Biochemical and Clinical Approaches in Evaluating the Prognosis of Colon Cancer

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Abstract. *Background: Colorectal adenocarcinoma is a common malignant neoplasm in the Western world. To achieve optimal treatment results, the risk estimation of recurrence should be as accurate as possible. Materials and Methods: Tissue material from tumour and normal mucosa was taken from six patients and was analysed to screen aberrantly expressed genes using cDNA microarray. Selected up-regulated genes were chosen for further analysis by immunohistochemistry. For this purpose a tissue array material of 114 colorectal cancer patients was obtained. In addition to the routinely used proliferation marker Ki-67, the analysed proteins included securin and CDC25B. Results: Processes such as cellular defense, cell structure, motility and cell division were found to be notably represented among the most deregulated genes. A significant portion of the overexpressed genes included those functioning in the cell cycle. Immunohistochemical stainings of securin and CDC25B showed a consistent expression pattern with that of cDNA microarray analysis. There was no statistical association between the studied proliferation markers and survival. Instead, there was a significant association between the Dukes' class and the histological grade (p=0.04), but not between histological grade and survival. The survival of Dukes' B patients was significantly poorer if no regional lymph nodes were studied compared with the Dukes' B patients with even a single lymph node was studied (p=0.04, hazard ratio 2.7). Conclusion: Tumour stage is superior in estimating the prognosis of patients with colonic cancer compared with the grading of cell cycle regulators or histological grade of the cancer. The study of regional lymph nodes is essential to identify the patients who would benefit from adjuvant chemotherapy.*

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Colorectal cancer is one of the most common malignant neoplasms in the Western world. During the last years the prognosis has improved due to improvements in diagnostics, surgery and oncology. The prognosis in stage I colon cancer (Dukes' A) is excellent and no additional treatment is needed after surgery. The challenge is the treatment of stage II-III (Dukes' B-C) colon cancer patients, whose prognosis can be improved by adjuvant treatments (1, 2). The prediction of treatment efficacy in disseminated disease (stage IV) would also be beneficial. The markers that may predict the efficacy of chemotherapy have been identified (3). However, new prognostic factors would be helpful to focus the intensive adjuvant chemotherapy on high-risk patients. Microarray techniques have yielded promising results in developing new treatments and the estimation of prognosis in some tumours (4, 5), for instance in haematological malignancies (5).

In this study, cDNA microarray was used as a screening method in a small group of colon cancer patients to identify up- and down-regulated genes. Selected up-regulated genes involved in cell cycle regulation were chosen for further analyses. The association between their expression level and survival of colon cancer patients was studied in a tissue array material of 114 colon cancer patients using immunohistochemistry. In addition, clinical and routine histological characteristics of these patients were analysed.

Materials and Methods

Patients. The material was obtained from colon cancer patients treated in Turku University Central Hospital, Finland. Fresh frozen tissue material was obtained from tumours of six patients with colon cancer. This was used for cDNA microarray to screen aberrantly expressed genes. Tissue samples from corresponding normal mucosa from the same patients were used as reference material. The TNM stage at the time of diagnosis was T1N0M0 for one patient, T2N0M0 for one patient, T3N0M0 for two patients, T3N2M0 for one patient and T3NxM1 for one patient. The detailed characteristics of the patients are reported in Table I. The study applied a cDNA microarray including approximately 4,000

probes of genes with a proven or suspected role in cancer, including genes involved in cell cycle and proliferation, development, transcription, signalling, redox-reactions, metabolism and tumour suppression, as well as genes related to cellular and extracellular structures ("Cancer Chip", the Turku Centre for Biotechnology, University of Turku and Abo Akademi University, Turku, Finland).

For further analysis with paraffin-embedded tissue array material, a population of 114 colon cancer patients, treated during 1993-2000 was collected. Because Dukes' classification had been used at that time for staging, it was also used for the material in the current study. The Dukes' class was A for 14 patients, B for 62 patients, C for 21 patients and D for 17 patients. The study had research approval from the Ethical Committee of Turku University Hospital, approval for using paraffin material from the National Authority for Medicolegal Affairs, and informed consent was obtained from each patient of the cDNA microarray group. The research was carried out in accordance with the Declaration of Helsinki.

