

Prognostic Estimation of Survival of Colorectal Cancer Patients With the Quantitative Histochemical Assay of G6PDH Activity and the Multiparameter Classification Program CLASSIF1

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Prognosis of colorectal cancer patients that show similar histopathology may vary substantially. An attempt was made to improve prognosis by the self-learning classification program CLASSIF1, based on automated multiparameter analysis of quantitative histochemical and clinical parameters of 64 colorectal carcinomas and adjacent normal mucosae. The histochemical parameters applied were the oxygen-insensitivity assay of glucose-6-phosphate dehydrogenase (G6PDH) activity, a valid discriminator between normal and cancerous mucosae, and related parameters CuZn- and Mn-superoxide dismutase (SOD) levels, and lipid peroxidation (LPO) capacity. Data were processed on the basis of a postoperative follow-up of minimally 32 and maximally 56 months. CLASSIF1 selected the parameters oxygen insensitivity of G6PDH activity, CuZn-SOD and Mn-SOD levels, LPO capacity, lymph node metastasis, Dukes' stage, and age for the highest prognostic value. On the basis of these selected parameters, CLASSIF1 correctly predicted favorable outcome in 100% of the surviving patients and fatal outcome in 64% of the deceased patients. G6PDH activity appeared to be the major information carrier for CLASSIF1. On the basis of G6PDH activity parameters alone, 96% of the surviving patients and 55% of the deceased patients were correctly classified. In comparison, estimation of prognosis on the basis of Dukes' stage alone resulted in 71% correctly classified surviving patients and 61% of patients who died. It is concluded that the self-learning classification program CLASSIF1, on the basis of quantitative histochemical and clinical parameters, is the best prognostic estimator for colon cancer patients yet available. *Cytometry (Comm. Clin. Cytometry)* 38:176–183, 1999. © 1999 Wiley-Liss, Inc.

Key terms: colorectal carcinoma; glucose-6-phosphate dehydrogenase; quantitative histochemistry; multiparameter analysis; prognosis

The varying courses of development of colorectal cancer have prompted many attempts to identify factors that are predictive for survival of patients with colorectal cancer. Dukes' classification (1) is such a prognostic indicator. Other prognostic factors include depth of penetration (2), invasion in blood vessels (3) or lymph nodes (4), and histological grading (5). However, prognosis of patients with colorectal carcinomas that show similar histopathological appearance may vary considerably because of unknown specific biological characteristics of the individual tumors (6).

Activity of glucose-6-phosphate dehydrogenase (G6PDH), the regulatory enzyme of the pentose shunt pathway, increases in early stages of malignancy before any morphological changes are apparent (7,8). G6PDH activity can be demonstrated histochemically in cryostat sections by reduc-

tion of a tetrazolium salt into its water-insoluble colored formazan, and this is a quantitative measure of activity (9). When the histochemical reaction is performed with neotetrazolium as tetrazolium salt in the absence of oxygen in the incubation medium, formazan production gives a

Abbreviations: AMI, average multiplicity index; ARI, average recognition index; CLASSIF1, self-learning classification program; CuZn-SOD, copper, zinc-superoxide dismutase; G6PDH, glucose-6-phosphate dehydrogenase; LPO, lipid peroxidation; Mn-SOD, manganese-superoxide dismutase; PBS, phosphate-buffered saline; RA, residual activity.

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proper reflection of G6PDH activity in all cell types (10). However, in the presence of oxygen, formazan deposition is prevented in normal epithelia but not in cancerous epithelia of the colon (11–13), stomach (11), breast (14), and bronchus (15). This so-called oxygen insensitivity of cancer cells is developed in a gradual manner during the carcinogenetic process (13). Metabolic backgrounds of the oxygen sensitivity of the histochemical G6PDH reaction in normal mucosae and oxygen insensitivity in cancerous mucosae have been described (16). On the basis of these studies, we concluded that discrimination between normal and cancerous mucosae is based on rapid inactivation of G6PDH in normal cells by lipid peroxidation (LPO) products in the presence of oxygen during the histochemical assay. Sufficient levels of endogenous superoxide dismutase (SOD) and LPO capacity facilitate generation of these LPO products during the histochemical reaction in oxygen, leading to rapid inactivation of G6PDH and thus to lack of staining. A series of metabolic changes in cancer cells such as decreased levels of SOD (17) and LPO capacity (16) and elevated levels of G6PDH activity (13,16) slows down this inactivation process, and thus staining is observed in cancer cells.

