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Tissue Microarray FISH Applied to Colorectal Carcinomas with Various Microsatellite Status*

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Colorectal carcinoma is etiopathologically heterogenic. It may develop through a sequence of mutations leading to chromosome instability or be a result of defects in DNA repair mechanisms manifested by microsatellite instability of varying degrees. Colorectal carcinoma can thus be classified into microsatellite-stable (MSS), highly microsatellite unstable (MSI-H) and intermediate low-level microsatellite unstable (MSI-L) groups. Fluorescent hybridization *in situ* (FISH) is a method of detecting specific sequences of nucleic acids that is based on specific bonding of a fluorescent marker-associated probe and specific DNA fragment. The material consisted of 146 non-selected cases of colorectal carcinoma patients operated on at First Chair of General Surgery, Collegium Medicum, Jagiellonian University in Cracow, Poland. Following a standard histopathological evaluation, tissue microarrays were prepared using a Tissue MicroArray Builder, and FISH was performed employing probes specific for chromosomes 1, 8, 17 and 18. Microsatellite instability was evaluated in frozen material using the PCR reaction with gel and capillary electrophoresis. The mean number of signals obtained for chromosome 1 in the entire material was 2.06, while the corresponding mean values in the MSS group equaled 2.07, in the MSI-L group – 2.07, and in the MSI-H group – 2.01. The mean number of signals for chromosome 17 in the entire material was 2.1, in the MSS group – 2.11, in the MSI-L group – 2.13, and in the MSI-H group – 2.01. The number of signals for chromosome 18 in the entire material was 2, in the MSS group – 2, in the MSI-L group – 2, and in the MSI-H group – 2. The means number of signals for chromosome 8 in the entire material was 2.07, in the MSS group – 2.08, in the MSI-L group – 2.01, and in the MSI-H group – 2. These differences are not sufficient for distinguishing colorectal carcinoma molecular forms.

Introduction

According to current opinions, the pathogenesis of colorectal carcinoma (CRC) is not uniform. The majority of cases are associated with chromosomal instability, whereas some 10–20% of cases are related to DNA repair defects and microsatellite instability (MSI). It has been also propounded that carcinomas with microsatellite instability in some loci (MSI-low, MSI-L) may be a result of the „third path" of carcinogenesis in the colon [7, 11].

Hybridization consists in a specific bonding of a probe and the search for DNA or RNA fragments. This method is used for detecting of mRNA for particular proteins, viral nucleic acids, gene location in metaphase chromosomes and interphase nuclei, quantitative and structural chromosomal aberrations and point mutations [4, 10].

Material and Methods

The material consisted of non-selected cases of colorectal carcinomas from patients operated on at First Department of General Surgery, Collegium Medicum, Jagiellonian University, Cracow, Poland. Immediately after operation, samples were transferred fresh to Chair of Pathomorphology and as-

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sessed grossly. Sections were taken from the peripheral part of tumors, with exclusion of ulcerated and necrotic areas, and uninvolved intestinal wall. The resultant specimens were frozen at –80°C and stored for molecular analysis. The remaining part of the material was fixed in 10% buffered formalin overnight. From it, sections were taken according to the routine protocol [17]. The sections were routinely processed using automatic tissue processors (Shandon, Astmoor, UK) and embedded in paraffin. Four μm-thick sections stained with hematoxylin-eosin were prepared from paraffin blocks for histopathological assessment. The evaluation followed routine protocols and the stage was determined according to the TNM classification [1].

Microsatellite analysis was performed according to the previously published protocol [9, 12, 18, 19]. Briefly, DNA was extracted from fresh-frozen tumors and corresponding non-neoplastic tissue (QIAamp DNA Mini Kit, Qiagen GmbH, Hilden, Germany), and PCR-amplified with a screening panel of five microsatellite markers: APC, p53, BAX, BATR II and BAT-26. PCR was performed in 20 μl of the reaction mixture containing: 2 μl DNA template (100 ng), 2 μl STR buffer (Promega, Madison, USA), 0.5 μl of each primer (10 nM), 1 U Taq polymerase (Fermentas Inc., Burlington, Canada). The amplicons were electrophoresed on 6% polyacrylamide gel at 50 W for 1.5 h and visualized using routine silver staining. All the cases demonstrating any, even single, genetic alterations at any marker of the screening panel were subjected to further analysis with an extended panel of nine microsatellite markers (Microsatellite Instability RER/LOH Assay Kit, Applied Biosystems, Foster City, USA) and the PCR products were visualized using capillary electrophoresis with an ABI PRISM 310 Analyzer (Applied Biosystems, Foster City, USA). The kit contains nine primer sets flanking microsatellite loci linked to tumor-suppressor genes: MSH2 (D2S123), DCC (D18S35), APC (D5S346). MLH1 (D3S1611), NM23, HPC1 (D1S2883), MET (D7S501), a dinucleotide marker linked to p53, and a pentanucleotide marker linked to the same gene. The results were analyzed by the Genescan and Genotyper Software (Applied Biosystems, Foster City, USA). A locus was deemed unstable when an electrophoregram of a PCR product derived from the tumor differed from that of normal matching tissue by the presence of at least one new peak with the length corresponding to 2 bp or 5 bp. A case was included into the MSI-low group when showing genetic instability at more than one, but not more than 40% of loci. The tumors were classified as MSI-high when MSI was detected at 40% or more loci analyzed in a given case. Additionally, as the literature strongly supports the high specificity of the BAT-26 marker in respect to the MSI-high phenotype, all tumors with instability at BAT-26 locus were included in the MSI-high group. The remaining cases were classified as microsatellite-stable (MSS) carcinomas. Tumor allele showing at least a 50% reduction in band/peak intensity in comparison with the corresponding band/peak of normal mucosa was assessed as LOH.

