Distribution of molecular markers in sporadic colorectal cancer, adjacent and distant mucosa

Přemysl Frič[1], Vlasta Sovová[2], Zdeněk Roth[3], Eva Šloncová[2], Petr Kocna[4], Adam Jirásek[5], Jaroslav Čermák[6]

Dedicated to Professor Zdeněk Mařatka, MD, DSc, on the Occasion of his 90th Birthday

[1] Second Department of Medicine, Central Military Hospital, Postgraduate Institute of Medicine and Department of Gastroenterology and Hepatology, First Faculty of Medicine, Charles University, Praha, Czech Republic

[2] Institute of Molecular Genetics, Czech Academy of Sciences, Praha, Czech Republic

[3] Department of Biostatistics and Informatics, State Health Institute, Praha, Czech Republic

[4] Institute of Clinical Biochemistry and Laboratory Diagnostics, First Faculty of Medicine, Charles University, Praha, Czech Republic

[5] Institute of Pathology, First Faculty of Medicine, Charles University, Praha, Czech Republic

[6] Department of Surgery, First Faculty of Medicine, Charles University, Praha, Czech Republic

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Abstract *Purpose: Molecular markers of carcinomatous transformation of the colonic mucosa have been predominantly determined in the tumour tissue. Their behaviour in the neighbouring mucosa has been only seldom followed and data on comparison of their distribution in individual locations are rare.*

Methods: In 53 consecutive subjects with sporadic colorectal cancer the frequency and intensity of expression of p53, c-Src protein kinase activity, c-erbB2, EGF-r, PCNA and arginase activity were followed in the tumour tissue, the adjacent (less than 2 cm) and distant (more than 5 cm from the tumour margin) mucosa.

Results: Frequency and intensity of the expression of molecular markers displayed two different distribution patterns. A decreasing pattern (the highest value in the tumour and the lowest in the distant mucosa) was observed in p53, arginase and to some extent also in c-Src protein kinase activity. An increasing pattern was found in c-erbB2 and EGF-r. Logistic regression analysis of frequency expression gave the following data: 1. p53, c-erbB2 and arginase displayed significant differences among tumour and mucosal locations. 2. In the case of arginase the frequency of expression between both mucosal locations was also significantly different. 3. Individual subjects differed in their ability to express EGF-r and arginase. Intensity of expression (paired t-test: 2-tailed) was significantly different among tumour and adjacent as well as distant mucosa in p53, c-erbB2 and arginase. In c-Src protein kinase activity the intensity of expression differed significantly in both mucosal locations. In relation to Dukes staging (stages AB versus CD) the intensity of expression was significantly different (non-paired t-test: 2-tailed) only in PCNA (distant mucosa) and arginase (adjacent mucosa).

Conclusions: Different distribution patterns of frequency and intensity of the expression of individual molecular markers in colorectal cancer and neighbouring mucosa reflect the complex character of carcinomatous transformation of the epithelial cells. The results may be exploited in prospective prognostic studies with the use of molecular markers.

Key words: *Molecular markers, distribution, colorectal cancer, adjacent and distant mucosa, frequency and intensity of expression, logistic regression analysis*

Frič P, Sovová V, Roth Z, Šloncová E, Kocna P, Jirásek A, Čermák J. Distribuce molekulárních markerů ve sporadickém kolorektálním karcinomu, přilehlé a vzdálené sliznici. Folia Gastroenterol Hepatol 2004; 2 (2): 62 - 71.

Souhrn. *Molekulární markery rakovinné transformace střevní sliznice byly dosud studovány hlavně v nádorové tkáni. Jejich chování v okolní tkáni bylo sledováno zřídka a data o jejich distribuci v různých lokalizacích jsou vzácná.*

Metody: V souboru 53 nemocných se sporadickým kolorektálním karcinomem byla sledována četnost a intenzita exprese p53, c-Src proteinkinázy, c-erbB2, EGF-r, PCNA a aktivity arginázy v nádorové tkáni, přilehlé sliznici (do 2 cm od okraje nádoru) a vzdálené sliznici (více než 5 cm od okraje nádoru).

Výsledky: Molekulární markery vykazují dva typy distribuce četnosti a intenzity exprese. Sestupný typ (nejvyšší hodnoty v nádorové tkáni a nejnižší ve vzdálené sliznici) byl zjištěn u p53, arginázy a do určité míry také u c-Src proteinkinázy. Vzestupný typ byl pozorován u c-erbB2 a EGF-r.

Logistická regresní analýza četnosti exprese poskytla tyto údaje: 1. p53, c-erbB2 se významně liší v nádoru a ve slizničních lokalizacích. 2. Frekvence exprese arginázy se významně liší také při srovnání obou slizničních lokalizací. 3. Jednotliví nemocní se významně liší ve schopnosti exprimovat EGF-r a arginázu. Intenzita exprese (párový t-test oboustranný) p-53 c-erbB2 a arginázy se významně liší v nádoru, přilehlé i vzdálené sliznici. c-Src proteinkináza se významně liší v obou slizničních lokalizacích. Ve vztahu k Dukesově stagingu nádorů (stadia AB vs CD) byla intenzita exprese (nepárový t-test oboustranný) významně rozdílná pouze u PCNA (ve vzdálené sliznici) a u arginázy (v přilehlé sliznici).