In the present study, we investigated which quantitative histochemical, histopathological, and/or clinical parameters or their combinations are the best prognostic indicators for survival of colorectal cancer patients, using the newly developed classification program CLASSIF1, that allows a standardized self-learning and simultaneous classification of multiple measurements (18–21). Our parameters included the quantitative histochemical insensitivity assay of G6PDH activity, quantitative histochemical assays to determine expression of CuZn-SOD, Mn-SOD, and LPO capacity, and clinical and histopathological information on patients, including Dukes' stage, age of patients, lymph node metastasis, blood vessel invasion, and differentiation grade.

MATERIALS AND METHODS

Tissue Specimens

Sixty-four samples of human colorectal carcinomas with adjacent histologically normal mucosal tissue were obtained from the Department of Pathology, Københavns Kommunes Hospital (Copenhagen, Denmark). After resection, the tissue samples were immediately frozen in liquid nitrogen and stored at -80°C until used. Serial cryostat sections were cut at a cabinet temperature of -25°C on a motor-driven cryostat (Bright, Huntingdon, UK) at a low but constant speed, mounted on clean glass slides, and stored at -80°C until used. Pathological examination of the sections was performed after hematoxylin-eosin staining by two pathologists (U.H., J.-Y.S.). Follow-up of the patients lasted minimally 32 and maximally 56 months after surgical resection. When the cause of death was not related to colorectal cancer, patients were excluded from the present study. The cohort of patients who were included consisted of 41 men (median age, 74 years; range, 56–88 years) and 23 women (median age, 75 years; range, 41–86 years). The following patient information was

also obtained: location of the carcinoma in the colorectum, Dukes' stage, presence of lymph node metastasis and/or blood vessel invasion, differentiation grade of the tumor, and survival time.

Enzyme Histochemistry

Incubation media for demonstration of G6PDH were prepared according to Van Noorden and Frederiks (22), and consisted of 100 mM phosphate buffer (pH 7.45) containing 18% (w/v) polyvinyl alcohol (hot water-soluble, weight average Mr 70,000–100,000; Sigma Chemical Co., St. Louis, MO), 10 mM glucose-6-phosphate (Boehringer, Mannheim, Germany), 0.8 mM NADP (Boehringer), 4.5 mM neotetrazolium chloride (Polysciences, Northampton, UK), and 0.45 mM 1-methoxyphenazine methosulfate (Serva, Heidelberg, Germany). Incubation media were poured into glass vials and equilibrated for at least 10 min at 37°C in an atmosphere of either nitrogen or oxygen, using a tonometer in order to avoid formation of gas bubbles in the viscous media (23). The flow rate of the gases was 500–800 ml/min. Immediately before incubation, sections were taken from the storage cabinet at -80°C and air-dried for 5 min at 37°C . After placing plastic rings around the sections, media were poured onto the sections and coverslips were placed on top of the rings in order to keep the incubation media saturated with either oxygen or nitrogen. After 10 min of incubation at 37°C , the reaction was stopped by rinsing off the viscous polyvinyl alcohol-containing medium from the sections in 100 mM phosphate buffer (pH 5.3, 60°C). Afterwards, sections were mounted in glycerol jelly.

Immunohistochemistry

Commercially available sheep polyclonal antibodies against human CuZn-SOD and Mn-SOD (The Binding Site, Birmingham, UK) were used. CuZn-SOD and Mn-SOD expression was detected in serial 6- μm -thick cryostat sections, as described previously (17). Briefly, sections were fixed in 4% formaldehyde for 2 min and then incubated in a graded ethanol series at room temperature for 10 min. After one rinse in phosphate-buffered saline (PBS; pH 7.4), endogenous peroxidases were blocked in PBS containing 0.3% H_2O_2 and 0.1% NaN_3 (15 min). Sections were rinsed in PBS and preincubated in PBS containing 10% normal rabbit serum, and were subsequently incubated for 60 min with the primary antibodies against CuZn-SOD and Mn-SOD (1:100). Sections were rinsed in PBS and then incubated with horseradish peroxidase-labeled rabbit anti-sheep immunoglobulins (Dako, Glostrup, Denmark; 1:200 in PBS, 30 min at room temperature). After three rinses in PBS, sections were incubated for 10 min at room temperature in 50 mM Tris-HCl buffer (pH 7.6) containing 3,3'-diamino-benzidine tetrahydrochloride (DAB; Sigma) and 0.3% hydrogen peroxide, rinsed with distilled water and mounted in glycerol jelly. In every run, negative controls were included by replacing the primary antibody with either nonimmune sheep IgG or by PBS.

