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Hypermethylation of 5′-Region of the Human Calcitonin Gene in Leukemias: Structural Features and Diagnostic Significance

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Abstract—Methylation of the 5′-region of the calcitonin gene was investigated in bone marrow and peripheral blood cells of 27 healthy volunteers and 25 leukemic patients. In all patients suffering from various forms of myeloid and lymphoid leukemia, hypermethylation of CpG sequences was observed in this region of the calcitonin gene. Cytosine hypermethylation in the CpG sequence did not involve cytosines of adjacent CpNpG sequences (where N is any nucleoside). The 5′-region of the calcitonin gene lacked CpNpG methylation both in healthy controls and in leukemic patients; this apparently represents specific "non-alternative" type of CpG methylation in the extended DNA sequence. Methylation of the calcitonin gene was monitored in 18 leukemic patients during malignant progression and medical treatment. Hypermethylation of the calcitonin gene was not observed on long-term clinical hematological remission. In ten patients characterized by unstable (or incomplete) remission hypermethylation of the calcitonin gene persisted through the whole period of observation. In relapses, hypermethylation of the calcitonin gene appeared again and in six patients, this "molecular relapse" being registered 1-8 months before onset of clinical and laboratory signs of disease progression. The leukemia-specific hypermethylation of CpG sequences of the 5′-region of the calcitonin gene is a promising prognostic and diagnostic marker of leukemias and might be useful for monitoring of this disease.

Key words: DNA, methylation, calcitonin gene, CpG and CpNpG sequences, leukemia, prognostic and diagnostic marker

The first reports of a relationship between altered enzymatic methylation of DNA and malignant growth were published more than 20 years ago [1, 2]. However, only recently it has been clearly recognized that tumor transformations determine not only classic genetic changes, but also epigenetic modifications related to DNA methylation [3]. Tumor cells simultaneously exhibit two opposite phenomena: hypomethylation of both genes and the whole genome and hypermethylation of certain genes; the latter requires increased level of DNA-methyl transferase activity which was actually detected; however, in some studies its assay was not methodologically correct [3, 4]. Each component of this "methylation imbalance" makes a significant contribution to development of a malignant tumor by causing chromosomal instability, mutations, activation of protooncogenes, and silencing of tumor and apoptotic suppressor genes [3]. Special attention is now given to analysis of hypermethylation of tumor suppressor genes, which might help to find reliable markers for early diagnostics of various specific forms of malignant tumors. Good evidence also exists that abnormal methylation of some genes may highly correlate with progression of specific malignant tumors. However, in some cases the role of these hypermethylated genes in tumor transformation and their functions in carcinogenesis remain unclear [3, 4]. In the case of leukemias, methylation status of the calcitonin

Abbreviations: ALL) acute lymphoid leukemia; AML) acute myeloid leukemia; CML) chronic myeloid leukemia; MDS) myelodysplastic syndrome.

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gene may provide valuable prognostic and diagnostic information. Hypermethylation of the calcitonin gene was found in some kinds of malignant transformation including leukemias [5-8]. However, data on quantitative correlation between various forms of leukemias and hypermethylation of the calcitonin gene are contradictory, and usually such correlation is observed in 50-90% cases of this disease [5, 9-12]. It is possible that such variations may be (at least partially) attributed to different resolution capacity of methods employed for analysis of DNA methylation [10, 11].

It should be noted that studies of most functions of enzymatic DNA methylation have focused only on CpG type of methylation. Although cytosine methylation in human DNA preferentially occurs in CpG sequences, such modification also occurs in symmetric (CpNpG) [13] and asymmetric (CpN) sequences (where N is any nucleoside) [14-16]. It is possible that various types of DNA methylation play various roles in the cell, but until recently this problem has not been investigated yet.

So, in the present study we have investigated site-specific (CpG and CpNpG) types of methylation of the 5′-region of the human calcitonin gene in patients with various forms of myeloid leukemias and acute lymphoid leukemias. We have also evaluated the possible importance of calcitonin gene methylation pattern as prognostic and diagnostic markers of leukemias. Special attention has been given to monitoring of the calcitonin gene methylation in 18 leukemic patients during development of malignant process, chemotherapy, and transplantation treatment. In this paper, we consider biochemical aspects of site-specific types of calcitonin gene methylation in leukemic patients. A detailed characteristic of clinical material was described in our previous publications [17, 18].

MATERIALS AND METHODS

DNA samples were obtained from nuclei-containing peripheral blood cells and bone marrow of patients with various forms of leukemia and also from healthy volunteers.

DNA was isolated by phenol–chloroform extraction after overnight incubation of cells at 37°C in 0.1% SDS with proteinase K (100 μg/ml; Sigma, USA) followed by subsequent treatment with RNase A (100 μg/ml; Sigma) at 37°C for 2 h as described earlier [19].

Restriction endonucleases *Hpa*II and *Msp*I were purchased from New England BioLabs (USA); *Eco*RII and *Bst*NI were isolated at the Pushchino Branch of the Institute of Bioorganic Chemistry using previously described methods [20, 21].

DNA cleavage by restriction endonucleases was carried out under conditions optimal for catalytic activity of each particular enzyme. DNA (8-12 μg) was cleaved by either restriction endonucleases *Hpa*II (12 U/μg of DNA) and *EcoR*II (20 U/μg of DNA), recognizing internal methylated cytosine in the CCGG and CCWGG sequences, or by restriction endonucleases *Msp*I (20 U/μg of DNA) and *Bst*NI (15 U/μg of DNA), which are insensitive to this type of methylation. The reaction was carried out at 37°C (or 55°C in the case of *Bst*NI) for 16-18 h.