Purification of total RNA. The samples used in the cDNA microarray study were dissected by a pathologist, fresh-frozen in liquid nitrogen within 10 min after surgical removal and stored at –70˚C. The total RNA was isolated by the guanidine isothiocyanate / acid phenol method (6) and further purified using RNeasy kit (Qiagen, Hilden, Germany). The concentration and the purity of RNA were determined spectrophotometrically (Ultrospec 1100pro, Amersham Pharmacia Biotech, Uppsala, Sweden). The quality of RNA was analysed by agarose gel electrophoresis by evaluating the integrity of ribosomal RNAs.

cDNA microarray labelling and hybridization. Twenty-five μg of tumour and reference RNAs were fluorescently labelled (CyDye, Amershambiosciences, Buckinghamshire, England) with Cy5 and Cy3, respectively, as described previously (7) with minor modifications. Four hundred units of Superscript II reverse transcriptase (Gibco BRL Life Technologies, Rockville, MD, USA) were used in cDNA synthesis. Hybridisation was performed in the volume of 80 μl under LifterSlips (Erie Scientific Company, Portsmouth, NH, USA) at 65˚C overnight. Each sample was hybridised once.

Pre-processing and statistical analysis of cDNA microarray data. For each sample, the raw data consisted of ~4,080 transcripts, where the expression ratio between study case and reference case was determined for each transcript. The expression ratios were first log2-transformed and then intensity-normalised with a locally weighted scatterplot smoothing (LOWESS; 8). There were three technical replicate spots for each transcript on the array. The quality of each spot was determined visually and data were pre-processed by calculating the signal over the whole data so that only spots with acceptable quality were taken into account. When there were three proper values, the median was used to calculate the expression ratio of the transcript in question. In case of two proper values, their mean was used and in case of only one proper value the value was used as such. If no proper value was found, then the transcript was marked as missing value for the sample under study. Only transcripts with more than three proper signal values among the six samples were subjected to statistical analyses. For each transcript, the pre-processed log-ratios were compared to zero with one-sample Student's *t*-test. A transcript was considered up-regulated if its mean value was larger than 0.5 and the *p*-value of the *t*-test was less than 0.05. Similarly, a transcript was considered down-regulated if its mean value was less than –0.5 and the *p*-value was less than 0.05. To explore functions of the genes identified, we used web-based databases Entrez Gene and Online Mendelian Inheritance in Man at National Center for Biotechnology Information (http://www.ncbi.nih.gov), as well as Gene Ontology Annotation at European Bioinformatics Institute (http://ebi.ac.uk/ GOA).

Table I. *Characteristics of colorectal cancer patients included in screening of aberrantly regulated genes using cDNA microarray (n=6) and those included in tissue array analyses (n=114), all treated in the Turku University Central Hospital.*

Characteristics	cDNA microarray	Tissue array
Gender (M/F)	3/3	46/68
Median age		
years (range)	74 (31-82)	69 (35-89)
Grade		
1	1	26
2	4	68
3	1	20
Dukes' class		
A	2	14
B	2	62
C	1	21
D	1	17
TNM-class		
T1-2N0-XM0 (Stage I)	2	14
T3-4N0-XM0 (Stage II)	2	62
T3-4N1-2M0 (Stage III)	1	21
T3-4N0-2M1 (Stage IV)	1	17
Time of diagnosis	2003-2004	1993-2000
All patients	6	114

Immunohistochemistry. Tissue microarrays (TMAs) were constructed, as described previously (9). Briefly, TMAs were prepared from formalin-fixed and paraffin-embedded archive material by selecting representative areas of colorectal cancer for 3-mm thick tissue cylinders. Specimens gathered on TMAs included a specimen pair, one from a cancerous tissue area and the other from a normal mucosal area from each patient. The TMAs and blocks representative of carcinoma tissue used in cDNA microarray study were cut at 5 μm and stained with human monoclonal antibodies for securin (Abcam, Cambridge, United Kingdom), CDC25B (Abcam) and Ki-67 (DakoCytomation, Glostrup, Denmark). Automated stainer was used in Ki-67 immunohistochemical staining. Securin and CDC25B were stained manually. Antigen retrieval was performed by heating two times for 7 minutes in microwave oven in 10 mM sodium citrate, pH 6. Endogenous peroxidase activity was blocked incubating the slides in 0.3% hydrogen peroxide in TBS. Primary antibodies were applied at the following concentrations: securin and CDC25B 1:20, Ki-67 1:100. Detection was performed using biotin-avidin reaction (Vectastain ABC reagent, Vector Laboratories, Burlingame, CA, USA) and diaminobenzidine chromogen.

