INTRODUCTION

Fragile histidine triad gene (FHIT) has been mapped to chromosomal region 3p14.2 (1). It spans the t(3:8)+(p14.2;q24) translocation breakpoint found in familiar renal cell carcinoma and encompasses the most common fragile site of the human genome, FRA3B (2). FHIT contains an open reading frame of 444 bps encoding a protein of 147 amino acids, which is ubiquitously expressed in human tissues. The FHIT protein is known as a human diadenosine triphosphate hydrolase that cleaves the diadenosine substrate into adenosine diphosphate (ADP) and adenosine monophosphate (AMP) (3). FHIT protein may also be involved in the regulation of cell cycle and/or DNA replication (4). FHIT gene has been proposed to be a candidate tumor suppressor gene involved in various types of human cancer, however, quantification of FHIT mRNA was seldom performed. Aim: To investigate loss of heterozygosity (LOH) at FRA3B, expression of FHIT gene at the mRNA and protein levels in sporadic colorectal carcinoma (CRC) and benign colon adenoma. Materials and Methods: FHIT mRNA was quantified by the validated real-time PCR (QPCR) in tumor samples of 84 CRC patients and mucosal biopsies of 15 adenomas, in comparison to 37 control patients, whereas subgroup of 57 CRC, 10 adenoma and 10 control cases were selected for immunohistochemical (IHC) detection of the native FHIT protein and LOH determination at FRA3B. Results: Higher level of FHIT mRNA was found in 86% of CRC (P<0.001) and 60% of adenomas (P=0.016). IHC showed comparable results to QPCR (P=0.003), revealing the strongest presence of FHIT protein in Dukes' C/D stages (P<0.001) and N1/N2 lymph nodes metastasis in CRC (P=0.04). FHIT gene expression and Dukes' and G staging were positively correlated in CRC as analyzed by QPCR and IHC. Deletion analysis of the fragile FRA3B site revealed the highest LOH frequency at D3S1234 in 32.5% of CRC informative cases, however, LOH did not correspond to QPCR, IHC or clinical-pathological variables. Conclusion: Our data suggest that reduction or absence of the FHIT gene expression is not a prerequisite for colorectal cancer development and progression.

Key words: FHIT gene expression, QPCR, IHC, colorectal cancer, colon adenoma
MATERIALS AND METHODS

Patients

In the prospective study specimens were obtained from northern and north-eastern Poland from 2005 to 2008. The study was approved by local ethical committees. Informed, written consent regarding the use of tissue and blood samples was obtained from all CRC patients before surgery, or in case of control and adenoma patients, before colonoscopy. Demographic and clinical data were recorded by analysis of patients charts including an interview at the time of enrollment. None of the patients included in this study had family history of CRC. The CRC group consisted of 84 patients (56 men and 28 women, mean age 67±10.2, range 37-89 years). CRC patients had neither suffered from a second neoplastic disease nor had undergone previous chemo- and radiotherapy. Tumor stages according to Duke’s classification and histopathological G grade (23, 24) are presented in Table 1.

Biopsies of benign colon adenoma were obtained from 7 male and 8 female patients (mean age 57±10.7 years, range 40-74 years). The location of adenomas was: ascending colon - 4, transverse colon - 2, descending/sigmoid colon - 4, rectum - 5. Histopathological findings revealed no dysplastic/malignant cells in any of the analyzed adenoma cases, thus all adenomas were qualified as well differentiated (G0) in G grading. CRCs in any of the analyzed adenoma cases, thus all adenomas were qualified as well differentiated (G0) in G grading. CRCs and adenomas from anal location were not included in the study.

The control group consisted of 37 patients (21 males and 16 females, mean age 52±14.9, range 31-76 years) who underwent colonoscopy as a part of a routine surveillance for CRC. The specimens were obtained from different colonic locations except for anal canal and anus. These patients had no CRC in family history and presented normal mucosal histology. None of the control patients was on medication at the time of investigation.