Electrophoresis of products of DNA digestion was carried out in 0.8% agarose gel (in the case of *Hpa*II and *Msp*I restrictases) or in 6% polyacrylamide gel (in the case of *Eco*RII and *Bst*NI restrictases) at the ratio acrylamide/*bis*-acrylamide 19 : 1. In both cases TBA buffer (0.089 M Tris, 0.089 M boric acid, 1 mM EDTA, pH 8.3) containing ethidium bromide (0.5 μg/ml) was used. The electrophoresis was carried out at voltage 3 V/cm. Separation of DNA fragments was monitored in the UV (310 nm).

Digestion products were transferred onto Hybond-N^+ (Amersham, England) nylon membrane by electroblotting using a Mini Trans Blot apparatus (BioRad, USA). Southern blot hybridization analysis of DNA was carried out using slightly modified protocol [19].

DNA probe. A fragment of 5′-region of human calcitonin gene (1.7 kb) kindly provided by Dr. S. Baylin (Johns Hopkins University Oncology Center, Baltimore, USA) was used as the hybridization probe. The probe was cloned as *Eco*RI-*Hind*III fragment in pUC18 plasmid and isolated by electrophoresis followed by subsequent purification by phenol–chloroform extraction.

The DNA probe was labeled by the method of random multiple priming using Multiprime DNA Labelling System (Amersham) according to the supplier's protocol and using [α-^{32}P]dATP (5000 Ci/mmol) produced by Physico-Energetic Institute (Obninsk, Russia). The resulting probe had specific activity $5\cdot10^8$-$1\cdot10^9$ pulse/min per μg of DNA.

Pre-hybridization, hybridization, and washing of membranes were carried out at 65°C using the supplier's recommendations. Membranes exposed for radioautograph preparation were then analyzed on a Phosphoimager radioactivity analyzer (Molecular Dynamics, USA). DNA samples were analyzed twice. For verification of complete cleavage of DNA by *Hpa*II and *Eco*RII restrictases, DNA samples were hydrolyzed by higher concentration of these enzymes (25-30 U/μg of DNA) and the hybridization pattern remained the same.

RESULTS

Effect of methylation state on the *Hpa*II-digestion pattern of the 5′-region of the calcitonin gene. According to Baylin et al. [5], Fig. 1 shows the restriction map of the calcitonin gene and various patterns of digestion of 5′-region of this gene by restriction endonucleases *Msp*I and *Hpa*II depending on methylation of this region. Digestion

of the 5′-region of the calcitonin gene by restriction endonuclease *Msp*I produces several restriction fragments. Three of these fragments (of 0.5, 0.6, and 1.0 kb) were detected after hybridization with the DNA probe (Fig. 2, lane *2*). Since restriction endonuclease *Msp*I is insensitive to methylation of internal cytosine of CCGG sequence, the *Msp*I pattern of blot-hybridization of the calcitonin gene was the same in healthy volunteers and leukemic patients. In this study, *Msp*I-blot-hybridization analysis was used twice as specific internal control for all DNA preparations (Fig. 3, lane *2*; Fig. 4, lane *6*). Digestion of the calcitonin gene by restriction endonuclease *Hpa*II, which cannot cleave CCGG sequences methylated by internal cytosine, revealed the presence of an additional fragment of 2.0 kb due to partial methylation of cytosine in the specific sequence CCGG M_5 in the 5′-region of the calcitonin gene; this is the normal pattern for healthy tissues [5] (Fig. 1; Fig. 2, lane *1*). In this study, a similar pattern of *Hpa*II-blot hybridization of the calcitonin gene with the DNA probe was observed in all 24 control samples of DNA from peripheral blood and in three DNA samples obtained from bone marrow cells of healthy donors (Fig. 2, lane *1*). With the hypermethylated state of the CCGG sequence of the 5′-region of the calcitonin gene, *Hpa*II-blot hybridization analysis revealed the appearance of additional (abnormal) restriction fragments of 1.2, 2.2, 2.6, and 3.1 kb (Figs. 1, 3, and 4). (Since the restriction pattern of the calcitonin gene below 1.0 kb was poorly informative, these fragments of the restriction pattern are not shown in Figs. 3 and 4.)

Hypermethylation of the 5′-region of the calcitonin gene in chronic myeloid leukemia (CML). The hypermethylation of this gene by abnormal *Hpa*II-fragments was clearly recognized in four patients at the terminal stage (blast crisis) and in the case of relapse of this disease after allogenic transplantation of bone marrow (Fig. 3, lanes *1-5*).

Hypermethylation of the 5′-region of the calcitonin gene in acute myeloid leukemia (AML). This was found in peripheral blood and bone marrow cells of all six primary patients (Fig. 3, lanes *6*, *10-14*). Similar changes in the methylation pattern of this gene were also found in two patients with relapse of this disease (Fig. 3, lanes *9* and *16*). This form of leukemia was characterized by maximal hypermethylation of the 5′-region of calcitonin in tumor cells: registration of abnormal *Hpa*II-fragment of 3.1 kb and (in one case) even larger fragment of 3.3 kb (Fig. 3, lane *13*). During clinical and hematological remission abnormal fragments almost disappeared (Fig. 3, lanes *15* and *17*). This suggests low degree of hypermethylation of