Table II. *Most deregulated genes in colorectal cancer compared with paired normal mucosa in cDNA microarray material. The aberrantly expressed genes are presented according to the grouping by Adams et al. (16). Observed fold change of gene expression included.*

Cellular process	Overexpression		Underexpression		
	Gene	Change	Gene	Change	Portion
Cell division and DNA synthesis (4.4%)[a]	CA9	4.1	UBA52	2.5	24.1%[b]
DNA synthesis / replication,	GRO1	3.7	PTPRB	2.3	
apoptosis, cell cycle,	S100A11	3.2			
chromosome structure	UBCH10	2.7			
	MYC	2.6			
	CDC25B	2.3			
	PTTG1	2.2			
	IER3	2.0			
	CDK4	2.0			
	CSF1R	1.9			
	TK1	1.9			
	CCND1	1.9			
Cell signalling / communication (12.4%)[a]	EPHB3	2.3	HSD11B2	4.8	10.3%[b]
adhesion, intracellular transducers,	CD44	2.0	PTPRB	2.3	
channels / transport proteins, receptors,	THY1	1.9	MAOA	2.3	
effectors / modulators, metabolism,					
protein modification					
Cell structure and motility (8.1%)[a]	COL1A1	4.1	KRT20	6.1	19.0%[b]
cytoskeletal, extracellular matrix,	S100A11	3.2	KRT8	4.4	
microtubule associated proteins /	FN14	3.0	GSN	3.8	
motors	CD44	2.0	MYL6	3.1	
	MMP3	1.9	SYNPO2	2.6	
	BGN	1.9			
Cell / organism defense (11.9%)[a]	CA9	4.1	IGL	19.0	29.3%[b]
DNA repair, stress response,	GRO1	3.7	CA1	10.5	
carrier proteins / membrane transport,	GRO3	3.1	CA2	8.7	
immunology	GRO2	2.5	MT1G	3.9	
	IFITM2	2.4	MT1L	3.2	
	ISG15	2.0	HLA-C	2.9	
	CSF1R	1.9	B2M	2.8	
	GPX2	1.9	MT1E	2.6	
			MT1H	2.1	
Gene / protein expression (21.9%)[a]	UBCH10	2.7	EGR3	5.3	12.1%[b]
RNA polymerases, RNA processing,	MYC	2.6	PIGF	3.4	
transcription factors, ribosomal proteins,	PTTG1	2.2	EEF1A1	2.8	
tRNA synthesis / metabolism,			UBA52	2.5	
translation factors, protein turnover,					
post transcriptional modification / targeting					
Metabolism (16.4%)[a]			CKB	4.4	3.4%[b]
amino acid, cofactors, lipid, nucleotide,			CKMT2	2.3	
sugar / glycolysis, TCA / energy,					
protein modification, transport					
Unclassified (24.8%)[a]			MST1	2.6	1.7%[b]

Distribution of genes in different groups (%): [a]in the study by Adams *et al.* (16); [b]in the current study.

Analysis of immunohistochemical reactions. For securin, CDC25B and Ki-67, three high power fields from each TMA-sample were studied for the fraction of positive nuclei. Each cancerous sample was compared with its paired control taken from normal mucosal area. To be classified as positive, the fraction of positive cells was 20% for CDC25B and Ki-67 12% for securin and at least double compared with its paired control.

Statistical analysis of clinical and immunohistochemical factors. Main statistical analysis was performed using survival analysis. The time from surgery to death or to end of follow-up (5 years) was analysed using Kaplan-Meier and Cox regression. In Kaplan-Meier, several classification variables were compared. In Cox regression, hazard ratios were calculated to estimate the magnitude of a possible difference between categorical variables, which were analysed using

Chi-square-test or Fisher's exact-test. Logistic regression was used to analyse binary response variables (for example death caused by disease within 5 years or not) using logit-link and cumulative logit-link in ordinal response situation. In both logistic regression modelling types, several classification variables were explanatory factors. Results from logistic regression are expressed using odds ratios and cumulative odds ratios. All results (both survival analysis and logistic regression) are expressed using univariate modelling. Cox regression was also analysed adjusting with age and gender. In this multivariate model, neither age nor gender was statistically significant, and those variables were removed from the final model. A p-value less than 0.05 was considered significant. The statistical analyses were carried out using SAS/STAT(r) software, Version 9.1.3 SP3 of the SAS System for Windows.