CRC samples were obtained during surgical hemicolectomy, whereas benign adenoma and control group specimens were collected during colonoscopy. In CRC, 10 x 10 x 10 mm samples were cut out from macroscopically altered tumor tissue by an experienced pathologist no later than 20 min after tumor resection, placed in sterile vials, quick-frozen in liquid nitrogen, and stored at -85°C. In benign adenoma cases, whole lesion was cut out from the colon, followed by tissue fragmentation. For this study, we obtained three 2 x 2 x 2 mm fragments of each adenoma for molecular assays, whereas rest of the tissue was sent for histological examination. In control patients, one 2 x 2 x 2 mm biopsy was fixed in 10% formalin for routine histological examination, whereas two specimens from the closest location were collected for RNA and IHC analyzes.

For LOH assessments 1 ml blood samples of 57 CRC, 10 benign adenoma cases and 10 control patients were obtained before colonoscopy or tumor resection and stored on ice in sterile vials containing EDTA.

Nucleic acids extraction

RNA was isolated using Total RNA kit (A&A Biotechnology, Gdynia, Poland) based on the phenol-chlorophorm-isoamyl alcohol and silica membrane technique (25) from 2 x 2 x 2 mm fragments of adenoma, tumor or whole-sized mucosal biopsies of control patients. Isolated RNA was stored at -85°C. 2 µl of RNA was reversibly transcribed using M-MLV Transcriptase and oligo-dT15 primer (Promega, Madison, WI, USA) in a total volume of 25 µl and stored at -25°C. DNA was isolated from 2 x 2 x 2 mm fragments of tumor tissue, from half-sized adenoma biopsies and from 1 ml of peripheral blood using the anion exchange membrane method with Sherlock AX kit (A&A Biotechnology, Gdynia, Poland) based on the phenol-chlorophorm-isoamyl alcohol and silica membrane technique (25).

Biopsies of adenoma and CRC were snap frozen in liquid nitrogen, and stored at -85°C. Isolated RNA was stored at -85°C. 2 µl of RNA was reversibly transcribed using M-MLV Transcriptase and oligo-dT15 primer (Promega, Madison, WI, USA) in a total volume of 25 µl. DNA was isolated from 2 x 2 x 2 mm fragments of tumor tissue, from half-sized adenoma biopsies and from 1 ml of peripheral blood using the anion exchange membrane method with Sherlock AX kit (A&A Biotechnology, Gdynia, Poland) based on the phenol-chlorophorm-isoamyl alcohol and silica membrane technique (25). From RNA, cDNA was reversibly transcribed using M-MLV Transcriptase and oligo-dT15 primer (Promega, Madison, WI, USA) in a total volume of 25 µl. DNA was isolated from 2 x 2 x 2 mm fragments of tumor tissue, from half-sized adenoma biopsies and from 1 ml of peripheral blood using the anion exchange membrane method with Sherlock AX kit (A&A Biotechnology, Gdynia, Poland) based on the phenol-chlorophorm-isoamyl alcohol and silica membrane technique (25). From RNA, cDNA was reversibly transcribed using M-MLV Transcriptase and oligo-dT15 primer (Promega, Madison, WI, USA) in a total volume of 25 µl.
Biotechnology, Gdynia, Poland) according to manufacturer's protocol, and stored at -25°C.

Quantitative PCR analysis

The expression rate of the *FHIT* gene was analyzed by Real-time PCR using iQ Cycler (Bio-Rad, Hercules, CA, USA) with Sybr®Green I as a fluorophore. *FHIT* (GeneBank acc. # NM_002012.1) expression ratio was determined by the comparative method (Livak's 2^−ΔΔCT equation) (26) in relation to the mean rate of two housekeeping genes *ACTB* (GeneBank acc. # NM_01101.2) and *RPL32* (NM_000994.3) which have constant expression in CRC (27, 28). Primers' sequences are presented in Table 2.