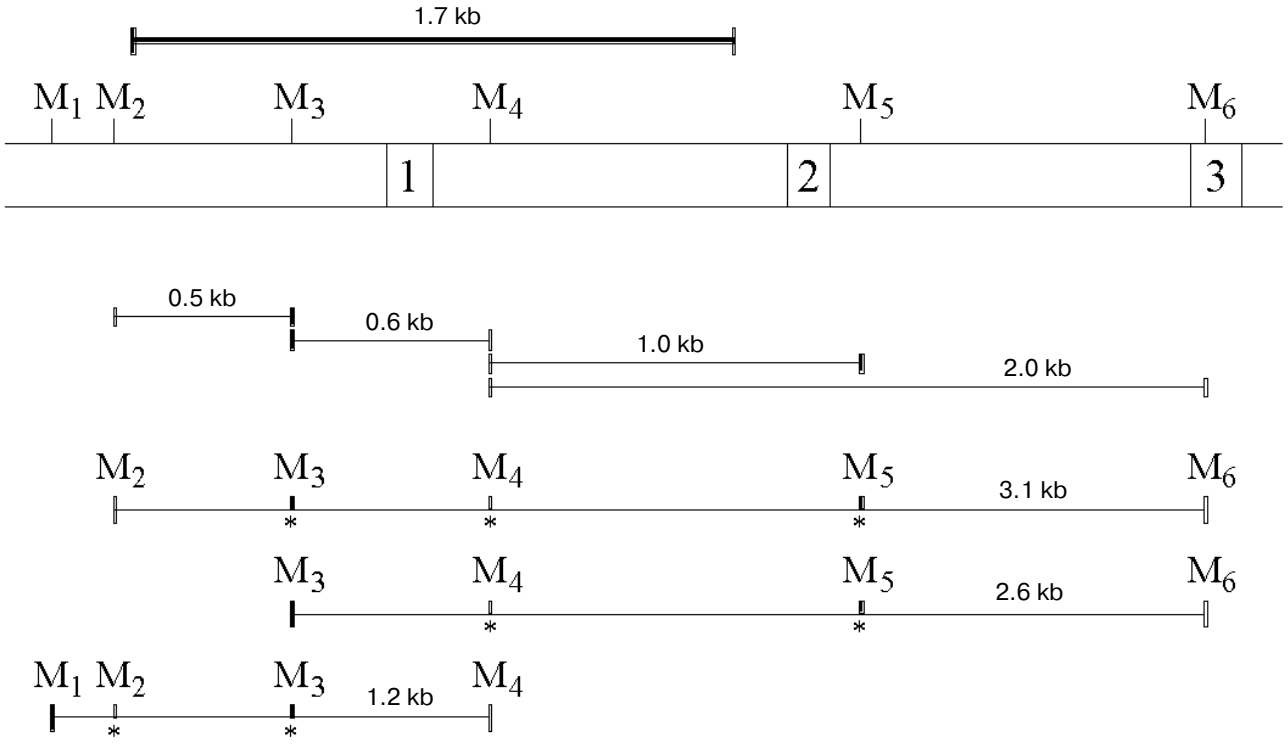

Fig. 1. Restriction map and possible variants of digestion of the 5′-region of the human calcitonin gene by restriction endonucleases *Hpa*II and *Msp*I. Restriction endonuclease *Msp*I always cleaves fragments of 0.5, 0.6, and 1.0 kb irrespectively to methylation state of this region. Restrictase *Hpa*II cleaves the same fragments in absolutely non-methylated 5′-region of the calcitonin gene. Fragments of 1.2, 2.0, 2.6, and 3.1 kb (with indicated methylation positions) are cleaved only by restrictase *Hpa*II under conditions of various methylation degree at the methylation points M_2-M_5. Marked regions 1-3 show exons. The solid line shows the size and position of the hybridization probe.

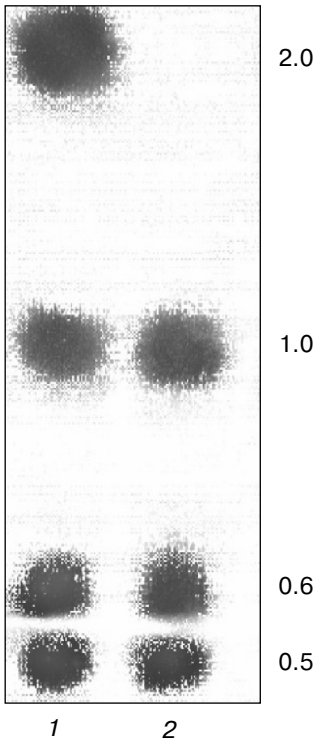

Fig. 2. Digestion of the 5′-region of the human calcitonin gene from normal bone marrow and peripheral blood cells by restriction endonucleases *Hpa*II (*1*) and *Msp*I (*2*). Size of fragments is shown as kb on the right.

5′-region of the calcitonin gene. Abnormal *Hpa*II-fragments were not found in bone marrow cell DNA from a patient with AML in the state of long-term remission for 9 years (Fig. 3, lane *8*). This implies full "molecular remission". In three patients, it was possible to monitor time-course of changes of the methylation state of the calcitonin gene in dependence on the effectiveness of chemotherapeutic treatment. The pattern of pathological hypermethylation of the calcitonin gene in one patient with primary resistance to treatment with cytostatics remained unchanged after three courses of chemotherapy (Fig. 3, lanes *6* and *7*). However, in two other patients with favorable prognosis to chemotherapeutic treatment it normalized on remission (Fig. 3, lanes *15* and *17*).

Hypermethylation of the 5′-region of the calcitonin gene in myelodysplastic syndrome (MDS). MDS refers to a group of pathological states of bone marrow and peripheral blood, which finally transform into AML. In one patient with this syndrome the presence of abnormal DNA fragment of 3.1 kb was recognized in bone marrow cell DNA in the favorable period of clinical course fours months before syndrome transformation into acute leukemia (Fig. 3, lanes *18* and *19*, respectively). The hypermethylation of the 5′-region of the calcitonin gene may indicate existence of a stable clone of tumor cells and increase in its proliferative activity. Similar hypermethylation of the 5′-region of the calcitonin gene was also found in the other patient with MDS in the state of transformation of this syndrome into AML (Fig. 3, lane *20*).

Our data on hypermethylation of the 5′-region of the calcitonin gene in patients with AML and on possible hypermethylation of this region in patients with MDS are consistent with observations by other authors [7, 8, 22]. Progression of AML and MDS was accompanied by hypermethylation of the 5′-region of the calcitonin gene in peripheral blood and bone marrow cells.