Results

cDNA microarray. Sixty-nine and 89 among the approximately 4,000 studied transcripts were found up- and down-regulated in tumour compared with normal colonic mucosa, respectively. The 48 most over- or under-expressed genes, ranked by the magnitude of expression change, are presented in Table II, together with their proposed cellular functions. A significant portion of the over-expressed genes are concerned with cell division. These included, for example, UBCH10, CDC25B, CDK4, CCND1, MYC and IER3. Several genes involved in cell structure and motility or host and cellular defense were also differently expressed in tumour tissue.

Confirmation of up-regulated genes on protein level. The up-regulation of pituitary tumour-transforming 1 gene (PTTG1, also known as securin) and CDC25B were confirmed at protein level in paraffin material obtained from the same patients from whom the cDNA microarrays were done. The fraction of positive cells was higher in tumour area compared with the normal mucosa corresponding to the results obtained from cDNA microarray. The reaction was only nuclear with CDC25B antibody, while there was an additional cytoplasmic reaction with securin antibody.

Tissue array. Securin and CDC25B were chosen for further analyses to compare their expression level with survival in a tissue array material of 114 colon cancer patients. In addition, Ki-67, a gold standard for proliferation, was included in the analysed markers. There was an association in positive reactions between CDC25B and securin ($p<0.01$, χ^2-test), but not with Ki-67. There was no association between the expression levels of the proliferation markers and survival (Table III). Positive reactions with securin and CDC25B in tissue array slides are demonstrated in Figures 1a-b.

Tumour stage. Tumour stage had a profound effect on survival. In a tissue array material of 114 patients, the five-year-survival was 92.9% for Dukes' A patients (13 out of 14), 64.5% for Dukes' B patients (40 out of 62), 42.9% for Dukes' C patients (9 out of 21) and 5.9% for Dukes' D patients (1 out of 17), (Table III, Figure 2).

Table III. *Disease-free survival of patients treated in Turku University Central Hospital during the years 1993-2000, clinical and cell biological characteristics. Patients in Dukes' B class were subdivided by TNM-classification into groups with or without studied regional lymph nodes.*

Parameter	Disease-free survival (5 years)	Disease-free survival % (5 years)
Dukes' class		
A	13/14	92.9%
B	40/62	64.5%
C	9/21	42.9%
D	1/17	5.9%
TNM-class		
T1-2N0-XM0 (stage I)	13/14	92.9%
T3-4N0M0 (stage II)	23/29	79.3%
T3-4NXM0 (stage II)	17/33	51.5%
T3-4N1-2M0 (stage III)	9/21	42.9%
T3-4N0-2M1 (stage IV)	1/17	5.9%
Grade		
1	15/26	57.6%
2	40/68	58.8%
3	8/20	40.0%
Cell biological markers		
Securin up-regulated / survived	17/29	58.6%
Cdc25B up-regulated / survived	22/37	59.5%
Ki-67 up-regulated / survived	41/80	51.3%
All patients	65/114	56.0%

Nodal status and Dukes' B patients. Dukes' B (stage II) patients were subdivided in groups where at least one lymph node was studied (Dukes' B(N0), 29 patients), or no lymph nodes were found (Dukes' B(Nx), 33 patients). The patients without studied lymph nodes had a significantly worse disease-free survival (17/33, 51.5%) compared with those with studied lymph nodes (23/29, 79.3%) ($p=0.04$, hazard ratio 2.7, confidence interval: 1.066-6.968). The results are summarised in Table III and Figure 3.

Histological grade. There was a slight tendency that patients with grade 3 cancer had a worse survival than patients with grade 1-2 cancer, but the difference was not statistically significant ($p=0.09$, hazard ratio 1.783). Instead, high histological grade was statistically more common in advanced tumours (Dukes' C *versus* B: $p=0.04$, cumulative odds ratio 2.955, confidence interval: 1.069-8.168; Dukes' D *versus* B: $p=0.05$, cumulative odds ratio 2.990, confidence interval: 0.998-8.954).