Hematoxylin-eosin stained sections were reviewed and in each case one section was selected that contained representative and well-preserved carcinoma tissue. Tissue microarrays were prepared from selected paraffin blocks using a Tissue MicroArray Builder (Histopathology Ltd., Pecs, Hungary). The tissue microarrays consisted of cylinders, 2 mm in diameter. Three μm thick sections were prepared from the paraffin blocks. Fluorescent hybridization in situ was performed using the FISH CEP VYSIS Assay kit (Vysis Inc., Downers Grove, USA) following the manufacturer's instruction. Briefly, the sections were deparaffinized and subsequently treated with 45% formic acid/0.3 % hydrogen peroxide solution for 15 minutes, washed in distilled water, and placed in Pretreatment Solution (Vysis Inc., Downers Grove, USA) for 10 minutes at 80°C, then washed again in distilled water. The slides were protease digested for 10 min at 37°C, washed in distilled water, air drained and dehydrated in series of alcohols. Subsequently, following the addition of DAPI solution, the digestion quality was controlled under a fluorescence microscope and DAPI was removed by rinsing. The hybridization solution contained 1 μl of probes, 2 μl of water and 7 μl of CEP Hybridization Buffer. The following probes were used: CEP1 – Spectrum Orange and CEP8 – SpectrumGreen, as well as CEP17 – SpectrumOrange and CEP18 – SpectrumGreen. The aliquot of 10 μl of probe solution was placed on the surface of each preparation, closed with a cover glass, sealed and dried at 37°C. Denaturation was carried out at 85°C for 1 minute in MP16 hybridization device (GENOS, Łódź, Poland). Hybridization was carried out at 37°C overnight. Following the removal of sealant and cover glass, the preparations were rinsed in post-hybridization Wash Buffer (2x sodium salt citrate, 0.3% NP-40, pH 7.0–7.5) at room temperature, and subsequently in post-hybridization Wash Buffer at 73°C for 2 minutes. The preparations were then air-dried, covered with 10 μl DAPI solution applied in drops and closed with coverslips. The preparations were stored in the dark at –20°C and warmed to room temperature for evaluation. The staining results were evaluated under an Axioscop microscope using a 100× PlanNeofluar lens (Carl Zeiss AG, Oberkochen, Germany). For each evaluated case and each probe, the authors counted the number of signals per cell, and calculated the following parameters:

index1=total number of signals/number of hybridization experiments,

index2=number of number of hybridization experiments with aneuploid number of signals,
index3=index2/number of number of hybridization experiments,

The statistical analysis was performed using the STATISTICA, v. 6.1PL package (StatSoft, Inc., Tulsa, USA). The significance level was set to 0.05.

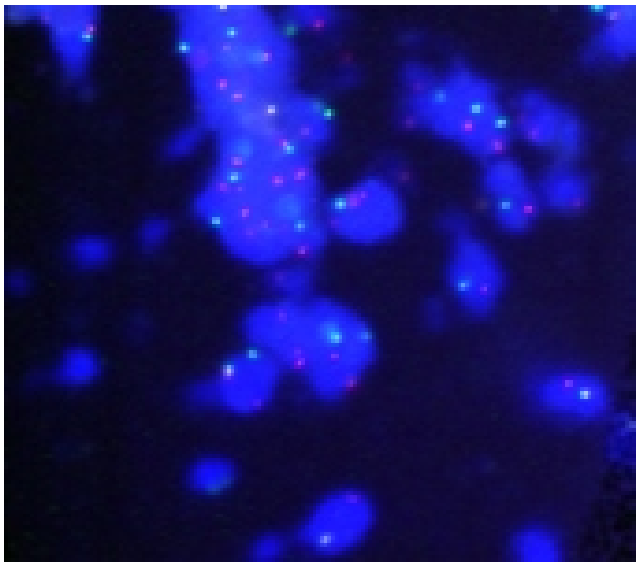

Fig. 1. An example of FISH result. Red signals are chromosome 17 probes, green – chromosome 18 probes; blue is the DAPI stained chromatin background. Lens magnification 100×.