Hypermethylation of CCGG sequence of the 5′-region of the calcitonin gene in acute lymphoid leukemia (ALL). In the case of ALL (as well as in the case of AML), the pattern of digestion of the 5′-region of the calcitonin gene by restriction endonuclease *Hpa*II is characterized by the presence of abnormal fragments related to hypermethylation of this region. After a course of chemotherapy hypermethylation of the 5′-region of the calcitonin gene was detected in bone marrow cells from a patient with clinically and hematologically diagnosed remission (Fig. 4, lane *1*). This suggests maintenance of proliferating activity of a malignant cell clone even when morphological parameters of bone marrow remained within the normal range in this period. Thus, even under conditions of clinical remission there were signs of "molecular relapse" of this disease. Abnormal *Hpa*II fragments (except poorly detectable fragment of 1.2 kb) disappeared three weeks after an additional course of chemotherapy (Fig. 4, lane *2*). However, four weeks later, these fragments appeared again (Fig. 4, lane *3*) and did not disappear up to clinical and laboratory confirmation of relapse of this disease diagnosed six months later (Fig. 4, lanes *4* and *5*). Thus, the pattern of "molecular relapse" appeared 5 months earlier than the first clinical signs registered by the increase in blast cells in the bone marrow. Although peripheral blood of this patient was characterized by low percentage of blast cells, there was detectable hypermethylation of the calcitonin gene registered by appearance of *Hpa*II-fragments of 1.2 and 2.6 kb, which obviously indicate initial and intermediate stages of hypermethylation of this region (Fig. 4, lane *5*). A similar pattern was also observed in the other patient with relatively low percentage of blast cells (3%) in peripheral blood (Fig. 4, lane *23*). This patient was characterized by extremely aggressive progression of this disease which was resistant to chemotherapy and which was accompanied by maximal methylation of the 5′-region of the calcitonin gene recognized by abnormal *Hpa*II-fragment of 3.1 kb in bone marrow cells (Fig. 4, lane *22*). Detection of an additional fragment of 1.2 kb in cell samples from this patient suggests proliferation of tumor cells (Fig. 4, lane *22*). With a similar hypermethylation pattern of the 5′-region of the calcitonin gene in corresponding DNA samples (Fig. 4, lanes *22* and *23*) there was inconsistence between

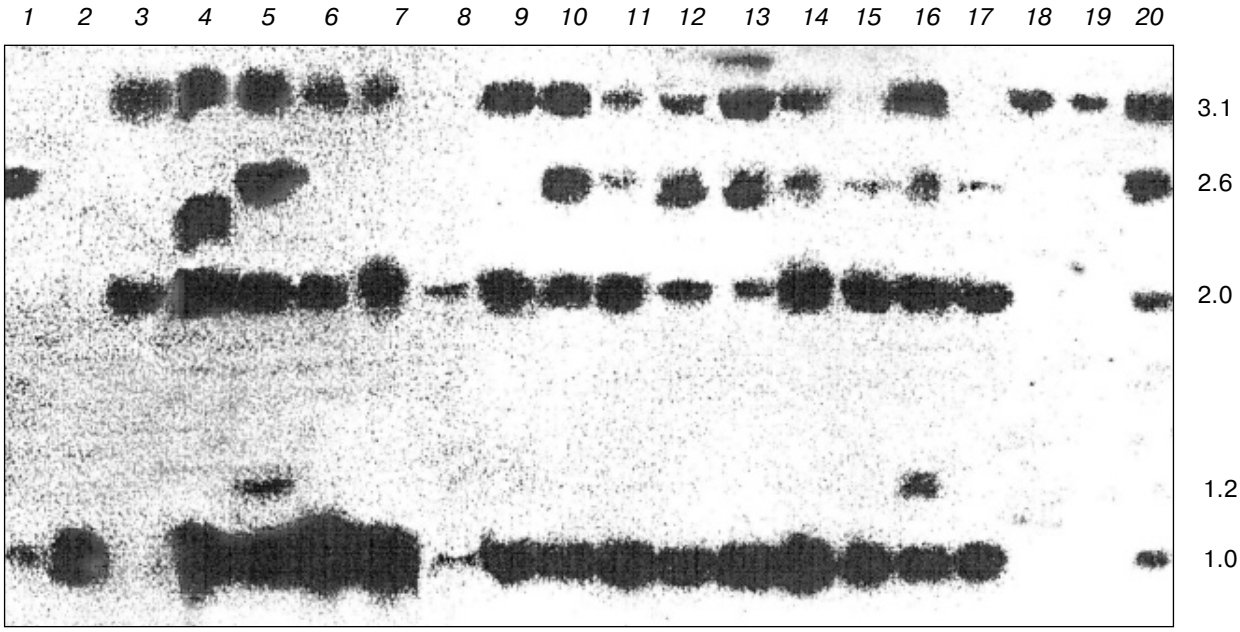

Fig. 3. Digestion of the 5′-region of the human calcitonin gene from bone marrow (BM) and peripheral blood (PB) cells of patients with various forms of myeloid leukemia by restriction endonuclease *Hpa*II (with exception of lane *2*, which shows the digestion pattern by restriction endonuclease *Msp*I): *1*) DNA of PB cells from patient with chronic myeloid leukemia (CML) relapsed after BM transplantation; *2*) the same as *1*, but DNA was digested by restriction endonuclease *Msp*I; *3*) DNA of PB cells from a patient with terminal stage of CML; *4*) DNA of PB cells from a patient with terminal stage of CML; *5*) DNA of PB cells from a patient with terminal stage of CML; *6*) DNA of BM cells from a patient with acute myeloid leukemia (AML), primary investigation; *7*) DNA of BM cells from the same patient (as in *6*) seen on the 27th day, appearance of tolerance to treatment with cytostatic preparation; *8*) DNA of BM cells from a patient with AML after nine years of full remission; *9*) DNA of PB cells from a patient with relapse of AML; *10*) DNA of PB cells from a patient with AML, primary investigation; *11*) DNA of PB cells from a patient with AML, primary investigation; *12*) DNA of BM cells from a patient with AML, primary investigation; *13*) DNA of PB cells from a patient with AML, primary investigation; *14*) DNA of BM cells from a patient with AML, primary investigation; *15*) DNA of BM cells from the same patient (as in *14*) but after one month, full hematological remission; *16*) DNA of BM cells of a patient with relapse of AML; *17*) DNA of BM cells from the same patient (as in *16*) but after 27 days, repeated remission; *18*) DNA of BM cells of a patient with myelodysplastic syndrome (MDS); *19*) DNA of PB cells of the same patient (as in *18*) but after 4 months, AML; *20*) DNA of PB cells from a patient during transformation of MDS to AML. Size of fragments is shown as kb on the right.