Závěr: Rozdílné typy distribuce četnosti a intenzity exprese jednotlivých molekulárních markerů v nádorové tkáni a sousední sliznici odrážejí komplexní charakter maligní transformace kolonocytů. Uvedené nálezy lze aplikovat v prospektivních prognostických studiích nádorových markerů.

Klíčová slova: *molekulární markery, distribuce, kolorektální karcinom, přilehlá a vzdálená sliznice, četnost a intenzita exprese, logistická regresní analýza*

Colorectal carcinogenesis is characterized by cascades of complex processes taking part at various levels with individual timing and duration. This fact has important consequences for early diagnosis and prognosis. For this purpose, considerable attention was paid to different molecular markers in the tumour tissue. Their behaviour in the surrounding mucosa and comparison of their distribution among the tumour and different mucosal locations was infrequently followed. The studies of molecular markers limited to the tumour tissue have contributed little to individual prognosis (21). In our opinion the knowledge of the distribution pattern of molecular markers in the tumour and the surrounding mucosa might be helpful in this respect.

This retrospective study is concerned with the frequency and intensity of the expression and activity of several molecular markers in consecutive series of subjects with sporadic colorectal cancer. The distribution of these markers was determined in the tumour tissue as well as in the adjacent and distant mucosa. The series included the expression of a tumour-suppressor p53, oncogenes (c-Src protein kinase, c-erbB2, EGF-r) and markers showing cell proliferation: proliferating cell nuclear antigen (PCNA) and arginase activity.

Inactivation of tumour-suppressor p53 usually involves somatic mutation of a p53 allele or a viral oncoprotein binding to p53 protein. Somatic mutation is considered a late event in colorectal carcinogenesis (12). Nevertheless, some prostaglandins, overexpression of which is associated with colorectal carcinomas, adenomas and inflammatory conditions, alter p53 protein location and its function (22). This type of inactivation of the wild type p53 offers a basis for its involvement in earlier colorectal oncogenesis.

The expression of two members of c-erbB family, epidermal growth factor receptor (EGFr) and c-erbB2, is regularly analysed in mammary carcinomas. Nevertheless the presence of EGFr on colonocytes, a great and still growing number of EGFr ligands and a high tyrosine kinase activity of heterodimers EGFr/erbB2 indicated that it may also be valuable to analyse the expression of these two receptors in colorectal tumours and surrounding mucosa.

The activity of c-Src protein was followed on the bases of the data showing an increase of c-Src tyrosine kinase activity at early stage of colorectal carci-

nogenesis (3, 4). The expression of PCNA, which is a DNA polymerase δ accessory protein complexing with cyclin D and cyclin-dependent kinases, and the activity of arginase were chosen as markers showing proliferation activity of the analysed tissues.

Material and methods

Specimens of the cancer tissue and the neighbouring mucosa were obtained from 53 consecutive patients (23 men and 30 women, aged 40 - 75 years) operated at the First Department of Surgery of the University Hospital. Informed consent was obtained from all subjects in context with surgical procedures. Immediately after removal the colon was cut thorough and laid out. Samples were taken from three different locations: 1. from the cancer tissue (CA) free of secondary changes (bleeding, ulcer or necrosis), 2. from the adjacent mucosa (less than 2 cm from the macroscopic tumour margin - AM), 3. from the distant mucosa (more than 5 cm from the tumour margin - DM). Mucosal samples assigned for methods using tissue sections were laid out with the mucosa upward on a gelatine plate. Samples were fixed in 10% neutral formol solution (for histology and determination of p53, c-erbB2, EGF-r and PCNA) or rapidly frozen at -70 °C up to the time of analysis (for the determination of c-Src protein kinase activity and arginase activity). The different number of analyses of the individual markers was caused by insufficient quantity of available tissue.