Results

The investigated material consisted of 146 colorectal carcinomas originating from 62 females and 84 males; the mean age was 64.81 years (SD 10.78). The tumors were classified as stage pT1 in six cases, pT2 in 53, pT3 in 79, and pT4 in eight patients. Metastatic deposits in the lymph nodes were noted in 62 cases, involving one to three lymph nodes (pN1) in 35 individuals, and four or more (pN2) in 49 patients. The mean number of investigated lymph nodes was 20.45 (SD 11.16). The mean number of metastatic lymph nodes was 3.95 (SD 6.54). The mean percentage of lymph nodes with metastatic deposits was 19.89 (SD 26.81). Remote metastases at the time of surgery were noted in 17 cases. In 17 patients, the procedure was not microscopically radical (pR1). In 80 cases, no angioinvasion was observed (pL0V0), in 23 patients, small blood vessels were involved (pL1V0), in 23 individuals, only large vessels were involved (pL0V1), while in 20 patients, both small and large vessels were involved (pL1V1). The tumors were well differentiated (G-I) in 36 cases, moderately differentiated (G-II) in 93, and poorly differentiated (G-III) in 17 patients.

The MSS phenotype was seen in 116 cases, MSI-L in 16, and MSI-H in 14 patients. The MSI-H carcinomas were more frequently situated on the right side ($p<0.01$); they also more commonly contained intratumoral lymphocytes ($p<0.001$). These tumors contained more solid and mucus producing areas. The mean number of signals for chromosome 1 in the entire material was 2.06 (SD 0.25), in the MSS group – 2.07 (SD 0.28), in the MSI-L group – 2.07 (SD 0.17), and in the MSI-H group – 2.01 (SD 0.02). The mean number of signals for chromosome 17 in the entire material was 2.1 (SD 0.26), in the MSS group – 2.11 (SD 0.27), in the MSI-L group – 2.13 (SD 0.27), and in the MSI-H group – 2.01 (SD 0.03). The mean number of signals for chromosome 18 in the entire material was 2 (SD 0.17), in the MSS group – 2 (SD 0.19), in the MSI-L group – 2 (SD 0), and in the MSI-H group – 2 (SD 0). The mean number of signals for chromosome 8 in the entire material was 2.07 (SD 0.23), in the MSS group – 2.08 (SD 0.26), in the MSI-L group – 2.01 (SD 0.03), and in the MSI-H group – 2 (SD 0.02). The mean Index1 value in the entire material was 2.05 (SD 0.16), in the MSS group – 2.06 (SD 0.18), in the MSI-L group – 2.05 (SD 0.08), and in the MSI-H group – 1.98 (SD 0.07). The mean value of Index2 in the entire material was 1.87 (SD 2.37), in the MSS group – 1.8 (SD 2.3), in the MSI-L group – 2.31 (SD 2.68), and in the MSI-H group – 1.93 (SD 2.67). The mean Index3 value in the entire material was 1.18 (SD 1.09), in the MSS group – 1.21 (SD 1.08), in the MSI-L group – 1.2 (SD 1.09), and in the MSI-H group – 0.89 (SD 1.17). Differences between FISH results in MSS, MSI-L and MSI-H carcinomas were, nevertheless, statistically non-significant.

Discussion

Hybridization *in situ* is a cytochemical technique that allows for detecting a single gene or its fragment in the chromatin, or its specific expression at the site of its natural occurrence. The process of hybridization consists in forming specific bonds between purine and pyrimidine bases of the probe and investigated nucleic acid. The probe is a specific DNA or RNA fragment coupled with an appropriate detection system [4, 10]. The method is used for example in endocrinology to detect mRNA for a given hormone, in detecting mRNA for specific proteins, viral nucleic acids, in investigating gene location in metaphase genes and interphase nuclei and in detecting quantitative and structural chromosomal aberrations and some point mutations [4, 10]. Probes employed in hybridization are labeled with radioactive and non-radioactive markers. The latter group includes direct labeling with such markers as for example fluorochromes, or indirect detection using haptens, as well as enzymes such as peroxidase and alkaline phosphatase

[10]. In the FISH method, probes are labeled with fluorochromes, such as fluorescein, rhodamine, resurfin, hydroxycoumarin, and many others [4, 10].