percentage of blast cells in bone marrow and peripheral blood (56 and 3%, respectively). Although percentage of blast cells in peripheral blood was low, clear registration of the abnormal fragment of 3.1 kb suggests the presence of a clone of malignant cells characterized by significant proliferating activity.

Detection of abnormal *Hpa*II-fragments in bone marrow cell DNA in acute lymphoid leukemia (ALL) may be explained by the fact that in most cases there was a repeated remission in patients with this disease and this remission is usually rather short and transforms into a new clinical relapse.

At the phase of unstable remission the patient with ALL was characterized by hypermethylation of the 5′-region of the calcitonin gene three months after the beginning of chemotherapy (Fig. 4, lane *9*). This observation was consistent with laboratory analyses demonstrating slightly increased percentage of blast cells (7.5 versus normal 5%). One month after the course of additional intensive chemotherapy, the pattern of methylation of the 5′-region of the calcitonin gene normalized (Fig. 4, lane *10*) and clinical and laboratory parameters of bone marrow normalized as well. However, four months later there was "molecular relapse" of this disease, which was observed during maintenance therapy with normal clinical parameters (Fig. 4, lane *11*); this "molecular relapse" appeared eight months before onset of clinical manifestations.

In the patient with ALL characterized by documented hematological remission the abnormal restriction fragment of 2.6 kb in bone marrow cell DNA was detected five months before the clinical relapse (Fig. 4, lane *12*). This suggests "molecular relapse" of this disease. It should be noted that after onset of clinical relapse the pattern of hypermethylation of the 5′-region of the calcitonin gene remained unchanged (Fig. 4, lane *13*). In the other patient with ALL with documented hematological remission the "molecular relapse" was also found 60 days before the appearance of clinical manifestations of relapse (Fig. 4, lane *14*). In a patient with unstable remission of ALL the "molecular relapse" was observed in peripheral blood and bone marrow cells one month

