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Epigenetic Modulation Expressed as Methylation Changes in DNA from Primary School Children of Two Different Geographical Environments II

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In previous work, proportions of the phenotypic characteristics attributable to the HSR (Hand Skill Relative, OMIM 139900) gene were found modified in primary school children from one of two distinct geographical regions of La Rioja province (Argentina). HSR gene has been described subjected to epigenetic regulation, and one of the main molecular mechanisms whereby this regulation is performed is by methylation of DNA. Thus, it was of interest in the present work to analyze in these children the possibility of changes in the DNA methylation patterns. 40 children from the two regions (20 from Region 2, and 20 from Region 1, considered control) were randomly selected from the original total sample of 547 children, and blood samples were taken to analyze the DNA methylation patterns by capillary electrophoresis technique. The phenotypic characterization for HSR also was performed on the children sample. Results showed that children from Region 2 had significantly altered the writing/reading capacity with a proportion about 65% of writing disturbances over control children of Region 1. No statistical differences were found in the other two phenotypic traits linked to the HSR gene (brain asymmetry and handedness). Molecular analysis of the methylation patterns revealed a significantly higher ratio of non-methylated to methylated cytosine in DNA from children of Region 2, compared to those of Region 1 (control). The present results suggest that the altered proportions of phenotypic characteristics found in children of Region 2 are supported by altered DNA methylation patterns, suggesting an environmental epigenetic modulation of DNA expression.

Keywords: Trace Elements, Epigenesis, Methylation Patterns, DNA, HSR, Cytosine/Methyl Cytosine Ratio.

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1. INTRODUCTION

Much attention has been given recently to the epigenetic processes regulating gene expression.[11, 17, 19, 22, 28, 33] The main reason of this increasing interest is that epigenesis appears as a different mechanism offering a non-mendelian inheritance, permitting the interesting feature that adaptive changes induced by the environment can be inherited.[22, 28] Although epigenetic processes are viewed in slightly different perspectives by the specialists,[22] all of these points of view have in common that the basic molecular mechanism of inheritance relies on changes in DNA not linked to changes in the gene sequence.[17] At least, one important molecular mechanism whereby epigenetic processes are mediated is through the action of the DNA methyltransferase modifying cytosine to 5-methylcytosine.[16, 18] The formation of methylcytosine by this enzymatic process in DNA, it is viewed as a strong suggestive "marker" for epigenetic expression. One of the most studied mechanisms whereby epigenesis is expressed is *imprinting*.[4, 7, 17, 28, 34] Imprinting follows a paternal or maternal expression pattern in gene inheritance. In some instances, the activation to expression of the imprinted gene is commanded by the paternal origin, such as the case of the *Igf2* gene, or by the maternal origin, such as *Igf2r* and *H*19 genes of

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the mouse.[2, 3, 8] The most conspicuous and interesting fact about imprinted genes is that they represent a very small fraction of the whole genome (about 1%) and most of them are expressed in the central nervous system.[7, 28] This fact suggests that the complex functioning of the brain is dynamically regulated by epigenetic processes during the continuous environment-central nervous system interactions. One phenotypic expression linking laterality, brain asymmetry and cognitive abilities is the recent description of the *HSR* (Hand Skill Relative, OMIM 139900) gene.[13, 14, 27] Our laboratory has been interested to study this gene; which it is thought that controls handedness, brain asymmetry, writing-reading capacity and susceptibility to schizophrenia.[13, 14, 21, 27, 29] All of these characteristics, although apparently not related to themselves, are expressions involving different aspects of laterality and cognitive functions, which are reasons why the study of this gene offers interesting perspectives. Other important feature about *HSR* was to be found imprinted, and consequently under epigenetic regulation.[14] In a previous study of our laboratory on this gene in primary school children of the two regions of La Rioja, province of Argentina, it was found a differential phenotypic expression for three out of four characteristics attributed to the *HSR* gene (handedness, brain asymmetry and writing-reading capacity[29]). Children were grouped according to their geographical origin into Region 1 and Region 2. The modified proportion of the phenotypic expression of *HSR* was observed in those children living in Region 2, a geographical region enriched with mines and mineral deposits.[29] It is not known if these environmental conditions have a relationship with the altered phenotypic pattern of the *HSR*, but it was speculated that if epigenetic modulation is involved, then molecular methylation of DNA cytosine might occur. Thus, the object of the second part of our work was to evaluate the possibility to find differences in the methylation patterns of the DNA in children from both regions of La Rioja.

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2. MATERIALS AND METHODS

2.1. Subjects

Of the initial 547 children studied from all the schools,[29] 20 subjects from region 1 and 20 from region 2 were randomly sampled for blood extraction procedures, using a random digits table for the selection.[32] Informed consent forms approved by the Bioethical Committee of the "Hospital de Clínicas José de San Martín," Universidad de Buenos Aires, and signed both by parents and children were used. As already described,[29] children were tested with validated psychological tests in order to evaluate their psychological abilities, emotional status and intelligence quotient. The following tests were applied:

(1) The projective color test of Lüscher, evaluating the presence of psychopathological alterations;[23]

(2) the projective drawing test of Wartegg which evaluates emotional maturation,[6] and

(3) the progressive matrix test of Raven for measuring the intelligence quotient.[30]

2.2. Procedures to Evaluate the Putative Phenotypic HSR Gene Expression

Three methods were used in order to determine the phenotypic expression attributable to the *HSR* gene, as already described in details elsewhere.[29] Briefly,

(1) handedness was evaluated by using the Edinburgh Hand Inventory,[26] which consists of 12 different spontaneous tasks to be performed by the skillful hand of the subject;

(2) brain asymmetry was evaluated indirectly by visual inspection of the scalp-hair whorl direction; and

(3) writing–reading capacity was evaluated by analysis of writing disturbances of a written text of a standardized validated text aurally exposed to the children.[29]

From the 317 children from schools of region 1, and of 230 from region 2, previously to any inspection of the three variables studied, 20 children per each region were randomly selected as mentioned before. Thus, two new groups were formed. Blood was extracted to analysis of DNA, and blood samples were immediately frozen for later chemical determinations.