To increase the performance of special staining in histopathology, the method of tissue microarrays may be used. The technique consists in combining materials from several cases into a single paraffin block. Following the selection of representative fields for each case, cylinders 0.6–2 mm in diameter are cut from original paraffin block. Subsequently, the obtained cylinders are placed in a previously prepared, new paraffin block. The position of each cylinder is to be recorded. Histological sections may be prepared from the thus obtained tissue microarray. This allows for performing any reactions and special staining procedures, especially immunohistochemical, as well as for using the FISH technique. Thus, the labor expenditure is decreased, similarly as is the cost of reagents. The last feature is especially attractive then using the expensive FISH method. It is also possible to use the available material more sparingly, since single original paraffin block may be source of material for several tissue microarrays. Thanks to this technique, investigations of large groups of cases may be performed and numerous markers may be analyzed simultaneously [8, 14].

Colorectal carcinomas constitute a heterogeneous group of tumors. These include familial cancers, associated with such syndromes as familial adenomatous polyposis and hereditary nonpolyposis colorectal cancer (HNPCC), as well as sporadic cases, which constitute the majority [3, 6, 11, 22, 28]. With respect to pathogenesis, two basic mechanisms of colorectal cancer development are proposed. The first mechanism is analogous to that present in adenomatous polyps and associated with chromosomal instability triggering major anomalies in the genome. Such anomalies may be manifest in classic cytogenetic tests as chromosome mutations. In consequence, carcinomas with chromosome instability are often DNA-aneuploid. Ooi et al. assessed the incidence of aneuploidy in colorectal carcinomas as occurring in approximately 50% of cases [13]. The incidence rates of alterations involving particular chromosomes are various. Obviously, the rate depends in a great degree on the employed method of evaluation. Classic cytogenetic methods detect changes involving entire chromosomes, while methods as FISH or PCR based detection are capable of detecting point mutations, which cannot be otherwise identified. In hybridization studies the results depend also on the selection of a type of marker. The use of centromere probes in the present study has been dictated by the aim of assessing a loss or addition of entire chromosomes rather than evaluation of changes within specific oncogenes, which may be easily tested in a reliable way using other methods. In colorectal carcinoma frequently encountered chromosome changes include trisomy of chromosome 7 (70%) and monosomy of chromosome 18 (30%) [13]. Zhou et al. noted loss of heterozygosity in chromosome 1 in approximately 1/3 of sporadic colorectal cases [29]. Praml et al. investigated numerous microsatellite markers situated at 1p and estimated the incidence of mutations as more than 80% [16]. Deletions in chromosome 18 may occur in 3/4 of colorectal carcinomas [25], while Sasaki [20] quotes the incidence of such deletions as 1/3 of cases. Ooi et al. found monosomy of chromosome 18 only in a single case of the 26 investigated using the FISH method [13]. Vogelstein assessed the incidence of deletions at 17p as approximately 75% [25], while by FISH, the incidence rate of changes in chromosome 17 may be below 50% [21]. The appearance of chromosome abnormalities is a reflection of serious impairment of cell division control mechanisms. The inactivation of repair mechanisms facilitates the occurrence of the carcinomatous phenotype. Changes in specific regions of the genome are also associated with deletion or amplification of specific genes. Thus, the gene encoding p53 protein is situated within 17p locus [2], while DCC is located on chromosome 18 [24].

The other path of colorectal carcinoma development is associated with a more subtle genome instability, which is expressed by alterations involving short, repetitive sequences, especially microsatellite DNA [5, 11]. Changes of this type do not involve any major areas within the genome and, therefore, their presence may be detected solely by molecular methods, chiefly based on PCR. Thus, carcinomas with microsatellite instability are in the majority of cases DNA-euploid. When compared to carcinomas with chromosome instability, tumors with microsatellite instability are characterized by their more proximal location, more frequent occurrence of lower degrees of differentiation and mucus generation, as well as the presence of numerous lymphocytes that infiltrate the tumor [5, 22, 28]. In addition to these two well-known categories, there are colorectal carcinomas with microsatellite instability detectable on some loci only; tumors of this type are characterized by changes detected in less than 40% of the examined microsatellite loci. The group has been termed “MSI-L”, although its place in the classification remains unclear [15, 23, 27]. The DNA repair systems involved appear to be distinct [26].

It can be concluded that colorectal carcinomas with various degrees of microsatellite instability show some differences in chromosomal counts obtained by the FISH method, but these are not sufficient for distinguishing carcinoma categories.

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