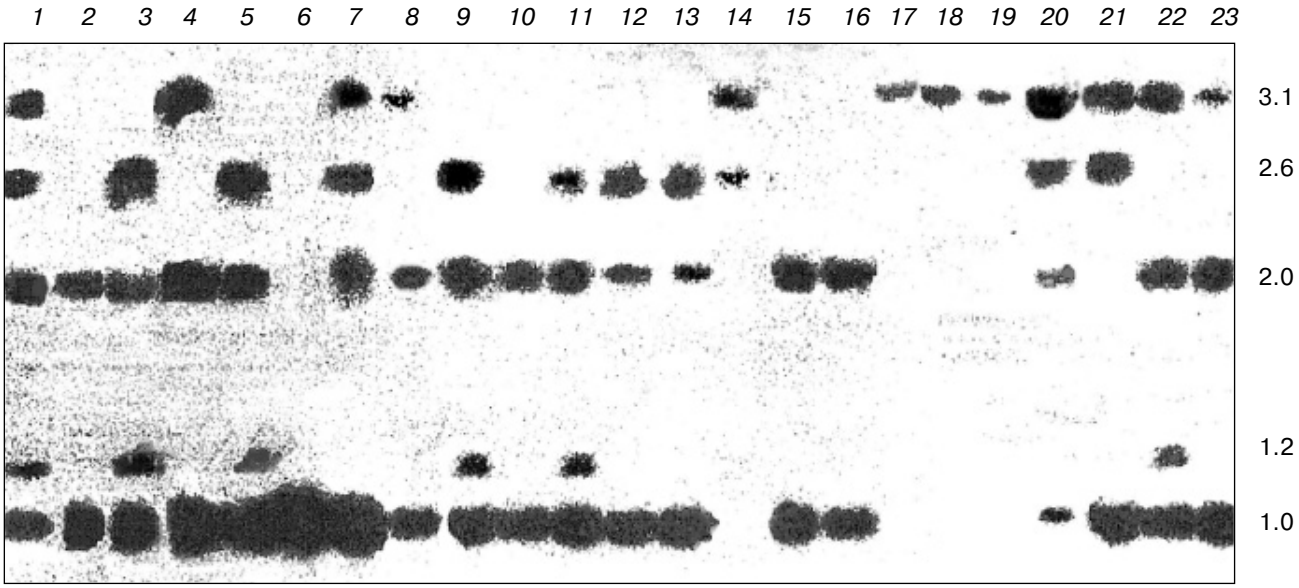

Fig. 4. Digestion of the 5′-region of the human calcitonin gene from bone marrow (BM) and peripheral blood (PB) cells of patients with various forms of acute lymphoid leukemia by restriction endonuclease *Hpa*II (with exception of lane *6*, which shows the digestion pattern by restriction endonuclease *Msp*I): *1*) DNA of BM cells from a patient in hematological remission, "molecular relapse"; *2*) DNA of BM cells of the same patient (as in *1*) but 25 days after additional treatment with chemotherapy; *3*) DNA of BM cells of the same patient (as in *2*) but after 38 days, hematological remission; *4*) DNA of BM cells of the same patient (as in *3*) but seven months later, clinical relapse; *5*) DNA of PB cells from the same patient (as in *3*) but seven months later, clinical relapse; *6*) DNA of BM cells (digestion by restriction endonuclease *Msp*I) from a patient, primary investigation; *7*) DNA of BM cells from the same patient (as in *6*), primary investigation; *8*) DNA of BM cells from the same patient (as in *7*) but after 18 days, hematological remission; *9*) DNA of BM cells from a patient at the stage of unstable remission three months after the beginning of chemotherapy; *10*) DNA of BM cells from the same patient (as in *9*) but 27 days after additional course of intensive chemotherapy; *11*) DNA of BM cells from the same patient (as in *10*) but at the stage of hematological remission, 8 months before relapse of disease, "molecular relapse"; *12*) DNA of BM cells from a patient at the stage of hematological remission, "molecular relapse"; *13*) DNA of BM cells from the same patient (as in *12*) but after five months, clinical relapse; *14*) DNA of BM cells from a patient at hematological remission, 60 days before clinical relapse, "molecular relapse"; *15*) DNA of donor BM cells; *16*) DNA of BM cells of a patient nine months after bone marrow transplantation; *17*) DNA of BP cells from a patient at unstable repeated remission; *18*) DNA of BM cells from the same patient as in *17*; *19*) DNA of BM cells of the same patient (as in *17* and *18*) but after one month, the second clinical relapse; *20*) DNA of PB cells from a patient, primary investigation; *21*) DNA of PB cells from a patient, primary investigation; *22*) DNA of BM from a patient, tolerant to cytostatic treatment; *23*) DNA of PB cells from the same patient (as in *22*). Size of fragments is shown as kb on the right.

before clinical registration of the second relapse of this disease (Fig. 4, lanes *17-19*). In all the cases, the pattern of hypermethylation of the 5′-region of the calcitonin gene remained unchanged. This pattern was characterized by the absence of normal *Hpa*II fragments of 1.0 and 2.0 kb. This suggests especially hard progression of this disease and significant reduction in normal cells in blood and bone marrow. In the case of positive result of autologous transplantation of bone marrow to a patient with acute lymphoblastic leukemia, we did not register hypermethylation of the 5′-region of the calcitonin gene nine months after the transplantation (Fig. 4, lanes *15* and *16*). In the other patient transition of acute development of this disease into clinical remission was accompanied (at the 18th day) by normalization of methylation pattern of the 5′-region of the calcitonin gene in bone marrow cells; the phase of remission was characterized by almost total disappearance of abnormal *Hpa*II fragments (Fig. 4, lanes *7* and *8*).

Our observations on hypermethylation of the 5′-region of the calcitonin gene in ALL are consistent with data from other laboratories [10-12].

Methylation of CCWGG sequences of the 5′-region of the calcitonin gene in various forms of leukemia. DNA from nucleus-containing cells of peripheral blood and bone marrow from patients with various forms of myeloid and lymphoid forms of leukemia was used for analysis. DNA from peripheral blood leukocytes of healthy volunteers was used as control. For each type of leukemia, we analyzed DNA from two patients. In these DNA samples, we initially analyzed the methylation state of internal cytosine in CCGG sequences of the 5′-region of the calcitonin gene. In all leukemic patients, this region was characterized by hypermethylation of CpG.

Figure 5 shows the *Bst*NI/*Eco*RII restriction map of the 5′-region of the calcitonin gene. The 1.7 kb fragment of the 5′-region of this gene was used as the hybridization probe. Blot-hybridization analysis of the 5′-region of the

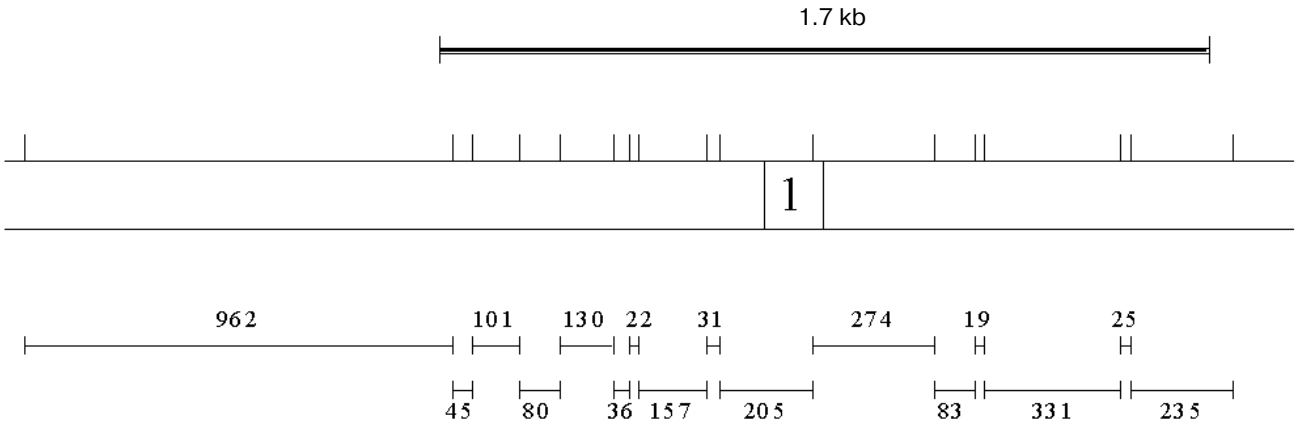

Fig. 5. Position of CCWGG sequence at the 5′-region of the human calcitonin gene. The position of the hybridization probe is shown at the top. The size of DNA fragments (as base pairs) cleaved by restrictase *Bst*NI is given on the bottom. The marked region shows exon 1.