Detection of p53, c-erbB2 and EGF-r. Formol fixed paraffine embedded tissue sections were dewaxed with a series of solvents (xylene, isopropanol, ethanol, water). Endogenous peroxidase was blocked by treatment with 3% hydrogen peroxide in 0.1% of natrium azide. Sections for p53 determination immersed in citrate buffer (pH 7.0) were pretreated in microwave oven at 67 °C for 7 min. After cooling, the sections were rinsed and immersed into TRIS-buffered saline (pH 7.6). Immunohistochemical assay included successive application of the following antibodies: 1. monoclonal antibody DO-1 (29) anti p53, anti-c-erbB2 antibody (Serva, Heidelberg, Germany) and anti-EGF-r antibody (Sigma, St Louis, Mo, USA) respectively; 2. peroxidase conjugated swine anti-mouse IgG (Sevac, Prague, Czech Republic) and 3. peroxidase conjugated rabbit anti-swine IgG (Sevac, Prague, Czech Republic). The substrate of the final reaction included 0.04% diaminobenzidine in 0.05 M TRIS buffer (pH 7.6) with 0.015% hydrogen peroxide. The nuclei were counterstained with Harris haematoxylin. Thereafter, the sections were treated by ethanol, acetone and xylene, mounted into solacryl and evaluated microscopically. Cell lines HT 29 and SKBR-3 were used as positive controls for p53 and c-erbB2 staining (7,16). The EGF-r antibody was titrated on the A431 cell line (26). The results were evaluated with the use of a semiquantitative scale: p53 - negative (0), positive (1); c-erbB2 and EGF-r - negative (0), slight (1) and intensive (2) positivity. In c-erbB2 analysis both the nuclear (N) and cytoplasmic (C) positivity was determined.

Detection of PCNA. PCNA was detected using the Dako-Epos kit (Dako, Glostrup, Denmark) in formol-fixed sections after deparaffinization (31). In cancer tissue the percentage of PCNA-positive nuclei was determined in 1000 tumour cells. In AM and DM the positivity was determined semiquantitatively: 0-negative, 1-positively reacting (proliferating) cells at the crypt bottom, 2-proliferating cells in the lower third of the crypt, 3-proliferating cells in the lower half of the crypt, 4-proliferating cells extending into the upper third of the crypt.

All slides were reviewed by two independent observers.

Analysis of c-Src protein-kinase activity. The procedure included preparation of p60-src immunoprecipitates and determination of protein kinase activity.

1) Preparation of immunoprecipitates: The samples were homogenized in liquid nitrogen and resuspended in 0.01 M TRIS-buffer (pH 7.6) enriched with 0.1 M NaCl, 0.001 M EDTA (pH 8.0), aprotinin (1μg/mL), phenylmethylsulfonyl fluoride (PMSF, 100 μg/mL) and 1% Nonidet P-40 (all Sigma, St Louis, Mo, USA). The lysates were incubated at 4°C for 30 min and centrifuged (10000 g, 5 min). Protein concentration of the supernatant was determined by bicinchoninic acid protein assay kit (Sigma). The samples were adjusted to contain 300 μg of protein and incubated with 3 mL of monoclonal anti-Src antibody 327 overnight at +4 °C (18). Afterwards rabbit anti-mouse IgG bound to Sepharose A was added and the samples were incubated at +4 °C for 90 min. The final steps include three washings in the NET buffer (0.15 M NaCl, 0.05 TRIS M, 0.005 M EDTA, 0.5% Nonidet P-40 and 0.002 M PMSF) and one wash in distilled water.
2) *Protein kinase activity* was assayed by a modified

method of Bolen et al. (3). Immunoprecipitates of p60c-Src were incubated in kinase buffer composed of 0.25 M HEPES (pH 7.6), 0.05 M $MnCl_2$, 0.05 M dithiothreitol and 1-2 mCi (gama-^{32}P) ATP. As substrates were used denaturated enolase and IgG-heavy chain of the immunoprecipitate. The reaction was stopped by adding the electrophoretic buffer and the samples were subjected to electrophoresis in 10% SDS-polyacrylamide gel. Thereafter the gel was washed, dried and applied to an X-ray film. Each gel was equipped with a positive control sample of a standard quality of v-Src kinase (immunoprecipitate of the v-Src product from cells transformed by Rous sarcoma virus). This internal standard of kinase activity enabled us to compare the results of the individual analyses. The activity of p60-Src was evaluated semiquantitatively: 0-negative, 1-slight, 2-moderate, 3-intensive positivity.

Arginase activity. A modification (13) of the photometric method of Konarska and Tomaszewski (15) was used. The activity was expressed in µM ornithine/min/g protein (U/g). Arginase activity ($x \pm SD$) in colonic mucosa of control subjects amounted to 83.2 ± 35.8 U/g protein. Arginase values higher than 176 U/g protein ($x + 2.58$ SD) are considered indicative of increased proliferative activity.

Staging of the tumours. Dukes staging was determined by the surgeon as well as the pathologist. The following number of individual stages were found: A - 1, B - 10, C - 38, D - 4. For this reason the stages A and B (cancer restricted to the colon) as well as stages C and D (cancer extending beyond the colon) were brought together.

Statistical analysis.

Frequency of expression. Relative values of expression of individual markers and 95 per cent confidence intervals were determined for all three locations (tumour, adjacent and distant mucosa). In addition the frequency of expression of individual markers was analyzed by logistic regression. This method enabled us to test the influence of location as well as the influence of patients, who may differ in their ability to express individual markers. The frequency of markers expression was compared among all three locations. In addition the pairwise comparison of all locations was performed using appropriate contrast variables (CA vs AM, CA vs DM, AM vs DM).