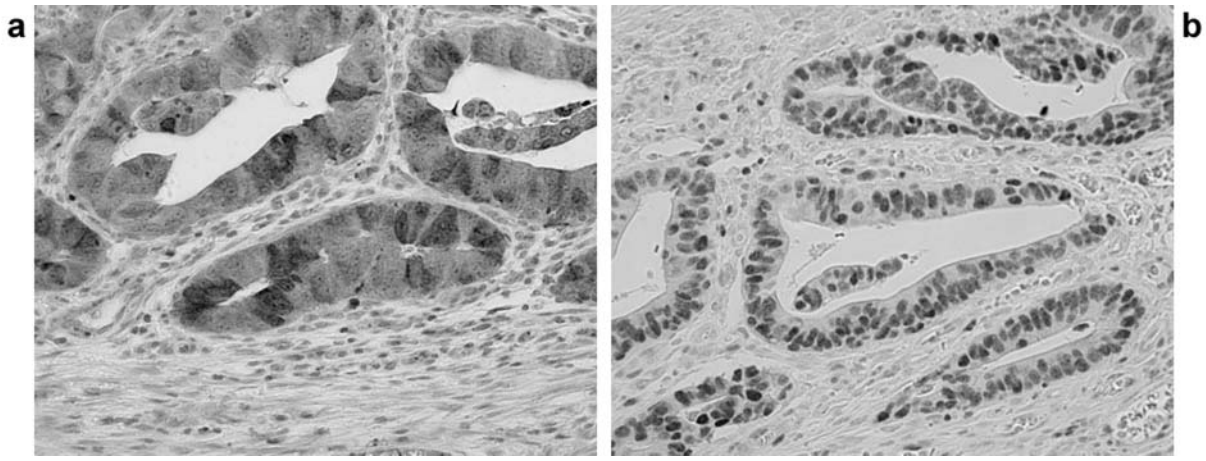

Figure 1. *Tissue array material. a) Securin in colorectal adenocarcinoma as shown by immunohistochemistry. The reaction is both nuclear and cytoplasmic. b) CDC25B in colorectal adenocarcinoma as shown by immunohistochemistry. The reaction is exclusively nuclear.*

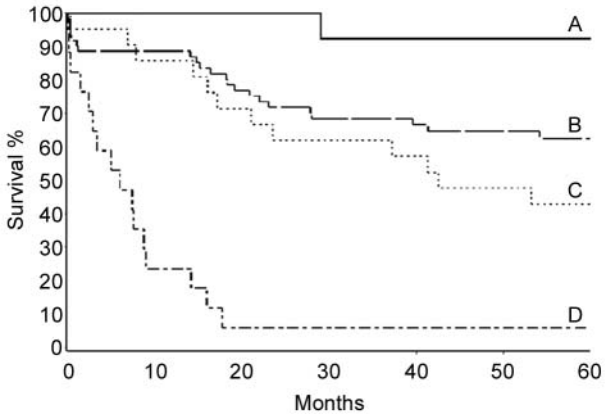

Figure 2. *Five-year survival of 114 patients included in tissue array material treated in Turku University Central Hospital between 1993-2000. Dukes' classification was used in staging.*

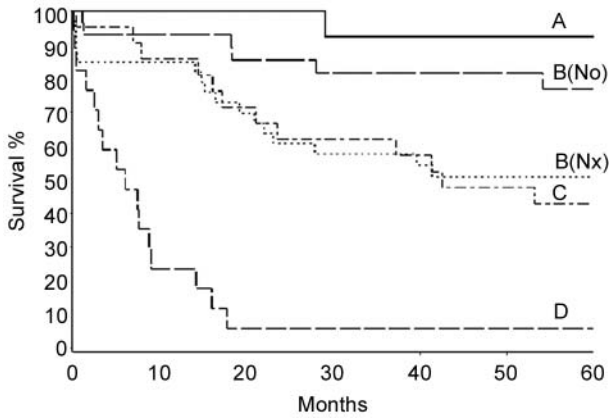

Figure 3. *Five-year survival of 114 patients included in tissue array material treated in Turku University Central Hospital between 1993-2000. Dukes' classification was used in staging. Additionally, Dukes' B patients where subdivided in groups were at least one lymph nodes was studied (Dukes' B(No)) or no lymph node were found (Dukes' B(Nx)). A statistically significant difference in survival was observed between these groups (p=0.04).*

The time of diagnosis. There was a tendency that patients diagnosed during 1993-1996 had worse survival than patients diagnosed during 1997-2000, but the difference was not statistically significant ($p=0.07$, log-rank test).

Discussion

The Human Gene Anatomy Project put together an assembly of 37 distinct organs and tissues transcriptomes at various developmental stages and disease states containing almost 90,000 distinct sequences. Of these, approximately 7,000 genes with known or putative functions were identified. These genes were classified into six broad categories: [1] cell division, [2] cell signalling, [3] cell structure and motility, [4] cell and host defense, [5] gene and protein expression and [6] metabolism. Each category was further divided into sub-role categories (Table II). A seventh broad category, unclassified, for proteins and genes with unassigned roles was also appointed (10). The most up- and down-regulated genes in our material included representatives of all the above mentioned cellular processes (Table II). Some genes appear in more than one category, such as the case in the original division by Adams *et al*. (10).