The components of the PCR reactions: 0.4 µl tissue cDNA (equivalent of 32 ng of total mRNA), 100 nM primers and real-time PCR iQ SybrGreen SuperMix (Bio-Rad, Hercules, CA, USA) were mixed to obtain a final volume of 17 µl. All reactions were performed in duplicate. For *FHIT*, *ACTB* and *RPL32*, the amplification profile was: 30-s denaturation at 95°C followed by 30-s annealing at 55°C for *RPL32*, and 30-s annealing at 60°C for *ACTB* and *FHIT*, 1-min elongation 72°C and 5-s fluorescence reading at 77-80°C for a total of 35 cycles. In order to avoid detection of possibly truncated amplicons we applied the 5-sec fluorescence reading step at the highest temperature at which the requested PCR product still existed as double-stranded DNA (Table 2). To confirm the size of detected *FHIT* cDNA (mRNA), dynamic melt-curve analysis and agarose-gel electrophoresis were used for all post-PCR reaction tubes. Data were automatically collected and analyzed by iCycler iQ Optical Software ver. 3.0a (Bio-Rad, Hercules, CA, USA).

Table 2. Primers’ sequences for the QPCR system.

<table>
<thead>
<tr>
<th>Gene</th>
<th>GeneBank acc. #</th>
<th>Primers’ sequences</th>
<th>T_anner/T_read, number of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>FHIT</td>
<td>NM_002012.1</td>
<td>5' GGTATCCTAGGAATACCTGCCTGC 5' GGTGAGAGAGGTCCCATGGAAATG</td>
<td>60/80°C, 40</td>
</tr>
<tr>
<td>ACTB</td>
<td>NM_001101.2</td>
<td>5' TGTGCCCATCTAGGGGGTATGC 5' GGTACATGGTGTGCGCCAGACA</td>
<td>60/80°C, 35</td>
</tr>
<tr>
<td>RPL32</td>
<td>NM_000994.3</td>
<td>5' TGACAACAGGGTGTGGTGAAGAT 5' GTTCTGAGGAACATTTGAG</td>
<td>54.5/77°C, 35</td>
</tr>
</tbody>
</table>

T_anner - annealing temperature; T_read - fluorescence reading temperature

Table 3. Associations of FHIT protein expression assessed by IHC with clinical, pathological and molecular features of CRC patients.

<table>
<thead>
<tr>
<th>variables</th>
<th>FHHIT protein expression*</th>
<th>n</th>
<th>negative/weak moderate 0-50%, score 0-6</th>
<th>strong/very strong 51-100%, score 7-12</th>
<th>P, χ²</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td></td>
<td>10</td>
<td>8</td>
<td>2</td>
<td>P=0.004</td>
</tr>
<tr>
<td>CRC</td>
<td></td>
<td>57</td>
<td>17</td>
<td>40</td>
<td>χ²=9.16</td>
</tr>
<tr>
<td>sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
<td>35</td>
<td>9</td>
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</tr>
<tr>
<td>F</td>
<td></td>
<td>22</td>
<td>8</td>
<td>14</td>
<td>χ²=0.73</td>
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<tr>
<td>Dukes stage</td>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td></td>
<td>11</td>
<td>5</td>
<td>6</td>
<td>P=0.007 b</td>
</tr>
<tr>
<td>B</td>
<td></td>
<td>17</td>
<td>8</td>
<td>9</td>
<td>χ²=7.25</td>
</tr>
<tr>
<td>C</td>
<td></td>
<td>23</td>
<td>3</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
<td>6</td>
<td>1</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>Differentiated (G stage)*</td>
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<td></td>
<td></td>
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</tr>
<tr>
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<td>4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>well / G0</td>
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<td>1</td>
<td>2</td>
<td>P=0.54</td>
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<tr>
<td>moderate / G1</td>
<td></td>
<td>14</td>
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<td>9</td>
<td>χ²=0.38</td>
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<tr>
<td>poorly / G2</td>
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<td>40</td>
<td>11</td>
<td>29</td>
<td></td>
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<td>Lymph node metastasis</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td></td>
<td>28</td>
<td>13</td>
<td>15</td>
<td>P=0.007</td>
</tr>
<tr>
<td>positive</td>
<td></td>
<td>29</td>
<td>4</td>
<td>25</td>
<td>χ²=7.25</td>
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<tr>
<td>Genomic alteration*</td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>31</td>
<td>7</td>
<td>24</td>
<td>NS</td>
</tr>
<tr>
<td>positive</td>
<td></td>
<td>26</td>
<td>7</td>
<td>19</td>
<td></td>
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<tr>
<td>mRNA expression*</td>
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</tr>
<tr>
<td>CRC&lt;control</td>
<td></td>
<td>8</td>
<td>5</td>
<td>3</td>
<td>P=0.02</td>
</tr>
<tr>
<td>CRC&gt;control</td>
<td></td>
<td>49</td>
<td>12</td>
<td>38</td>
<td>χ²=4.94</td>
</tr>
</tbody>
</table>