Histochemical Detection of LPO

The procedure for histochemical detection of LPO was a modification of the method described by Thomas et al. (24). Cryostat sections were dried (5 min, room temperature), fixed in 5% trichloroacetic acid, and rinsed in distilled water. Sections were then incubated (60 min, 37°C) either in a medium containing 50 mM Tris-maleate buffer (pH 7.4), 150 mM KCl, and 1 mM EDTA which served as control, or in a medium containing 50 mM Tris-maleate buffer (pH 7.4), 150 mM KCl, 25 μ M ascorbic acid, 1 mM ADP (Boehringer), and 15 μ M FeCl₃ as a pro-oxidant system. In our procedure, reduced NADP (NADPH) was replaced by ascorbic acid, which resulted in comparable production of LPO (data not shown). Sections were then incubated overnight at room temperature in distilled water containing 0.9% NaCl, 0.1% (w/v) naphthoic acid hydrazide (Janssen Chimica, Beerse, Belgium) dissolved in dimethylsulfoxide (final concentration, 10% (v/v)), and 5% (v/v) acetic acid. Unreacted naphthoic acid hydrazide was removed by four rinses with 15% (v/v) dimethylsulfoxide in 1 mM HCl. Sections were then incubated in medium containing 100 mM phosphate buffer (pH 6.5) and 0.1% (w/v) Fast Blue B (Serva) for 20 min at room temperature. Before being mounted in glycerol jelly, the sections were rinsed for 5 min with running tap water.

Cytophotometry

Glucose-6-phosphate dehydrogenase activity.

Formazan production was measured with a Vickers M85a scanning and integrating cytophotometer (Vickers, York, UK). All measurements were made at 585 nm, the isobestic wavelength of the formazans of neotetrazolium (25). A Leitz NPL \times 6.3 objective (N.A. 0.50), a bandwidth setting of 65, and a scanning spot with an effective diameter of 3 μ m were applied for the measurements. The mask had a diameter of 63 μ m, and the area scanned in each measurement was thus 3,119 μ m² (22). Three serial sections of each biopsy after incubation in the presence of nitrogen and three after incubation in the presence of oxygen were analyzed. Per section, five areas of cancerous and normal epithelial cells with highest activity in oxygen were selected for each measurement. In exactly the same areas, measurements were made in serial sections that had been incubated in the presence of nitrogen. Arbitrary cytophotometric machine units were converted into amounts of substrate (μ moles) converted per cm³ weight of tissue per min according to Van Noorden and Frederiks (22). Control reactions were performed by omitting substrate and coenzyme from the incubation media, and resulting control values were subtracted from the test values (22). Residual activity (RA) of G6PDH was calculated as percentage of the amount of formazan produced in the presence of oxygen in comparison with that in the presence of nitrogen after 10 min of incubation. We considered cells as oxygen-sensitive when the RA was <20% and oxygen-insensitive when the RA was >20% (11,13,26).

CuZn-SOD and Mn-SOD expression and LPO capacity. CuZn-SOD and Mn-SOD expression was measured as absorbance of polymerized DAB at 480 nm (17,22). LPO

products were measured at the absorbance maximum of 550 nm of the final reaction product (24). Of each biopsy, three serial sections were used. Per section, five measurements were taken in the same areas of normal mucosae and carcinomas that were selected for the oxygen-insensitivity assay. Reproducibility of the immunohistochemical detection methods of both forms of SOD was high, since differences in staining intensity in three consecutive sections of the same biopsy were negligible. The immunohistochemical assay of CuZn-SOD and Mn-SOD expression was validated for quantitative purposes by Van Driel et al. (17). All tissues were treated identically and exposure to all reagents, including DAB and H₂O₂, was kept constant to ensure that the amount of polymerized DAB as marker for both SODs (test minus control) was proportional with section thickness (4–12 μ m) (17). This was in agreement with previous quantitative immunohistochemical methods, on the basis of measurement of polymerized DAB (27–30).

