et al. Tyrosine kinase receptor B (TrkB) expression in colorectal cancers

© 2020 The Authors. APMIS published by John Wiley & Sons Ltd on behalf of Scandinavian Societies for Medical Microbiology and Pathology
highlights anoikis resistance as a survival mechanism of tumour budding cells. Histopathology 2015;66:715–25.

8. Wang XC, Tian LL, Jiang XY, Wang YY, Li DG, She Y, et al. The expression and function of miRNA-451 in small non-small lung cancer. Cancer Lett 2011;310:203–9.

9. Wang YN, Zeng ZL, Lu J, Wang Y, Liu ZX, He MM, et al. CPT1A-mediated fatty acid oxidation promotes colorectal cancer cell metastasis by inhibiting anoikis. Oncogene 2018;37:6025–40.

10. Frankel A, Rosen K, Filmus J, Kerbel RS. Induction of anoikis and suppression of human ovarian tumor growth in vivo by down-regulation of Bcl-X(L). Cancer Res 2001;61:4837–41.

11. Patankar M, Vayrynen S, Tuomisto A, Makinen M, Eskelinen S, Karttunen TJ. Micropapillary structures in colorectal cancer: an anoikis-resistant subpopulation. Anticancer Res 2018;38:2915–21.

12. Patankar M, Eskelinen S, Tuomisto A, Makinen MJ, Karttunen TJ. KRAS and BRAF mutations induce anoikis resistance and characteristic 3D phenotypes in Caco2 cells. Mol Med Rep 2019;20:4634–44.

13. Vayrynen JP, Vornanen J, Tervahartiala T, Sorsa T, Bloigu R, Salo T, et al. Serum MMP-8 levels increase in colorectal cancer and correlate with disease course and inflammatory properties of primary tumors. Int J Cancer 2012;131:E463–74.

14. Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: open source software for digital pathology image analysis. Sci Rep 2017;7:16878–017-17204-5.

15. Kleinbaum DG, Klein M. Survival Analysis: A Self-learning Text. Springer-Verlag: New York, NY, 3rd ed., 2012.

16. Elseragy A, Salo T, Coletta RD, Kowalski LP, Haglund C, Nieminen P, et al. A proposal to revise the histopathologic grading system of early oral tongue cancer incorporating tumor budding. Am J Surg Pathol 2019;43:703–9.

17. Bosman FT, Carneiro F, Hruban RH, Theise ND. WHO Classification of Tumours of the Digestive System, 4th ed. Geneva, Switzerland: WHO Press, 2010.

18. Chen F, Zhang L, Wu J, Hao F, Ren X, Zheng J, et al. HCRP-1 regulates EGFR-AKT-BIM-mediated anoikis resistance and serves as a prognostic marker in human colon cancer. Cell Death Dis 2018;9:1176-018:1217-2.

19. Eisenhoffer GT, Rosenblatt J. Bringing balance by force: live cell extrusion controls epithelial cell numbers. Trends Cell Biol 2013;23:185–92.

20. Aoudjit F, Vuori K. Integrin signaling in cancer cell survival and chemoresistance. Chemother Res Pract 2012;2012:283181.

21. Beaumier M, Noel D, Thibodeau S, Bouchard V, Harnois C, Beaulieu JF, et al. Integrin/Fak/Src-mediated regulation of cell survival and anoikis in human intestinal epithelial crypt cells: selective engagement and roles of PI3-K isoform complexes. Apoptosis 2012;17:566–78.

22. Hamidi H, Ivaska J. Every step of the way: integrins in cancer progression and metastasis. Nat Rev Cancer 2018;18:533–48.

23. Campbell FC, Loughrey MB, McClements J, Deevi RK, Javadi A, Rainey L. Mechanistic insights into colorectal cancer phenomics from fundamental and organotypic model studies. Am J Pathol 2018;188:1936–48.

24. Weibel ER. Stereological principles for morphometry in electron microscopic cytology. Int Rev Cytol 1969;26:235–302.

25. Lino-Silva LS, Salcedo-Hernandez RA, Herrera-Gomez A, Padilla-Roschiano A, Ramirez-Jaramillo M, Herrera-Goeoepfert RE, et al. Colonic cribriform carcinoma, a morphologic pattern associated with low survival. Int J Surg Pathol 2015;23:13–9.

26. Montironi R, Cimadomare A, Gasparrini S, Mazzucchelli R, Santoni M, Massari F, et al. Prostate cancer with cribriform morphology: diagnosis, aggressiveness, molecular pathology and possible relationships with intraductal carcinoma. Expert Rev Anticancer Ther 2018;18:685–93.

27. Qu Y, Lin H, Zhang C, Li K, Zhang H. Cribriform pattern in lung invasive adenocarcinoma correlates with poor prognosis in a Chinese cohort. Pathol Res Pract 2019;215:347–53.

28. Barresi V, Reggiani Bonetti L, Ieni A, Caruso RA, Tucci G. Histological grading in colorectal cancer: new insights and perspectives. Histol Histopathol 2015;30:1059–67.

29. Chandler I, Houlston RS. Interobserver agreement in grading of colorectal cancers-findings from a nationwide web-based survey of histopathologists. Histopathology 2008;52:494–9.

30. Ueno H, Kajiwara Y, Shimazaki H, Shintoh E, Hashiguchi Y, Nakanishi K, et al. New criteria for histologic grading of colorectal cancer, a morphologic pattern associated with low survival. Int J Surg Pathol 2015;23:13–9.

31. Rogers AC, Winter DC, Heeney A, Gibbons D, Lugli A, Kirsch R, Ajioka Y, Bosman F, Cathomas G, Dawson H, et al. Recommendations for reporting tumor budding in colorectal cancer based on the ITBCC 2016. Mod Pathol 2017;30:1299–311.

32. De Smidt L, Palsmans S, Andel D, Govaere O, Boeckx B, Smeets D, et al. Expression profiling of budding cells in colorectal cancer reveals an EMT-like phenotype and molecular subtype switching. Br J Cancer 2017;116:58–65.