a - scoring method of IHC staining pattern according to Hao et al. (13) as described in Methods
b - statistically significant difference between Dukes' A+B and Dukes' C+D groups, Fisher's test
c - non-significant differences between adenoma+G0 and G1+G2 groups, Fisher's test
d - LOH in at least one of three studied markers
e - cases with different median level of FHIT than control group (value = 0.0054)
LOH assessments

In order to check for allelic deletions within the FHIT ORF (59.71-61.2 MBp), three microsatellite markers mapped in genomic fragile site FRA3B were chosen: D3S1300 (locus FRA3B; intron 5 of FHIT gene; 60.4 Mbp), D3S1481 and D3S1234 (loci 60.6 and 60.08 Mbp, respectively). PCR reaction mixture; 30 ng of genomic DNA and 200 nM primers for each microsatellite pair were mixed with 2 mM MgCl2 and 0.6 U of Taq polymerase (Fermentas, Vilnius, Lithuania) to a final volume of 15 µl. After separation in 6% denaturing acrylamide gel using Sequi-Gen II Sequencing Cell (Bio-Rad, Hercules, CA, USA) at 2000V for 1.5-4 h, gel was silver-stained (AgNO3, gel using Sequi-Gen II Sequencing Cell (Bio-Rad, Hercules, CA, USA), dried, scanned and analyzed using Gene Doc 2000 and Quantity One software (Bio-Rad, Hercules, CA, USA). LOH data were automatically compared between peripheral blood and malignant/adenoma tissue with respect to allele peak size, height and area ratio. Intensity or signal ratio differences of 40% or more were considered sufficient for positive LOH assignment.

Immunostaining of the FHIT protein

CRC tissue or colon biopsies that were stored at -80°C were used to obtain 12 µm-thick cryostat sections. Sections were incubated with rabbit primary polyclonal anti-FHIT antibodies (Polyclonal-ZR44; final dilution 1:500, Zymed Laboratories, San Francisco, CA, USA) overnight at 4°C. Then, sections were incubated with goat, anti-rabbit biotinylated polyclonal antibodies (Vectastain ABC Kit, PK-4001, Vector Labs., Burlingame, CA, USA) for 60 min at room temperature followed by treating with avidin-biotinylated horseradish peroxidase reagent for 10 min at room temperature (SuperPicTure Polymer Detection Kit, Zymed Laboratories, San Francisco, CA, USA). Immunostaining of the FHIT protein

As a negative control, the primary antibody was replaced with nonimmune serum in a similar dilution. The analysis of IHC reactions was performed in a blinded manner with respect to the clinical information. A scoring system related to the extent and intensity of immunostaining of enterocytes was used according to the method described by Hao et al. (13). The extent of positive staining was scored as 0, negative; 1, weak; 2, moderate; 3, strong by two independent observers (M.S. and Z.K.). The extent of positive staining, i.e. the relative number of immunoreactive enterocytes, was scored as 0 (<5%), 1 (5-25%), 2 (26-50%), 3 (51-75%), and 4 (>75% of all enterocytes in respective lesions). The final score was determined by multiplying the intensity score and extent score, yielding a range from 0 to 12. Scores 7-12 were defined as a strong/very strong immunostaining, whereas scores 0-6 as a negative/weak expression of the FHIT protein.

Statistical analyses

All statistical analyses were done using Statistica version 8.0 software (StatSoft Inc., Tulsa, OK, USA). In case of non-categorical variables Mann-Whitney U test or H-test of Kruskal-Wallis were used to compare median values of FHIT expression between groups. Spearman's test was used to assess correlations between mRNA values and clinical-pathological variables. Fisher's exact test was applied in order to assess associations between LOH, IHC and clinical-pathological variables. For all tests, the level of statistical significance was set at P<0.05. All plots were done using Microsoft Office Excel 2003.