Statistical Analysis

All statistical comparisons of RA and expressions of CuZn-SOD, Mn-SOD, and LPO products were performed with paired Student's *t*-tests at an α -level of 0.01. Multi-regression analysis was performed with JPM software (SAS Institute, Cary, NC). The independent prognostic value of parameters was tested by applying the multivariate Cox regression model. The level of significance was set at $P = 0.05$, and the domain of the correlation coefficients (r) was set at $r \leq 0.3$ and $r \geq 0.3$. Nonparametrical correlation coefficients were calculated by using Spearman correlation analysis.

Data Processing

The data that were used for the CLASSIF1 (Partec, Münster, Germany) evaluation consisted of five clinical parameters (Dukes' stage, age of the patient, lymph node metastasis, blood vessel invasion, and differentiation grade), 12 histochemical parameters, i.e., 6 for cancer tissue and 6 for adjacent normal tissue (G6PDH activity in the presence and in the absence of oxygen, RA of G6PDH, expression of CuZn-SOD and Mn-SOD, and LPO capacity), and 12 calculated parameters based on quantitative histochemical data, i.e., 6 ratios of cancer tissue and normal tissue in the biopsies and 6 differences between cancer tissue and normal tissue in the biopsies.

The CLASSIF1 triple-matrix classification algorithm (18,19) was used to extract prognostic information from the above data by self-learning. Assumptions with respect to parameter distributions were not made. In brief, the triple-matrix algorithm works as follows: symmetric upper and lower percentile values (e.g., 15% and 80%) are calculated for the reference value (= survivor) distribution of each database column (= parameter, e.g., Dukes' stage). All individual database column values are then expressed as the triple-matrix characters +, 0, or -, according to their position above, within, or below the respective reference percentile thresholds.

The CLASSIF1 program determines a so-called “representative classification mask” for each classification state, i.e., either a survivor or a nonsurvivor patient, by selecting the most frequent triple-matrix character for each data column. All patients are subsequently reclassified according to the highest coincidence of their triple-matrix characters with one of the just-determined classification masks of a survivor or a nonsurvivor patient. The classification results are introduced into a confusion matrix of the clinical outcome (dead or alive) on the ordinate and the CLASSIF1 prediction on the abscissa in order to evaluate the quality of the classification. The average recognition index (ARI) serves as a standardized evaluator of classification quality. It is determined as the ratio of the sum of the percentage of correctly classified patients (recognition percentages) in the diagonal boxes of the confusion matrix and the number of classification states. In the present study, this number was 2: survivor and nonsurvivor. When the classification is correct, all diagonal values of the confusion matrix are 100%, and all other values of the confusion matrix are 0%. In practice, the ARI should be >80% for clinical purposes (19).

During subsequent iterations, single data columns or combinations of data columns in all permutations up to a maximum of three columns are temporarily removed from the classification to check for classification improvement. Once improvement is no longer achieved, all database columns that alone or in combination with other columns did not improve the classification result, i.e., the noninformative “noise” parameters, are eliminated. This means that only informative data columns remain in the final classification masks of each classification state.

Multiple classifications, in which a patient is classified both as survivor and nonsurvivor, are caused by either a different biologically defined transition state between classification categories, or a classification error. Errors can be introduced when small data sets or inappropriate percentile pairs are used. The average multiplicity index (AMI) represents a standardized optimization indicator. Optimization can be obtained by variation of percentile pairs as well as by increasing the number of patients and/or the number of database columns. It is determined as the ratio between the total sum of recognition frequencies in the horizontal lines of the confusion matrix divided by the number of classification categories, i.e., 2 (survivor and nonsurvivor). An AMI of 1.00 indicates the absence of multiple classifications (thus none of the patients are classified as both survivor and nonsurvivor), while AMIs, e.g., of 1.10, 1.33, and 1.50 indicate double classification for every tenth, third, and second patient, respectively. This means, in practice, that ARIs are only acceptable for AMIs between 1.00–1.20.

The optimized classifier for each data set is determined by successive learning at different percentile pairs, e.g., 10/90%, 15/85%, 20/80%, 25/75%, and 30/70%, respectively. Optimized classifiers are suitable for the classification of new patients. New patients are classified according to the highest positional coincidence of their triple matrix

with the established reference classification masks of survivor and nonsurvivor patients.

