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ESTABLISHING THE ROLE OF METHYLATION OF DNA CYTOSINE IN HUMAN GENETIC INDIVIDUALITY

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UDC 577.224.23

KEY WORDS: genomic dactyloscopy; methylation; human HA-RAS 1 proto-oncogene

Three main trends of development have become formed in genetic dactyloscopy. The first, and the best studied at the present time, is the discovery of individual structural features of the genotype relative to number and size of mini-satellite DNA fragments [1, 8, 14], the second is establishment of sites of interaction of transcriptionally active chromatin with nuclear regulatory proteins [2, 11], and the third is analysis of the distribution of methyl groups in the promoter region of functioning genes [3, 12]. The data so far obtained suggest that the last two trends of genetic dactyloscopy may be closely interconnected during discussion of their molecular-biological mechanisms, for it has been observed that methylation of guanine bases, in the opposite, complementary, but transcriptionally inactive DNA chain prevents binding of the regulatory protein with its own site-specific region [2]. It must be expected that investigations in the region of DNA-protein interactions and DNA methylation constitute one common direction in the study of the functional features of the genotype of the cell, tissue, organ, or whole organism. The writers pointed out previously [3] that a DNA probe containing the 5′-flank of the human HA-RAS 1 oncogene exhibits polymorphism of the lengths of the restriction fragments (PLRF) of the HA-RAS 1 protooncogene, which has features of individual specificity for each patient with gastric carcinoma studied. The aim of the present investigation was to study molecular-biological phenomena determining this feature of the human genotype.

EXPERIMENTAL METHOD

Altogether 20 patients with carcinoma of the stomach and chronic gastriculcer were investigated. Chromosomal DNA was isolated by the phenol-detergent method [3] from white blood cells (nine samples), gastric mucosa [15], primary carcinomas [16] and metastases of carcinoma of the stomach in regional lymph nodes [7], and cells surrounding a chronic gastric ulcer (three samples). Molecular-genetic analysis of the structure of the HA-RAS 1 proto-oncogene was carried out by methods described previously [3]. Genomic DNA was hydrolyzed by enzymes Msp I and Bam HI ("Ferment," USSR). The EJ 6.6 probe (plasmid pEJ 7.6), carrying the full-scale human HA-RAS 1 oncogene [13], was used in the hybridization reaction. In this experiment hybridization was carried out under mild conditions, determined by the presence of triple SSC and a temperature of 60-62°C. The filters were washed and autoradiography carried out by the standard method [3].

Forensic Medical Examination Bureau, City Health Board of Leningrad City Council, and Laboratory of Biochemistry, Professor N. N. Petrov Research Institute of Oncology, Ministry of Health of the USSR, Leningrad. Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 112, No. 8, pp. 197-199, August, 1991. Original article submitted November 16, 1990.