calcitonin gene using *Bst*NI restrictase and the probe revealed expected nucleotide fragments of 962, 331, 274, 235, 205, 157, 130, 101, and 83/80 bp (Fig. 6). Relatively weak intensity of the radioactive signal of the fragment of 962 bp may be attributed to its insignificant overlapping with the hybridization probe and difficult (for this particular fragment) hybridization conditions. Conditions of electrophoresis and subsequent blot-hybridization of restriction digestion products did not allow us to localize smaller *Bst*NI fragments of this site of the calcitonin gene on the radioautogram. In all the cases, the pattern of digestion of the 5′-region of the calcitonin gene by *Eco*RII restrictase sensitive to the presence of 5-methyl-cytosine in the recognized sequence did not change from the corresponding *Bst*NI-pattern. This suggests lack of 5-methyl-cytosine in the CCWGG sequence in this region. (The latter means lack of methylation of this region of CpNpG type.) The CpNpG type of methylation of the

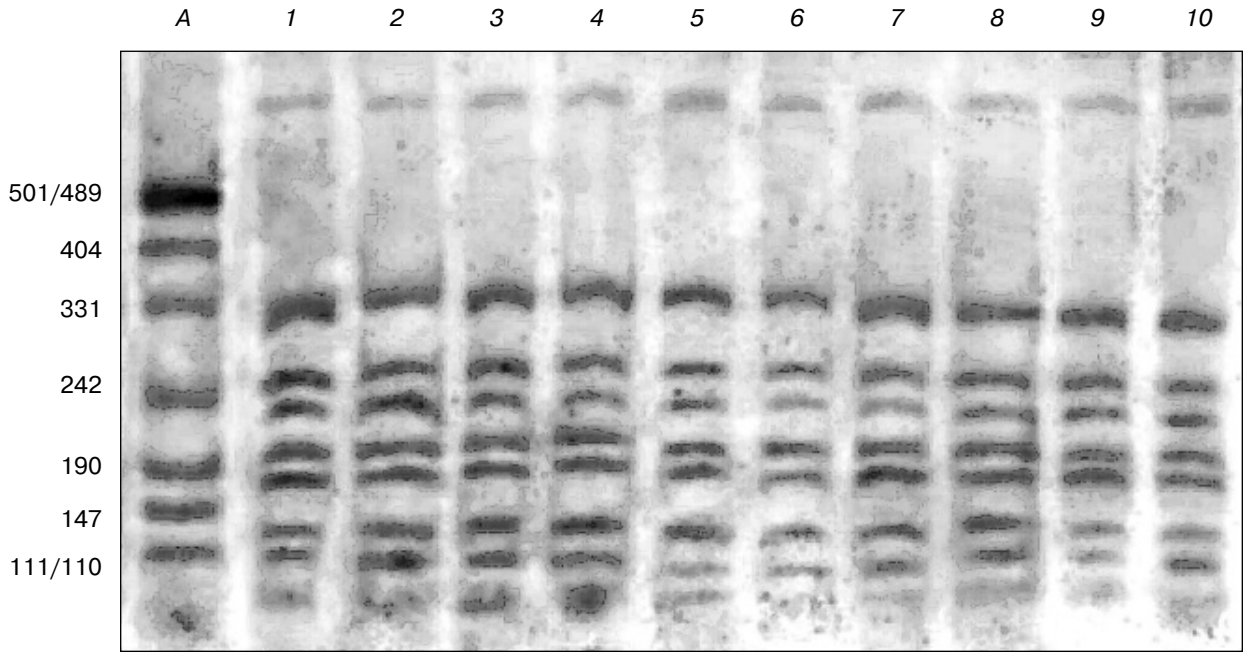

Fig. 6. Digestion of the 5′-region of the human calcitonin gene from bone marrow (BM) and peripheral blood (PB) cells from healthy volunteers and leukemic patients by restriction endonucleases *Eco*RII and *Bst*NI: A) pUC19/*Msp*I; *1*, *2*) DNA of BM cells from a patient with acute lymphoid leukemia (ALL), full remission; *3*, *4*) DNA of PB cells from a patient with relapse of ALL; *5*, *6*) DNA of BM cells from a patient with AML, primary investigation; *7*, *8*) DNA of PB cells from a patient with terminal stage of CML; *9*, *10*) DNA of PB cells from healthy volunteers; *1*, *3*, *5*, *7*, *9*) digestion by *Eco*RII; *2*, *4*, *6*, *8*, *10*) digestion by *Bst*NI. Size of fragments (as base pairs) is shown on the left.

5′-region of the calcitonin gene was not found in cells of peripheral blood and bone marrow isolated from healthy volunteers and patients suffering from various forms of leukemia.

DISCUSSION

In the present study, we analyzed site-specific methylation of the human calcitonin gene and its importance as a prognostic and diagnostic marker of leukemias. Special attention was given to monitoring of methylation of the calcitonin gene in leukemic patients during their medical treatment and development of malignant growth.

In accordance with observations by other authors [7-12, 22], hypermethylation of internal cytosine of CCGG sequences of the 5′-region of the calcitonin gene is a characteristic feature of tumor progression in various forms of myeloid and lymphoid leukemias. Under conditions of clinical and hematological remission, hypermethylation of the calcitonin gene was not detected. However, hypermethylation of the calcitonin gene persisted under conditions of incomplete remission (or when full remission was not achieved). In addition to previous studies, we found abnormal methylation of the calcitonin gene under conditions of clinical remission and resistance of leukemic patients to treatment with cytostatic and cytotoxic drugs. In some cases of various forms of leukemia, the appearance of abnormal *Hpa*II fragments reflecting hypermethylation of the 5′-region of the calcitonin gene resulted in the decrease or even complete disappearance of "normal" *Hpa*II fragments of 1 and 2 kb (Fig. 3, lanes *18* and *19*; Fig. 4, lanes *17-19*). In these cases, the leukemic process was characterized by heavy progression that was resistant to chemotherapeutic treatment.