The robustness of the classifier is tested by removing the first and subsequently every fifth survivor, and similarly the first and every tenth nonsurvivor patient from the entire group of patients, thus forming two smaller groups of patients who were classified with the reference classification masks.

RESULTS

Oxygen-Insensitivity Test

The histochemical method for detection of G6PDH activity was specific, since control incubations never resulted in significant formazan production.

In the absence of oxygen, mean G6PDH activity in normal mucosa (mean \pm SD, 3.1 ± 1.0 μ moles G6P converted per min per cm^3 of tissue) was significantly lower than in colorectal carcinomas (mean \pm SD, 4.9 ± 2.6 μ moles G6P per min per cm^3 of tissue; $P < 0.0001$). However, there was overlap of G6PDH activity in normal and cancerous tissue.

In all biopsies of histologically normal-looking mucosae adjacent to carcinomas, RA in oxygen was always <20% (Fig. 1; Table 1). All colorectal carcinomas showed RA >20%. Therefore, all carcinomas were considered oxygen-insensitive without exception (Fig. 1; Table 1). In fact, 63 out of 64 carcinomas showed RA >30%. Only one carcinoma showed a low RA of 21%. The oxygen-insensitivity assay based on the histochemical detection of G6PDH activity with neotetrazolium as final electron acceptor thus proved to be a good discriminator between normal mucosae and colorectal carcinomas.

SOD Expression and LPO Capacity

Both cytoplasmic CuZn-SOD and mitochondrial Mn-SOD were homogeneously distributed in the cytoplasm of epithelial cells of normal mucosae, whereas nuclei were unstained. Control reactions in which nonimmune IgG or PBS replaced the primary antibodies were negative. Colorectal carcinomas showed significantly lower expression of CuZn-SOD and Mn-SOD than adjacent normal mucosae (Table 1).

Both epithelial cells in normal mucosae and carcinomas were stained bluish after being incubated with the prooxidant system followed by staining LPO products. The final reaction product was localized exclusively in the cytoplasm; nuclei remained unstained. In colorectal carcinomas, capacity to undergo LPO was significantly lower than in adjacent normal mucosae (Table 1).

Expression of CuZn-SOD, but not of Mn-SOD, proved to be inversely correlated with oxygen insensitivity in carcinomas ($P = 0.0001$, $r = 0.52$). By means of Cox regression, a correlation between differences in RA in carcinomas and normal mucosae adjacent to the carcinomas and differences in CuZn-SOD expression ($P = 0.0007$, $\alpha = 0.05$) or differences in LPO capacity ($P = 0.04$, $\alpha = 0.05$) were found as well.