 0007-4888/91/0008-1176$12.50

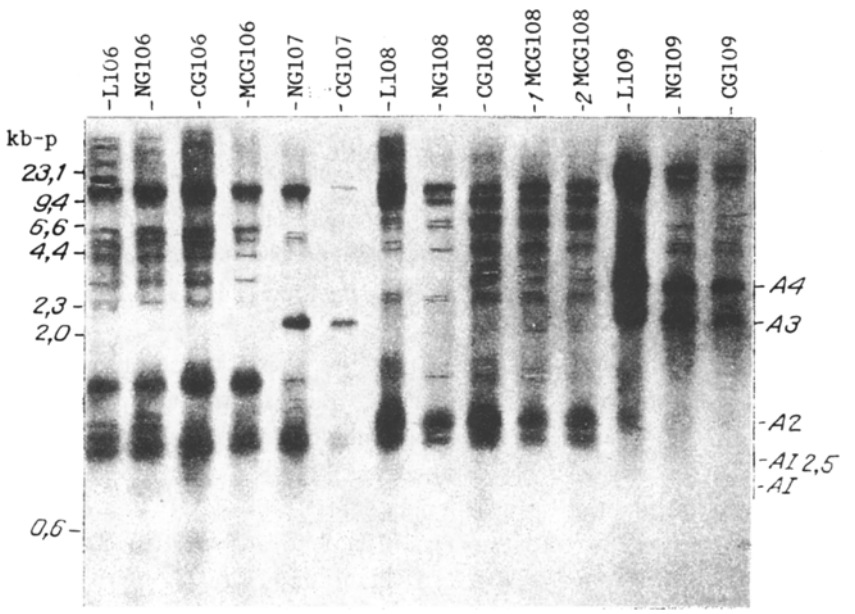

Fig. 1. Hybridization of the Eg 6.6. probe with preparations of genomic DNA, hydrolyzed by enzyme Msp I, and obtained from leukocytes (L), normal gastric mucosa (NG) and gastric carcinoma (CG), and also from metastases of gastric tumors (MCG) in regional lymph nodes of patients with gastric carcinom. Size (kb-p — kilobase-pairs) of HindIII restriction fragments of marker DNA of phage λ shown on left Msp I-restriction fragments of DNA, containing VTR of the corresponding allele (A) of proto-oncogene HA-RAS 1 on right.

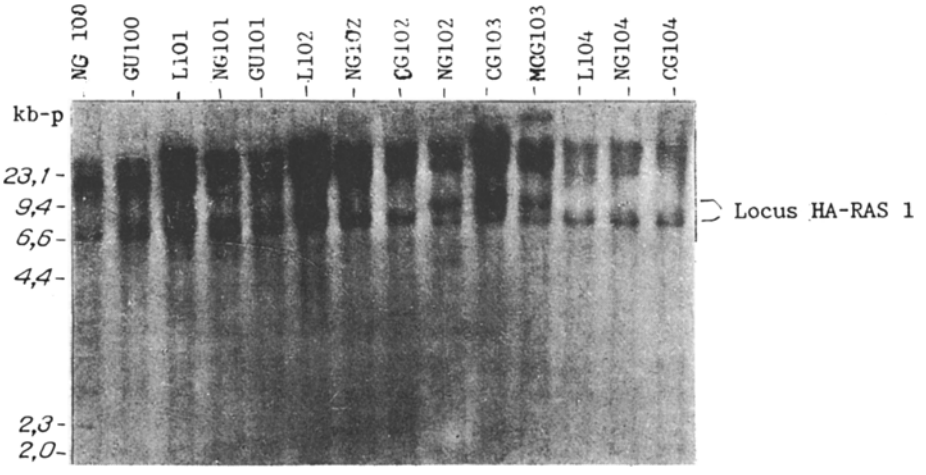

Fig. 2. Hybridization of probe Eg 6.6 with preparations of genomic DNA hydrolyzed by enzyme Bam HI. Size of HindIII-restriction fragments of marker DNA of phage λ shown on left. GU) Chronic gastric ulcer. Locus HA-RAS 1-restriction fragments corresponding to proto-oncogene HA-RAS 1 shown on right. Remainder of legend as to Fig. 1.

EXPERIMENTAL RESULTS

Preliminary analysis showed [3] that, of enzymes having the largest number of restriction sites within the 5′-flank of the HA-RAS 1 proto-oncogene (Mva I 11, Bcn I 17, Bsp RI 32), only the Bsp RI restriction endonuclease revealed a PLRF pattern characteristic of each individual. Bsp RI recognizes the GGCC sequence but does not degrade it if the internal cytosine is methylated in position 5 (5′-GG^{M5}CC-3′) [10]. Unlike Bsp RI, restriction endonucleases Mva I and Bcn I degrade DNA at definite sites for them (CC$_T$^{A}GG and CC$_G$^{C}GG) only if the internal cytosine does not contain a methyl group in position 4 [10]. In genomic DNA hydrolyzed by enzymes Mva I and Bcn I the size and number of the restriction fragments of the 5′-flank of the HA-RAS 1 proto-oncogene were found to the same for all patients studied. The results suggested that methylation of certain cytosine bases in position 5 in the human genome may have features of individual specificity.