Intensity of expression. With the exception of arginase activity and PCNA positivity in the tumour tissue, the intensity of expression of individual markers was evaluated using a semiquantitative scale, in which the numerical value increases with the intensity of positivity. The size of the series allows the processing of such data - although they are only integer values - as quantitative variables. Arginase values in the tumour and mucosa displayed an asymetric distribution. For this reason a logarithmic transformation of the data was performed and the geometric means obtained after backward transformation of logarithmic values are given. The logarithmic values of standard error of the mean (SE) cannot be subjected to this procedure and they correspond approximately to the coefficient of variance.

The significance of average values of individual markers in the tumour, the adjacent and distant mucosa was evaluated by paired t-tests (2-tailed). The intensity of markers in individual locations was also compared with Dukes staging of the tumours (stages AB vs CD) using the non-paired t-test (2-tailed).

Results

Frequency of expression (Tables 1 and 2). Individual markers display considerable differences in frequency of expression at individual locations (CA, AM, DM) as well as in the character of the distribution pattern.

p53 was expressed only in the tumour tissue. It was found in one third of the samples studied. Logistic regression analysis revealed highly significant differences in frequency of expression among individual locations ($p < 0.001$) as well as between the tumour and AM or DM ($p = 0.005$ and 0.011). The difference among patients to express the marker was not significant.

The frequency of *c-src protein kinase activity* was very high in CA tissue and slightly lower in AM and DM (91.7, 82.6 and 70.0 per cent). The differences among patients as well as among individual locations were not significant.

In spite of the exclusive membrane location of *c-erbB2* in breast carcinoma cell line SKBR-3 used as a positive control, colorectal tumours as well as normal colonocytes showed additional cytoplasmic and often also nuclear staining. The lowest frequency of both the nuclear and cytoplasmic c-erbB2 was found in the cancer tissue and the highest in DM. The

Table 1
Frequency of expression (per cent values and 95 per cent confidence interval) of p53, nuclear and cytoplasmic c-erbB2, EGF-r, PCNA and c-Src and arginase (ARG) activities in sporadic colorectal cancer (CA), adjacent (AM) and distant (DM) mucosa.

Marker	Location	N	Expression	
			%	95%-CI
p53	**CA**	40	32.5	18.6-49.1
	AM	41	0	0-8.6
	DM	36	0	0-9.7
c-src	**CA**	24	91.7	73.0-99.0
	AM	23	82.6	61.2-95.1
	DM	20	70.0	45.7-88.1
c-erbB2 (nuclear)	**CA**	40	30.0	16.6-46.5
	AM	41	58.5	42.1-73.7
	DM	30	69.4	51.9-83.7
c-erbB2 (cytoplasmic)	**CA**	40	62.5	45.8-77.3
	AM	41	95.1	83.5-99.4
	DM	36	94.4	81.7-99.3
EGF-r	**CA**	37	27.0	13.8-44.1
	AM	38	31.6	17.5-48.7
	DM	37	35.1	20.2-52.5
PCNA	**CA**	25	96.0	79.7-99.9
	AM	27	100.0	90.5-100.0
	DM	36	100.0	90.3-100.0
ARG	**CA**	50	76.0	61.8-86.9
	AM	50	32.0	19.5-46.7
	DM	50	20.0	10.0-33.7

Table 2
Logistic regression analysis of differences (p-values) of frequency expression of p53, nuclear and cytoplasmic c-erbB2, EGF-r, PCNA and c-Src and arginase (ARG) activities in sporadic colorectal cancer (CA), adjacent (AM) and distant (DM) mucosa. The differences were followed among patients (1) and different locations: CA vs AM vs DM (2), CA vs AM (3), CA vs DM (4), and AM vs DM (5). NA-not applicable.

Marker	p-values				
	1	2	3	4	5
p53	0.629	< 0.001	0.005	0.011	NA
c-src	0.103	0.175	0.936	0.092	0.312
c-erbB2 (nuclear)	1.00	< 0.001	0.001	< 0.001	0.585
c-erbB2 (cytoplasmic)	0.169	< 0.001	0.002	0.004	0.894
EGF-r	< 0.001	0.312	0.773	0.248	0.617
PCNA	0.660	0.229	0.434	0.434	0.991
ARG	< 0.001	< 0.001	< 0.001	< 0.001	0.025

cytoplasmic expression of the marker was distinctly more frequent than the nuclear expression in all three locations. The results of logistic regression analysis were identical for both the nuclear and cytoplasmic expression of the marker. The differences were highly significant among all three locations (nuclear, cyto-

plasmic: $p < 0.001$) as well as between CA vs AM ($p = 0.001$ and 0.02) and CA vs DM ($p < 0.001$ and $p = 0.004$). On the other hand, the differences among patients as well as between AM and DM were not significant.