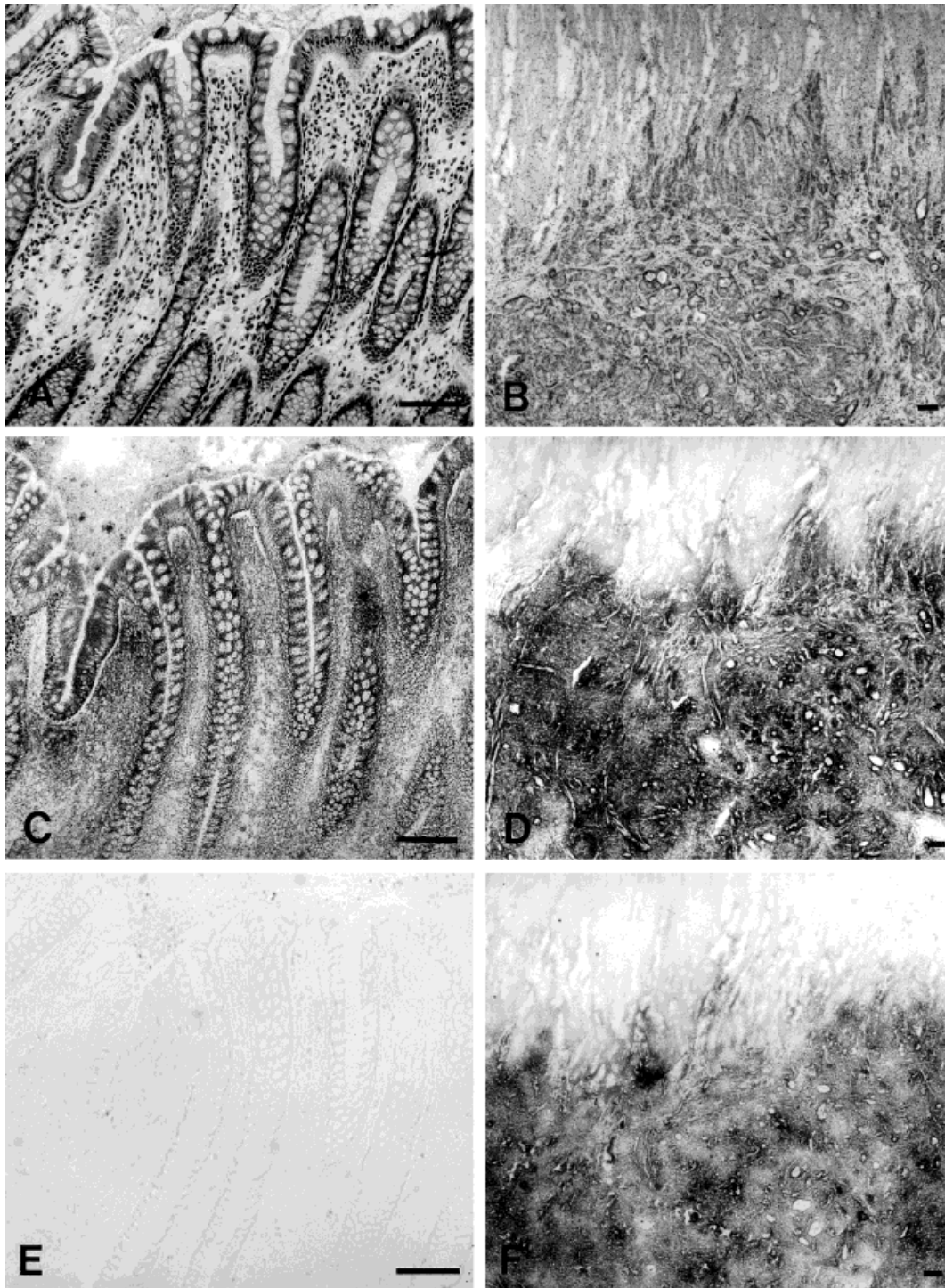


FIG. 1. Photomicrographs of serial sections of normal human colonic mucosae (A, C, E) and colon carcinoma infiltrating the muscularis propria (B, D, F), stained with hematoxylin and eosin (A, B), or for activity of glucose-6-phosphate dehydrogenase in the absence (C, D) or presence (E, F) of oxygen. Staining in C and D represents total activity of the enzyme, whereas lack of staining in E reflects oxygen sensitivity of normal cells. Staining in F reflects oxygen insensitivity of cancer cells. Bars, 100 μ m.

Table 1
Residual Activity of Glucose-6-Phosphate Dehydrogenase Expressed as Percentage and Expression of the Related Parameters CuZn- and Mn-Superoxide Dismutase and Lipid Peroxidation Capacity in Human Colorectal Mucosae and Carcinomas[†]

	Mean ± SD	Range	P-value*
Residual			
G6PDH activity			
Normal	8.6 ± 3.2 ^a	3.6–16.2	P < 0.0001
Carcinoma	49.1 ± 11.7	20.6–76.3	
CuZn-SOD			
Normal	8.9 ± 1.8 ^b	3.5–11.5	P < 0.0001
Carcinoma	6.7 ± 2.0	5.2–12.9	
Mn-SOD			
Normal	7.5 ± 1.3 ^b	4.4–10.4	P < 0.0001
Carcinoma	5.4 ± 1.2	2.4–7.9	
LPO capacity			
Normal	7.7 ± 1.9 ^b	4.8–12.7	P < 0.0001
Carcinoma	4.5 ± 1.8	1.9–10.7	

[†]SD, standard deviation; G6PDH, glucose-6-phosphate dehydrogenase; SOD, superoxide dismutase; LPO, lipid peroxidation; Normal, normal colon mucosa.

^aPercentage.

^bArbitrary machine units.

*Paired Student's *t*-test, α = 0.01.