"Molecular remission" reflecting normalization of the calcitonin gene methylation was characterized by significant reduction or complete disappearance of abnormal *Hpa*II fragments. This accompanied clinical remission and improvement in conditions of patients. "Molecular relapse" reflecting restoration of abnormal hypermethylation of the 5′-region of the calcitonin gene was observed 1-8 months before appearance of clinical and laboratory signs of the disease. Consequently, reversion of the normal pattern of methylation of the 5′-region to the "pathological" pattern may be considered as an early molecular stage of development of leukemias.

The search for primary changes of genome methylation related to carcinogenesis is one of the basic problems of epigenomics [23]. The solution of this problem may help not only better understanding of molecular mechanisms of carcinogenesis but also early diagnostics and more effective treatment of malignant tumors. However, at the present time no universal DNA-markers for prognosis and early diagnostics of the most widespread forms of human cancer are available. Perhaps, this reflects involvement of different abnormal genetic and biochemical programs underlying development of various forms of neoplastic cell transformation. If this assumption is correct, we may expect discovery of specific DNA-markers for certain types of tumors. These may include genes with altered methylation pattern. Hypermethylation of a representative group of tumor suppressor genes (including genes with documented functions in carcinogenesis) is firmly recognized [3, 6]. Methylation of some of these genes was observed in all (100%) cases of some types of tumors [24]. Abnormal methylation of genes with putative suppressor function in carcinogenesis may also correlate well with progression of specific malignant tumors [3, 6, 25]. In the case of leukemias, the methylation pattern of the human calcitonin gene may provide similar prognostic and diagnostic information. Data from different laboratories suggest that the (hyper)methylation pattern of the calcitonin gene may be used as a marker gene for monitoring of leukemia [7-12, 17, 18, 22]. It should be noted that there is a wide range of correlation values (43-94%) between cases of leukemia and hypermethylation of the calcitonin gene [9-12]. However, even in the cases of extremely low value of this parameter hypermethylation of the calcitonin gene is considered as an independent prognostic marker of increased risk of relapse of acute lymphoid leukemia [12]. Various forms of leukemic diseases may differ in this parameter and maximal positive correlation (reaching more than 90%) is recognized in the cases of acute lymphoid leukemias [10, 11]. We did not find leukemic patients who were negative in CCGG-hypermethylation. Variation of correlation values may be partially attributed to different resolution ability of methods used for analysis of DNA methylation [10, 11]. Further studies are required for subsequent evaluation of methylation pattern of the 5′-region of the human calcitonin gene as a diagnostic and prognostic marker of leukemias.

Structural–functional studies of enzymatic mammalian DNA methylation are mainly focused on CpG-type of methylation. However, it is firmly recognized that in mammalian cells DNA may be methylated not only by CpG sequences but also by CpNpG (where N is any nucleoside) and cells may methylate CpNpG *de novo* and also they may maintain this site-specific DNA modification [13]. This raises reasonable questions on the functional role of CpNpG-type of mammalian DNA methylation and also on specific genetic regions subjected to CpNpG-methylation. Studies of CpNpG-type of methylation of mammalian genes are at the beginning of the story and the human calcitonin gene represents an interesting object. The 5′-region of this gene represents a typical "CpG-island" [26]. This region may also be referred to "CpNpG-island" due to its enrichment with these sequences, which also includes CCWGG sequences (where W = A or T) [27]. These CCWGG sequences are potential targets for CpNpG-specific DNA methylation.

Until our study, methylation of CCWGG sequences of the 5′-region of the calcitonin gene had not been investigated.

During *de novo* DNA methylation in mammalian cells a "methylation wave" may cover not only adjacent CpG-site but also cytosine residues in non-palindrome sequences [14-16]. So we could expect that hypermethylation of CpG-sequences of the 5′-region of the calcitonin gene, which accompanies development of various forms of leukemia, may also involve adjacent CCWGG sequences. However, we failed to detect methylation of adjacent CpNpG sequences in this region of the calcitonin gene in all the samples from leukemic patients. Thus, in contrast to hypermethylation of CpG dinucleotides, CpNpG sequences of the 5′-region of the human calcitonin gene remain unmethylated both in normal conditions and during development of leukemia. Besides our observations [28], other authors also found some relationship between the ratio of CpG- and CpNpG-site-specific types of methylation of regulatory and promoter regions of eukaryotic genes. For example, in the primary culture of B-cell lymphoma and myeloma cells a promoter of a non-expressing *B-29* gene is methylated either at CG or CCWGG sequence (but not by both sequences simultaneously) [29]. The reversed relationship between methylation of CG and CCWGG sequences was also found for the proviral genome of Moloni mouse leukemia virus [30] and mitotic *myf-3* gene [31]. Our data obtained using clinical material not only suggest the existence of a strong reversed relationship between CpG and CpNpG types of methylation of specific genetic region, but they also imply the possibility of constant absence of CpNpG-specific methylation of certain genes under norm and malignant growth. Thus, a distinct and specific mode of methylation of the 5′-region of the calcitonin gene found in leukemic patients and healthy volunteers may represent "non-alternative" CpG-type of methylation of extended DNA stretches. The mechanism of inhibition of CpNpG specific methylation of the 5′-region of the calcitonin gene in nucleus-containing cells of peripheral blood and bone marrow from leukemic patients remains unclear. It is possible that such inhibition of CpNpG specific methylation reflects structural features of the 5′-region of the calcitonin gene and mutual positioning of CpG and CpNpG sequences in this region.

In the light of these latest data, we suggest the existence of marker genes that are specific for different forms of malignant tumors and which are characterized by different site-specific type of methylation. Subsequent extension of this study of the calcitonin gene methylation using a larger group of leukemic patients will allow better understanding of results of the present work.

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