RESULTS

FHIT mRNA expression in CRC and colorectal adenoma

FHIT mRNA levels were significantly up-regulated in tumor tissue of 86% (73/84) of CRC cases (P=0.00015; Table 1). The median FHIT mRNA level was at least 6 times higher in CRC than in control patients and in 30% of CRC cases FHIT ratio was at least 10-fold higher.

In adenoma 9/15 cases showed higher levels of FHIT mRNA as compared with control patients (P=0.016; Mann-Whitney U test). Moreover, a positive correlation was found between FHIT gene expression and the canonical CRC development pathway: normal mucosa (control group) ⇒ adenoma ⇒ CRC (R2 = 0.23, Spearman's test, P<0.05). We observed in CRC a positive correlation between FHIT mRNA level and tumor progression (Duke's A⇒D; R2 = 0.29; Spearman's test, Fig. 1A) and decreasing differentiation of malignant cells (G3⇒G2; R2 = 0.32; Spearman's test, Fig. 1B). No statistically significant relationships were found between FHIT mRNA ratios and sex, age, lymph node metastases or tumor location. Apart from expected 348 bp length PCR product of native FHIT gene, we found additional smaller amplicons of various sizes in 17/84 CRC cases, however, the presence of those bands was not associated with the occurrence of LOH at FRA3B or lower expression of native FHIT protein (data not shown). No differences were found between quantity of FHIT mRNA (QPCR) between those particular 17 cases with smaller amplicons and 67 CRC cases with native-length PCR product.

Loss of heterozygosity at the FHIT locus

DNA for LOH analysis was obtained from 57 CRC and 10 benign adenoma patients. Homozygotic and microsatellite instable cases at different microsatellite loci were treated as non-informative and were excluded from further analyses. The
highest occurrence of loss of heterozygosity was found in D3S1234 locus, in 32.5% (13/40) of informative cases. We found LOH in D3S1300 in 24% (10/42) and in D3S1481 in 11% (5/46) of all cases. No LOH within FRA3B was detected in analyzed adenoma samples. Although we did not observe any continuous deletions of all 3 microsatellite markers, we found simultaneous LOH at both D3S1300 and D3S1234 markers in 3 respective CRC biopsies.

FHIT mRNA levels were lower in LOH-positive cases (as analyzed by QPCR), however, the difference between FHIT ratios of patients with observed loss and retention of heterozygosity was not significant. Further analyses did not show differences between LOH and clinical-pathological variables (age, sex, Dukes' and differentiation stage and lymph node metastasis status).

**Immunohistochemical detection of the FHIT protein**

The presence of an immunoreactive FHIT protein in colonic enterocytes which reflected the expression of a native FHIT protein was evaluated in a semi-quantitative manner in mucosal biopsies obtained from 10 control, 57 CRC and 10 adenoma patients. A weak/moderate FHIT-immunoreactivity with respect to the staining intensity and number of immunopositive enterocytes was observed in control patients (8/10) (Fig. 3A), however, strong and very strong reaction prevailed in adenoma and CRC (Fig. 3B-D). We found that presence of FHIT protein correlated with tumor development (control<adenomas<CRC; R²=0.28, Spearman's test) and progression/invasiveness (control<adenomas<N0>N1; R²=0.38, Spearman's test). On the other hand, in regard to the progression of malignancy at a single cell level (differentiation G staging), we did not observe differences between groups. Moreover, no correlation between cellular presence of native FHIT protein and occurrence of deletions within the FRA3B genomic region was found. By comparing FHIT expression profile at the mRNA and protein levels, we found that IHC assays paralleled the QPCR results (p=0.003, Mann-Whitney U test). Sporadically, FHIT-immunostaining was observed within mucosal/submucosal smooth muscle cells and inflammatory mononuclear cells.