Prognostic Value of Histochemical, Histopathological, and Clinical Parameters

Classification of the 29 database columns of all 64 patients provided prognostic information with respect to patient survival at the time of surgery, as shown in Table 2A. CLASSIF1 identified 64.3% of the ultimate nonsurvivors, i.e., 27 out of the 42 patients with an AMI of 1.00, i.e., no confusion with survivors occurred. The survival prognosis was correct for all survivors (100%), but 35.7% of the nonsurvivor patients were classified as survivors, i.e., the predictive value was 73.7% (ARI was 82.1).

Classification was achieved with only 11 of the 29 parameters available (Table 3). The majority of parameters, i.e., 18 of the 29 parameters were noninformative with respect to patient prognosis and were eliminated. The parameters of age, Dukes' stage, and lymph node metastasis were selected from the clinical/histopathological parameter group. Four parameters derived from the G6PDH measurements, three from the SOD measurements, and one from the LPO measurement were selected for the final classification masks of survivors and nonsurvivors. While most of the histochemical parameters were decreased in nonsurvivors, the ratio of RA in carcinomas and normal mucosae increased as a consequence of the comparatively higher RA in cancer tissue of nonsurvivors, whereas the RA was diminished in normal mucosae of nonsurvivors (Table 3).

The CLASSIF1 classifier appeared to be robust because randomly reduced groups of patients (Table 2B,C) were classified similar to the whole patient group (Table 2A).

Besides the classification with the entire set of 29 parameters (Table 2A), various parameter subsets were separately used for patient classification to determine how

the histopathological, clinical, and histochemical parameters contributed to the prognostic value of the CLASSIF1 program. While classification with the use of Dukes' stage (ARI, 66.2%; Table 2D), histopathological and clinical parameters (ARI, 65.0%; data not shown), and SOD and LPO parameters (ARI, 74.0%; data not shown) on their own contained a certain degree of prognostic value, the G6PDH parameters (ARI, 75.1%; Table 2E) improved the classification result quite significantly. The G6PDH parameters alone classified equally as well as all histochemical parameters together, and the combined histopathological, clinical, and G6PDH parameters together or not with LPO or SOD (data not shown). Apparently, the G6PDH parameters contained a large amount of prognostic power. Although G6PDH parameters were the major information carrier, classification was improved to a small but important degree by using parameters of all classes (ARI, 82.1%; Table 2A), particularly because of the 100% prediction of survivors. The 11 selected parameters out of 29 (Table 3) were, in fact, all needed for optimum classification performance.

Combinations of histopathological, clinical, and histochemical parameters were far better prognostic indicators (ARI, 82.1%) than the traditional Dukes' stage as a single parameter (ARI, 66.2%). Therefore, CLASSIF1 classification is an important improvement in determining individual patient prognosis.

DISCUSSION

Until recently, Dukes' stage at the time of diagnosis and differentiation grade were the only prognostic indicators of clinical importance in colorectal cancer. The value of Dukes' stage but not of differentiation grade was confirmed by the CLASSIF1 classification (Table 2D). However, the present study shows that a distinct improvement was obtained by the combined classification of histopathological, clinical, and histochemical G6PDH, CuZn-SOD, Mn-SOD, and LPO parameters in cancer and normal colorectal tissue (Tables 2A, 3).

Therefore, the advantage of histochemical in combination with histopathological and clinical parameters resulted in a substantially improved predictive capacity for the establishment of single-patient prognosis in colorectal cancer. However, the increased practical efforts needed to obtain all data constitute a drawback. Therefore, we investigated how the number of histochemical parameters could be minimized, while keeping predictive value as high as possible. SOD and LPO parameters can be omitted without losing too much prognostic power. On the other hand, G6PDH parameters are essential to maintain predictive values of at least 96% and 55% for survivors and nonsurvivors, respectively (Table 2E).