The frequency of *EGF-r expression* was low in the tumour (27.0 per cent) and slightly higher in both mucosal locations (31.6 and 35.1 per cent). The differences among locations were not significant, whereas the ability of the patients to express the marker was significantly different ($p < 0.001$).

PCNA was expressed in all examined samples with the exception of one tumour. Logistic regression analysis did not reveal any significant differences either among patients or individual locations.

Arginase activity was increased most frequently in the CA samples (76.0 per cent). This value decreased with distance from the tumour. Nevertheless, even in DM 20.0 per cent of samples displayed increased arginase activity. Logistic regression analysis revealed significant differences among patients ($p < 0.001$), all three locations ($p < 0.001$) as well as in pairwise comparisons: CA vs AM ($p < 0.001$), CA vs DM ($p < 0.001$) and AM vs DM ($p = 0.025$).

Intensity of expression (Table 3). Similarly to frequency of expression the markers display considerable differences in intensity of expression at individual locations and in their distribution patterns.

The exclusive expression of *p53* in CA renders the difference of its intensity at this location in comparison with AM and DM highly significant ($p < 0.001$ and 0.001).

The activity of *c-Src protein kinase* was the same in CA and AM. A decrease in DM was significant only in comparison with the average intensity in AM ($p = 0.012$).

The average intensity values of nuclear and cytoplasmic *c-erbB2* behaved in a similar way as its frequency values. The lowest intensity values were found in the CA tissue and the highest in DM. The average values in CA and AM were significantly different (nuclear: $p = 0.009$, cytoplasmic: $p = 0.016$) and the same was true for CA and DM ($p = 0.001$ and 0.008). The difference of intensity values between AM and DM was not significant.

EGF-r displayed a similar distribution pattern as c-erbB2, but the average values at individual locations were not significantly different.

Comparison of the values of *PCNA* in both mucosal locations showed that the difference of intensity values in AM and DM was not significant.

Increased *arginase activity* was most prominent in CA and decreased with distance from the tumour. The differences of average values in CA and AM as well as in CA and DM were highly significant ($p < 0.001$), whereas the difference between both mucosal locations was not significant.

Relation of intensity of expression to Dukes staging (Table 4). Significant differences of average intensity values in Dukes cancers AB vs CD were found only with PCNA determination in DM (1.44 vs 1.96, $p = 0.038$) and with arginase activity in AM (76.6 vs 143.9, $p = 0.032$).

Discussion

Dawson and Filipe (6) were the first who paid more detailed attention to histologically normal mucosa adjacent to carcinoma. They coined the term "transitional mucosa" contrary to "normal mucosa" more remote from the tumour. They suggested that changes in transitional mucosa may reflect failure in the normal process of cell differentiation along the crypt. Subsequent reports described mostly changes of transitional mucosa at the cellular level. Boland and Kim (2) presented the opinion that transitional mucosa represents a premalignant antecedent to colonic adenocarcinoma and that it may result from the paracrine influence of tumour growth factors released by the tumour.

This study was concerned with distribution of a series of molecular markers in the tumour tissue as well as in adjacent and distant mucosa. The series included the expression of tumour-suppressor gene product (p53), oncogenes (c-Src protein kinase, c-erbB2, EGF-r) and markers showing cellular proliferation (PCNA, arginase activity). Individual markers displayed considerable differences in frequency and intensity of expression at individual locations. Two types of gradients may be distinguished. The expression of p53, c-src protein kinase and arginase activities displayed the highest values of frequency and intensity of expression in the tumour tissue, whereas in both mucosal locations the values were lower. c-erbB2 and EGF-r behaved in an opposite manner.

p53 protein was detected only in one third of the tumours. p53 was not found in any sample of adjacent or distant mucosa, although our modification of the histochemical detection showed to be very sensi-

Table 3
Intensity of expression of p53, nuclear and cytoplasmic c-erbB2, EGF-r, PCNA and c-Src and arginase (ARG) activities in sporadic colorectal cancer (CA), adjacent (AM) and distant (DM) mucosa (paired t-test: 2-tailed). ARG activity is given in log. values (ln) and geometric means (xg) after backward transformation of logarithmic data. NA-not applicable.