**DISCUSSION**

The FHIT gene is localized in the most fragile chromosome region of the human genome, FRA3B, and has been proposed as a putative tumor suppressor gene since its decreased expression was found in various human malignant diseases (2, 5-10) including cancers of the gastrointestinal tract (10-12). Moreover, decreased FHIT expression was found in many cancer cell lines (2, 4, 5, 7, 29, 30). Similarly to WWOX putative suppressor gene's mechanism of inactivation (locus FRA16D) (31), loss of heterozygosity (LOH) at FRA3B has been proposed to be a major factor associated with the decreased expression of the FHIT gene in various types of cancer (7, 10, 13-18). However, the extent of FHIT gene's suppression widely varied between reported studies due to the differences in applied methods, number of patients and their ethnic background (13-18). Earlier reports which showed FHIT gene down-regulation in CRC (12-18) and colorectal adenoma (32) were based on the analysis of the FHIT protein presence/absence by IHC or Western blot assays. Because FHIT transcripts are frequently abnormal (33, 34) due to deletions and insertions which result in inactive FHIT protein, the quantitative analysis of FHIT gene expression using only IHC is not possible. Some authors studied FHIT gene expression by reverse-transcription followed by nested PCR method (18, 35-37). This method was reported to be very
sensitive, e.g. in finding low amount of molecular target (cDNA) in a test-tube (4). However, nested-PCR is not a quantitative technique since the results are non-parametric and reflect only the presence or absence of an amplicon (38, 39). By selecting a very restrictive semi-quantitative analysis of the FHIT mRNA, we showed, in contrast to many previous reports, that FHIT gene expression was significantly up-regulated in CRC. Moreover, we found that the quantity of FHIT transcript increased with tumor progression and invasiveness. Our results are in line with the data of Thiagalingam et al. (20) who showed very high expression of a FHIT transcript which contained the complete coding sequence of the gene in 29/31 cell lines derived from human CRC (20). The opposite results of ours and Thiagalingam et al. (20) studies and previous reports may be caused by the use of different methodologies, differences in patient selection procedures, e.g. in the study by Hao et al. patients with familial adenomatosis polypl were included (13) and, possibly, different genomic structure of studied populations. The last option is supported by our finding that loss of heterozygosity at the fragile FRA3B site did not correspond to QPCR, IHC or clinical-pathological variables, whereas LOH at this site was found to be a major factor associated with decreased expression of the FHIT gene in various cancers (7, 10, 13-18).

Although our data which showed that immunohistochemical detection of the FHIT protein confirmed QPCR data are at variance with the observations of other authors (15-18), it has to be noted that significant differences in the immunohistochemical analyses of FHIT protein expression have been recently reported. E.g., Hao et al. (13) and Mori et al. (14) reported reduced expression of FHIT protein in 44% and 50% of CRC patients, respectively. However, Mady and Mehlman found in the series of 100 CRC patients that the majority of cases (69%) showed equal or higher expression of the FHIT protein in tumor tissue as compared with adjacent normal mucosa, and in only 8% of patients FHIT protein was not detected (19).

The presented results pose an intriguing question about the functional importance of the increased FHIT gene expression in our group of CRC and benign adenoma patients in a view of reports that showed lost or reduced FHIT protein expression in many preneoplastic lesions and in many types of human cancer (41). The studies of FHIT gene-deficient mouse and human tissue-derived and cancer-derived cells suggest that FHIT protein may promote apoptosis in response to various cell-damaging factors, possibly due to a strong activation of the ATR pathway following DNA damage (reviewed by Ishii et al. (41)). Results of a recent study have suggested that FHIT protein, encoded within DNA damage-susceptible FRA3B/FHIT chromosome fragile region, is necessary for protecting cells against DNA damage through modulation of cell cycle checkpoint proteins Hus1 and phosphoChk1 (42). According to
this approach enhanced expression of the FHIT gene in CRC tissue found by us and other authors (19), can be regarded as a way to protect cell against deleterious events such as DNA damage and cell cycle disturbances which accompany many neoplasia.

In conclusion, the results of this study suggest that reduction or absence of the FHIT gene expression is not a prerequisite for colorectal cancer development and progression.

Acknowledgment: The study was supported by the Polish Ministry of Science and Higher Education (grant no 2 P05B 083 26).

Conflict of interests: None declared.

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Received: August 5, 2009
Accepted: September 10, 2009

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