The present study demonstrates the remarkable relation between G6PDH activity and cancer. The diagnostic value of the oxygen insensitivity of the quantitative histochemical assay of G6PDH activity is proven here, with RA <20% for all normal colorectal mucosae and RA >20% for all cancerous colorectal mucosae. This confirms the outcome of previ-

Table 2

A: Prediction of Survival of 64 Colon Cancer Patients With the Use of the CLASSIF1 Classification Program Based on 29 Histochemical, Histopathological, and Clinical Parameters at the Percentile Range of 30–70; B, C: Robustness of the CLASSIF1 Classification Program as Tested With Two Randomly Selected Smaller Patient Populations at the Percentile Range of 30–70; D, E: Prediction of Survival of 64 Patients With the Use of the CLASSIF1 Classification Program, Based on Dukes' Staging Only or on G6PDH Parameters Only, Respectively*

	Clinical diagnosis	Number of patients	Percentage classified as alive (n)	Percentage classified as "dead" (n)
A: Full learning set				
ARI, 82.1%	Alive	22	100.0 (22)	0.0 (0)
AMI, 1.00	Dead	42	35.7 (15)	64.3 (27)
B: Reduced learning set				
ARI, 79.0%	Alive	17	100.0 (17)	0.0 (0)
AMI, 1.06	Dead	37	45.9 (17)	54.1 (20)
C: Unknown patients				
ARI, 70.0%	Alive	5	100.0 (5)	0.0 (0)
AMI, 1.00	Dead	5	60.0 (3)	40.0 (2)
D: Dukes' stage				
ARI, 66.2%	Alive	22	71.4 (16)	28.6 (6)
AMI, 1.00	Dead	42	39.0 (16)	61.0 (26)
E: G6PDH parameters				
ARI, 75.1%	Alive	22	95.5 (21)	4.5 (1)
AMI, 1.00	Dead	42	45.2 (19)	54.8 (23)

*ARI, average recognition index; AMI, average multiplicity index.

Table 3

Selected Classification Parameters (Classifiers) That Were Needed for Optimized Prediction With the Use of the CLASSIF1 Classification Program*

Selected parameter	Triple matrix characters ^a	
	Patients alive (survivors)	Patients dead (nonsurvivors)
Age	0	–
Dukes' stage	0	+
Lymph node metastasis	0	+
CuZn-SOD in carcinomas	0	–
Mn-SOD in carcinomas	0	–
RA in normal mucosa	0	–
Ratio of G6PDH activity in N ₂ in carcinomas and normal tissue	0	–
Ratio of RA in carcinomas and normal tissue	0	+
Difference of G6PDH activity in N ₂ in carcinomas and adjacent normal tissue	0	–
Difference of CuZn-SOD expression in carcinomas and adjacent normal tissue	0	–
Difference of LPO capacity in carcinomas and adjacent normal tissue	0	–

*SOD, superoxide dismutase; RA, residual activity; G6PDH, glucose-6-phosphate dehydrogenase; LPO, lipid peroxidation.

^aSeventy-nine iterations for mask improvement were performed.

ous studies in which oxygen insensitivity was used to discriminate between nonmalignant and malignant tissues (11–16,26). It has also been proven in the present study that oxygen insensitivity is inversely related to CuZn-SOD expression and LPO capacity. Both parameters have been implied to be involved in the biochemical backgrounds of the oxygen-insensitivity phenomenon (16). However, most importantly, the present study shows that G6PDH-

dependent parameters have a high prognostic value for survival of patients with colorectal cancer. The test is very easy to perform and takes only 20 min once cryostat sections are available. The incubation lasts 10 min and quantitative analysis takes not more than 10 min, with the image analysis equipment presently available (30,31).

The prognostic value was highest when CLASSIF1 selected the 11 parameters listed in Table 3, including 4 G6PDH-related parameters, 3 SOD-related parameters, 1 LPO-related parameter, and 3 histopathological and clinical parameters. CLASSIF1 correctly predicted fatal outcome in 64.3% of deceased patients, and recognized all patients who survived during the follow-up period of minimally 32 months and maximally 56 months. When reduced groups of randomly selected patients were tested, a similar prognosis was found as when the whole group of patients was used (Table 2). In our opinion, the results of these classification procedures show that the self-learning classification program CLASSIF1 is robust and is the best so far.

It is concluded that the CLASSIF1 classification program constitutes an important improvement in single-patient prognosis. Furthermore, the oxygen-insensitivity assay of G6PDH activity is a simple test, that has proven to be a good discriminator between nonmalignant and malignant epithelial cells besides its proven essential role in predicting survival of colorectal cancer patients. The outcome of this study opens up new potential for the establishment of individual patient prognosis for colorectal cancer.

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