marker	location	n	x (x_g)	SE	p
p53	CA	37	0.32	0.08	
	AM	37	0.00	0.00	< 0.001
	CA	32	0.31	0.08	
	DM	32	0.00	0.00	0.001
	AM	36	0.00	0.00	
	DM	36	0.00	0.00	NA
c-src	CA	23	1.65	0.17	
	AM	23	1.65	0.20	1.00
	CA	20	1.60	0.20	
	DM	20	1.15	0.23	0.083
	AM	19	1.74	0.23	
	DM	19	1.58	0.25	0.012
c-erbB2 (nuclear)	CA	37	0.49	0.13	
	AM	37	0.92	0.13	0.009
	CA	32	0.56	0.15	
	DM	32	1.19	0.16	0.001
	AM	36	1.14	0.15	
	DM	36	1.22	0.15	0.324
c-erbB2 (cytoplasmic)	CA	37	0 .81	0.12	
	AM	37	1.14	0.08	0.016
	CA	32	0.88	0.13	
	DM	32	1.31	0.11	0.008
	AM	36	1.22	0.08	
	DM	36	1.28	0.09	0.571
EGF-r	CA	36	0.33	0.10	
	AM	36	0.44	0.11	0.254
	CA	35	0.34	0.10	
	DM	35	0.66	0.18	0.078
	AM	37	0.43	0.11	
	DM	37	0.62	0.18	0.147
PCNA	AM	36	1.97	0.14	
	DM	36	1.83	0.13	0.324
ln ARG (ARG x_g)	CA	50	5.855 (349.0)	0.125	< 0.001
	AM	50	4.830 (125.2)	0.129	
	CA	50	5.855 (349.0)	0.125	< 0.001
	DM	50	4.671 (106.8)	0.108	
	AM	50	4.830 (125.2)	0.129	0.087
	DM	50	4.671 (106.8)	0.108	

tive. Similar restriction of p53 location to the tumour tissue was described by Connelly et al. (5), whereas Baytner et al. (1) found some positivity also in the adjacent mucosa. The low number of positive

Table 4
Intensity of expression of p53, cytoplasmic and nuclear c-erbB2, EGF-r, PCNA and c-Src and arginase (ARG) activities in sporadic colorectal cancer (CA), adjacent (AM) and distant (DM) mucosa related to Dukes staging (AB vs CD; non-paired t-test: 2-tailed). ARG activity is expressed in the same way as in tab. 3. NA-not applicable.

Marker	location	Dukes	n	x (x_g)	SE	p
p53	**CA**	**AB**	10	0.20	0.13	0.313
		CD	30	0.37	0.09	
	AM	**AB**	10	0.00	0.00	NA
		CD	31	0.00	0.00	
	DM	**AB**	7	0.00	0.00	NA
		CD	29	0.00	0.00	
c-src	**CA**	**AB**	5	1.40	0.40	0.544
		CD	19	1.68	0.19	
	AM	**AB**	5	1.60	0.51	0.909
		CD	18	1.67	0.23	
	DM	**AB**	4	1.00	0.41	0.717
		CD	18	1.67	0.23	
c-erbB2 (nuclear)	**CA**	**AB**	10	0.80	0.29	0.197
		CD	30	0.37	0.13	
	AM	**AB**	9	0.56	0.29	0.113
		CD	32	1.13	0.16	
	DM	**AB**	7	1.00	0.38	0.521
		CD	29	1.28	0.16	
c-erbB2 (cytoplas)	**CA**	**AB**	10	1.10	0.23	0.154
		CD	30	0.70	0.13	
	AM	**AB**	9	1.00	0.17	0.266
		CD	32	1.22	0.09	
	DM	**AB**	7	1.29	0.29	0.975
		CD	29	1.28	0.10	
EGF-r	**CA**	**AB**	8	0.25	0.16	0.642
		CD	29	0.35	0.11	
	AM	**AB**	7	0.71	0.29	0.278
		CD	31	0.35	0.12	
	DM	**AB**	7	1.57	0.65	0.124
		CD	30	0.40	0.13	
PCNA	**CA**	**AB**	7	55.0	8.5	0.375
		CD	18	65.3	7.4	
	AM	**AB**	9	1.78	0.32	0.547
		CD	28	2.00	0.15	
	DM	**AB**	9	1.44	0.18	0.038
		CD	27	1.96	0.16	
ln ARG (ARG x_g)	**CA**	**AB**	11	5.984 (397.0)	0.157	0.458
		CD	39	5.818 (333.6)	0.154	
	AM	**AB**	11	4.338 (76.6)	0.232	0.032
		CD	39	4.969 (143.9)	0.145	
	DM	**AB**	11	4.514 (91.3)	0.188	0.387
		CD	39	4.715 (111.6)	0.128	

tumours may be influenced by the varying character of p53 gene mutations and their location in different regions of the gene. This may introduce conformational changes of the final protein product, which are reflected in its detection by the antibody used. Direct detection of the gene mutation and not immunohisto-

chemical determination of the protein product in case of p53 gene mutation appears to be the more suitable method (9, 10, 25). According to Bolen et al. (3) and Cartwright et al. (4) the protein-tyrosine kinase activity of p60c-Src is significantly higher in the tumour tissue than in the adjacent normal mucosa. We found high frequency of expression of this marker in all three locations. The differences among them were not significant and the same was true for the ability of the probands to express this marker. The intensity of expression displayed a significant difference only between the adjacent and distant mucosa.