To test this hypothesis we replaced the enzyme Bsp RI by Msp I, which also is sensitive to methylation of the cytosine in position 5, but which recognizes the CCGG sequence, which is reversed compared with the nucleotide sequence degraded by the enzyme Bsp RI [10]. Also, as the DNA probe in the hybridization reaction we used the full-scale oncogene HA-RAS 1 (the probe Eg 6.6) and not the 5′-flank. The results of this experiment are given in Fig. 1, which shows the principal Msp I-restriction fragments, containing a variable tandem repeat — VTR (located on the 3′-flank of the HA-RAS 1 proto-oncogene), and additional fragments, whose spectrum is identical for all tissues of the same patient, and differs from the spectrum of Msp I-restriction fragments in DNA in the tissues of another patient. Incidentally, the size and number of these additional Msp I-restriction fragments are not disturbed even in DNA of malignant tumors and their metastases, which confirms the resistance of this genetic feature. Goelz and co-workers [6] also drew attention previously to the high stability of the presence of methyl groups attached to the outer cytosine in sequences CCGG (5′-^{M5}CCGG-3′) of the HA-RAS 1 proto-oncogene in the DNA of carcinomas of the human large intestine.

Restriction endonuclease Msp I does not hydrolyze the 5′-^{M5}CCGG-3′ sequence in which the outer cytosine is methylated, but Bsp RI does not hydrolyze the 5′-GG^{M5}CC-3′ sequence, in which the inner cytosine is methylated [10]. Hence it follows that both enzymes are sensitive to methylation of the 5′-terminal cytosine in 5′-^{M5}CC-3′ duplexes. It was these two enzymes which gave the "dactyloscopic" imprint of the genome of each patient tested. Consequently, in the human genome methylation of cytosine in position 5 has features of individual specificity only relative to the 5′-terminal cytosine, a component of 5′-^{M5}CC-3′ duplexes.

We know that active proto-oncogenes of the ras family in man are complementary to one another by not more than 30% [5]. A detailed analysis of the primary structure of the HA-RAS 1 oncogene, carried out according to data of Capon and co-workers [4], showed that most Msp I restriction sites are located in its 5′ promoter region, which consists of a set of CG- and GC-rich regions, binding Sp I transcription factor [7]. Promoters of this type are also present in some other genes that do not belong to the ras proto-oncogene family [7]. It can be accepted that the Msp I-restriction fragments which we found under mild hybridization conditions could belong not only to the proto-oncogene HA-RAS 1, but also to certain genes with a structurally analogous promoter.

To test this hypothesis hybridization of probe Eg 6.6 with genomic DNA, hydrolyzed by enzyme Bam HI, was carried out. The results are shown in Fig. 2. Besides restriction fragments ranging in size from 6.4 to 8.0 kb-p, specific for HA-RAS 1, in each DNA preparation studied eight additional Bam HI-restriction fragments were discovered, one of which was over 30 kb-p and the others were 16.0, 10.0, 5.5, 4.4, 3.6, 2.4, and 1.8 kb-p. Only the 3.6 kb-p fragment corresponds to pseudogene HA-RAS 2 [9], and the remainder probably belong to some other unique genes, for they are found in the form of clearly outlined bands. The results confirm the hypothesis that under the hybridization conditions used probe Eg 6.6 reveals additional genes, present in the human genome, and structurally related to protooncogene HA-RAS 1.

The investigation thus shows that methylation of the 5′-terminal cytosine in 5′-^{M5}CC-3′ duplexes in human DNA has features of individual specificity. This genetic feature is undisturbed even in DNA of malignant tumors of the human stomach, and it can be used in genetic dactyloscopy. The Eg 6.6. probe, containing sequences of the cellular oncogene HA-RAS 1, under mild hybridization conditions reveals the HA-RAS 1 proto-oncogene and genes structurally related to it.

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