Twenty-nine percent of the most deregulated genes in current colorectal carcinoma cDNA microarray study were involved in host and cellular defense. This is considerably more than expected from division by Adams *et al*. (10). For instance,

cellular defense against reactive oxygen species includes different enzymatic responses and DNA repair (11). Metallothioneins comprise a family of enzymes associated with protection against DNA damage, oxidative stress, apoptosis, metal ion homeostasis and detoxification. Aberrant expression of metallothioneins has been related to various human tumours, but there are quite few reports about expression of different isoforms. Use of metallothionein expression as a marker of tumour differentiation, cell proliferation and prognosis predictor is unclear (12, 13). Four metallothionein isoforms were identified to be down-regulated. Another group of enzymes of host defense are carbonic anhydrases, which have an important role in maintaining the pH homeostasis. Our finding of under-expressed carbonic anhydrase I and II, as well as over-expressed IX is in line with colorectal cancer literature (14, 15).

Of the most deregulated 48 genes, those functioning in cell division and DNA synthesis gained our special attention. As many as 12 out of 24 overexpressed genes could be related to this category, corresponding altogether to 14 out of the 48 (24%) most differently expressed genes; still, in average tissue only a minority of expressed genes is expected to belong to this class (10). Assessment of proliferation has become popular in histopathology as a means of predicting the behaviour of tumours. Proliferative activity can be used as a marker of clinical utility in identifying subsets of patients with poor prognosis (16, 17). We chose two genes – PTTG1 (securin) and CDC25B – for further evaluation as potential prognostic markers in colorectal cancer. There is some previous evidence for significance of these genes in human colorectal tumorigenesis (18, 19). Both of the chosen genes are related to cell cycle regulation. Securin and CDC25B were found to be overexpressed at protein level in part of studied colorectal tumours, but neither of them showed statistical significance for survival. In some studies, the up-regulation of CDC25B was linked to poor prognosis (19). There are at least five splicing variants of CDC25B, which seem to be differentially regulated in colorectal cancer (20). Of these, CDC25B2 has been linked to aggressiveness of non-Hodgkin's lymphomas (21) and to the grade of differentiation in colorectal cancer, but not with proliferative activity (20). Instead, it was suggested to be associated with the enhanced probability of micrometastasis (19). CDC25B2 was reported to be more stable than B1 or B3 (22) and its overexpression was related to greater activity as mitotic inducer (23). The antibody used in current study was raised using human full length CDC25B protein as immunogen (Abcam).

In our material, securin was up-regulated on the protein level in a group of cancer cases, but no prognostic significance on survival could be found. Securin was up-regulated in a much smaller group of patients than Ki-67, for instance. This may be linked to the fact that securin is expressed during anaphase of cell cycle (24), while Ki-67 is positive in nuclei during a broader phase of cell cycle (25, 26). Anyway, up-regulation of securin was linked to an adverse prognostic factor in many cancers (27, 28). In addition, the reason why the up-regulation of securin on protein level had a statistical association with the up-regulation of CDC25B, but not with Ki-67 remains to be elucidated.

As this study also demonstrated, the tumour stage is superior in estimating the prognosis of colorectal cancer patients compared with other factors. This study population was collected at the time when the crucial significance of the accurate regional lymph node status was not yet fully appreciated. Of the 62 Dukes' B patients of this material, no lymph nodes were found in 33 patients. The prognosis of this group of patients was significantly worse than those Dukes' B patients with even a single studied lymph node ($p=0.04$). Of course nowadays, only a macroscopic analysis of mesenterial fat tissue to assess the regional lymph node status is no longer acceptable. There are many studies where the minimum of the sampled regional lymph nodes is at least 12 (29), which is also the current consensus in Turku University Central Hospital.

The histological grade of colorectal cancer was linked to poor survival in several studies (30), although it did not reach to a statistically significant level in this study ($p=0.09$). However, high histological grade was statistically more common in advanced tumours ($p=0.04$). Probably, high-grade cell clones have had time to develop in advanced tumours. As with proliferation markers, a larger study population would probably have shown a difference in survival in groups of high histological grade and high expression of proliferation marker levels.

In conclusion, the cell proliferation markers CDC25B and securin or histological grade did not show a statistical significance as prognostic factors in colorectal cancer in this study. The accurate assessment of the pathological stage of the disease is essential for choosing an optimal treatment for each patient. This also concerns the sufficient sampling of regional lymph nodes to identify the patients with regional lymph node metastases.

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