Protein-tyrosine kinase of c-src participates in signalling events mediated by a variety of polypeptide growth factors including EGF-r and other members of the c-erbB family. The c-src product increases the oncogenic activity of EGF-r and the growth of tumour cells (19, 20, 24). c-erbB2, which displays large structural homology with EGF-r, behaves in a similar manner. c-erbB2 and EGF-r have also a similar distribution pattern. They exhibit the lowest frequency and intensity of the expression in the tumour and the highest in distant mucosa. This distribution pattern of EGF-r was mentioned already by Koenders (14). In spite of the similar distribution pattern c-erbB2 and EGF-r display differences in significance of frequency and intensity of the expression in individual locations as well as in the ability of patients to express both markers. The cytoplasmic and nuclear location of colorectal c-erbB2 was described also by Kay et al. (11). This finding may be due to several different protein products of this marker (28).

PCNA was expressed in all samples with the exception of one tumour. This may be probably due to a technical failure. We were unable to confirm a significant upward shift of proliferating cells in transitional mucosa described by Yoshikawa and Utsinomiya (31).

Arginase catalyzes the synthesis of polyamines in the early phase of global proteosynthesis. The expression of arginase gene in colorectal cancer cell line is stimulated through cAMP by mechanisms involving activation of protein kinase and appropriate transcription factors (30). Arginase activity is significantly increased in colorectal adenomas, carcinomas as well as in inflammatory bowel disease and may be considered a useful proliferative marker (13,17). Its activity is inversely proportional to the degree of differentiation of colorectal cell lines (27). In this series increased arginase activity was found in 76 per cent of cancers and the frequency decreased with distance from the tumour. Normal arginase activity in the remaining cancers might be due to their higher differentiation or to pronounced inability of tumour-infiltrating macrophages to produce this enzyme as suggested by Park et al. (23).

The intensity of expression related to Dukes staging of cancers AB versus CD was significantly different only in the case of PCNA (distant mucosa) and of arginase (adjacent mucosa). Intensity of expression appears to have more relation to tumour location. We have recently found that cytoplasmic c-erbB2, EGF-r and PCNA was significantly higher in sporadic cancer of the right than of the left colon. In addition cytoplasmic c-erbB2 revealed the same difference in adjacent and distant mucosa. These findings were also not related to Dukes staging (8).

From the methodological point of view it should be remembered that immunohistochemical analysis offers no information about the functional state of the detected receptors. A small amount of receptors with a high ligand-binding capacity may be more important for growth characteristics of the tumour cells than a large amount of receptors with low binding ability.

This retrospective study reflects the complex character of colorectal carcinogenesis in sporadic cancer. The recognition of the different distribution patterns of individual markers among the cancer tissue, the adjacent and distant mucosa as well as the knowledge of significant differences of individual probands to express some markers may be exploited in prospective prognostic studies with the use of molecular markers. Such studies should be aimed not only on the frequency and intensity of expression of molecular markers in the tumour tissue, but also on the evaluation of alterations of their distribution patterns and ability of patients to express the markers. These prospective studies would be of considerable importance for objective planning of post-surgical care in patients with sporadic colorectal cancer.

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REFERENCES

1. Baytner S, Mitmaker B, Gordon PH, Wang E. Immunohistochemical expression of mutant p53 oncogene in transitional mucosa adjacent to human colon cancer. Clin Invest Med 1983; 16: 379 - 385.
2. Boland CR, Kim YS. Transitional mucosa of the colon and tumor growth factors. Med Hypotheses 1987; 22: 237 - 243.
3. Bolen JB, Veilette A, Schwartz AM, DeSeau V, Rosen N. Activation of pp60c-src protein kinase activity in human colon carcinoma. Proc Natl Acad Sci USA 1987; 84: 2251 - 2255.
4. Cartwright CA, Meister AL, Eckhart W. Activation of pp60c-src protein kinase is an early event in colonic carcinogenensis. Proc Natl Acad Sci USA 1990; 87: 558 - 562.
5. Connelly JH, Bruner JM, Robey-Cafferty SS, Sabin A. p53 protein expression in transitional mucosa and adenocarcinomas of the colorectum. Mod Pathol 1992; 5: 537 - 539.
6. Dawson PA, Filipe MI. An ultrastructural and histochemical study of the mucous membrane adjacent to and remote from carcinoma of the colon. Cancer 1976; 37: 2388 - 2398.
7. Fogh J, Trempe G. New human tumour cell lines (p 115 - 141). In: Fogh J (ed): Human Tumour Cells In Vitro. New York: Plenum Press, 1975.
8. Frič P, Sovová V, Šloncová E, Lojda Z, Jirásek A, Čermák J. Different expression of some molecular markers in sporadic cancer of the left and right colon. Eur J Cancer Prev 2000; 9: 265 - 268.
9. Goh HS, Elnatan J, Low CH, Smith DR. p53 point mutation and survival in colorectal cancer patients: effect of disease dissemination and tumour location. Int J Oncol 1999; 15: 491 - 498.
10. Goh HS, Yao J, Smith DR. p53 point mutation and survival in colorectal cancer patients. Cancer Res 1995; 55: 5217 - 5521.
11. Kay EW, Mulcahy H, Walsh BC, Leader M, Donohue DO. Cytoplasmic c-erbB2 protein expression correlates with survival in Duke's B colorectal carcinoma. Histopathology 1994; 25: 455 - 461.
12. Kinzler KW, Vogelstein B. Lessons from hereditary colon cancer. Cell 1996; 87: 159 - 170.
13. Kocna P, Frič P, Zavoral M, Pelech T. Arginase activity determination. A marker of large bowel mucosa proliferation. Eur J Clin Chem Biochem 1996; 34: 619 - 623.
14. Koenders PG, Peters WH, Wobbes T, Beex LV, Nagengast FM, Benraad TJ. Epidermal growth factor receptor levels are lower in carcinomatous than in normal colorectal tissue. Br J Cancer 1992; 65: 189 - 192.
15. Konarska L, Tomaszewski L. A simple quantitative micromethod of arginase assay in blood spots dried on filter paper. Clin Chim Acta 1986; 154: 7 - 17.
16. Kraus MH, Popescu NC, Amsbaugh SC, King CR. Overexpression of the EGF receptor-related protooncogene erbB-2 in human mammary tumor cell lines by different molecular mechanisms. EMBO J 1987; 6: 605 - 610.
17. Leu SY, Wang SR. Clinical significance of arginase in colorectal cancer. Cancer 1992; 70: 733 - 736.
18. Lipsich LA, Lewis AJ, Brugge JS. Isolation of monoclonal antibodies that recognize the transforming proteins of avian sarcoma viruses. J Virol 1983; 48: 352 - 360.
19. Luttrell DK, Lee A, Lansing TJ, Crosby RM, Jung KD, Willard D, Luther M, Rodriguez M, Berman J, Gilmer TM. Involvement of pp60c-src with two major signaling pathways in human breast cancer. Proc Natl Acad Sci USA 1994; 91: 83 - 87.
20. Luttrell DK, Luttrell LM, Parsons SJ. Augmented mitogenic responsiveness to epidermal growth factor in murine fibroblasts that overexpress pp60c-src. Mol Cell Biol 1988; 8: 497 - 501.
21. McLeod HL, Murray GI. Tumour markers of prognosis in colorectal cancer. Br J Cancer 1999; 79: 191 - 203.
22. Moss PJ, Edes K, Fitzpatrick, FA. Inactivation of wild-type p53 tumor suppressor by electrophilic prostaglandins. Proc Natl Acad Sci USA 2000; 97: 9215 - 9220.
23. Park KG, Heys SD, Harris CI, Steele RJ, McNurlan MA, Eremin O, Garlick PJ. Arginine metabolism in benign and malignant disease of breast and colon: evidence for possible inhibition of tumor-infiltrating macrophages. Nutrition 1991; 7: 181 - 188.
24. Radinsky R, Risin S, Fan D, Dong Z, Bielenberg D, Bucana CD, Fidler J. Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma. Clin Cancer Res 1995; 1: 19 - 31.
25. Smith DR, Ji CY, Goh HS. Prognostic significance of p53 overexpression and mutation in colorectal adenocarcinomas. Br J Cancer 1996; 74: 216 - 223.
26. Sovová V, Fidlerová H, Trnka D, Šloncová E. Epidermal growth factor receptors in Rous sarcoma virus-transformed cells of different hosts. Folia Biol (Praha) 1994; 40: 291 - 299.
27. Sovová V, Šloncová E, Frič P. Differences of alkaline phosphatase and arginase activities in human colorectal carcinoma cell lines. Folia Biol (Praha) 1997; 43: 101 - 104.
28. Tannapfel A, Dworak O, Mansmmann U, Wittekind C. Cytoplasmic c-erbB2 and survival in Dukes's B colorectal carcinoma. Pathology 1995; 27: 199 - 202.
29. Vojtěšek B, Bártek J, Midgley CA, Lane DP. An immunochemical analysis of the human nuclear phosphoprotein p53. New monoclonal antibodies and epitope mapping using recombinant p53. J Immunol Methods 1992; 151: 237 - 244.
30. Wei LH, Morris SM Jr, Cederbaum SD, Mori M, Ignarro LJ. Induction of arginase II in human caco-2 tumor cells by cyclic AMP. Arch Biochem Biophys 2000; 374: 255 - 260.
31. Yoshikawa R, Utsinomiya J. Cell proliferation kinetics are abnormal in transitional mucosa adjacent to colorectal carcinoma. Br J Surg 1996; 83: 36 - 39.

Correspondence to:
Professor Přemysl Frič, MD, DSc, Central Military Hospital
and Postgraduate Institute of Medicine, CZ-169 02 Prague 6,
Czech Republic
E-mail: fricprem@uvn.cz