2.3. DNA Extraction Procedures

DNA from the blood samples was extracted by standard methods.[24] Briefly, samples were incubated over night at 37 °C with proteinase K (10 mg/ml). At the following day, RNAase (2 mg/ml) was added and samples were incubated for 2 hours at 37 °C. Later, precipitation of DNA material was performed with NaCl 5 M. Samples were centrifugated at 4 °C, 30 min and at 3500 rpm. Cold absolute ethanol was added, centrifuged and the resultant DNA preparation was washed three times with alcohol 70%. Finally, DNA recovered was stored in Tris base, pH 7.6-EDTA 0.1 mM buffer at −20 °C.

2′-Deoxyadenosine, 2′-deoxythymidine, 2′-deoxyguanosine, 2′-deoxycytidine and 5-methyl-2′-deoxycytidine were purchased from Sigma-Aldrich Química S.A. All the nucleosides were dissolved at 5 mM in Milli-Q grade water.

2.4. Genomic DNA Hydrolysis

DNA samples (3 μl, 0.5 μg/μl) were heated for 2 min in a boiling water bath and cooled rapidly in ice; 0.75 μl of 10 mM $ZnSO_4$ and 1.25 μl of nuclease P1 (Sigma-Aldrich Química S.A.); 200 units/ml in 30 mM $C_2H_3O_2Na$ were added and mixture were incubated for 16 h at 37 °C.

2.5. Cytosine and 5-Methyl Cytosine Separation and Quantification by Capillary Electrophoresis

The analysis of the hydrolyzed DNA samples was carried out as previously described.[12] The capillary electrophoresis (CE) instrument was a Capillary Ion Analyzer System (Waters, Milford, MA, USA). Data were processed by Millenium™ 2010 software (Waters). Deoxynucleoside standard solutions were daily prepared from stock solutions by appropriate dilution with water. Before their separation by CE, hydrolyzed DNA samples were carefully evaporated by means of a nitrogen stream and resuspended in 7 μL of water. Separations were performed in an uncoated fused-silica capillary (Polymicro Technologies, Phoenix, AZ, USA) (60 cm in length, 75 μm id, 365 μm od). The CE system temperature was held at 25 °C. Hydrostatic injection for 30 s (10 cm height difference) was used for introduction of samples. The separation voltage was 15 kV and UV-detection at 254 nm (mercury lamp) was performed. The running buffer and washing solutions were filtered through nylon membrane filters (0.45 μm, Micron Separations Inc., Westboro, MA, USA). The running buffer consisted of 48 mM $NaHCO_3$, pH 9.6, and 60 mM Sodium docecyl sulphate. Before each run, the capillary was washed with 1 M NaOH for 2 min, followed by 1 mM NaOH for 3 min, and running buffer for 3 min.

Results are expressed as the mean ± standard error of the mean of the non-methylated cytosine to methylcytosine concentration ratio (R_{NMM}) of children from both regions. No distinction between the sexes of children was made. Phenotypic results are expressed as proportions of children having a determined phenotypic characteristic with respect to the total of children examined.

2.6. Statistical Methods

For each phenotypic characterization, subjects were classified in two mutually exclusive traits. The statistical significance of the difference of proportions was evaluated using the χ^2 Distribution. The significance of the differences of the ratio of non-methylated/methylated cytosine between children from Region 1 and 2 in the molecular analysis of DNA was evaluated using the non-parametric Mann-Whitney Test for independent observations. A probability of less than 0.05 was considered significant. All calculations were performed using a domestic standard statistical pack (PdeB, Mendoza, Argentina, Version 2.5).

3. RESULTS

The main demographic characteristics of children from Region 1 and 2 are shown in Table I. No statistical differences were observed between these 2 groups.

The phenotypic characteristics attributed to the HSR gene in the selected sample of 20 children from Region 1 and 2, are shown in Figure 1. As previously described[29] a significant greater proportion of children from Region 2 had writing-reading disturbances compared to those from Region 1. No statistical differences in the proportions of children with clock-wise hair whorl or right handedness were found between groups.

Table I. Demographic data of school children of region 1 and 2.

Characteristic	Region 1 ($n = 20$)	Region 2 ($n = 20$)	Difference p
Age (years)[a]	10.3 ± 0.2	11.1 ± 0.3	n.s.
Raven score[a]	50 ± 2.6	42.5 ± 3.2	n.s.
Male (number)	10	11	n.s.
Female (number)	10	9	n.s.

[a]Mean ± standard error of the mean.

The molecular ratio of non-methylated cytosine to methylcytosine (R_{NMM}) from the DNA of children from Regions 1 and 2 is shown in Figure 2. Figure shows that R_{NMM} from DNA of children of Region 2 is about 14 times significantly higher than the R_{NMM} from children of Region 1 ($p < 0.01$, Fig. 2).

4. DISCUSSION

As shown in Table I, demographic characteristics between the two children groups (region 1 and region 2) were not statistically different. This is in complete agreement with previous results worked out with the complete sample of 547 children.[29] Since the present sample was selected at random from the original complete sample for the molecular studies, this result was to be expected. As previously mentioned,[29] the two groups can be considered similar under a demographic point of view. Thus, these features cannot contribute to explain the differences found in the

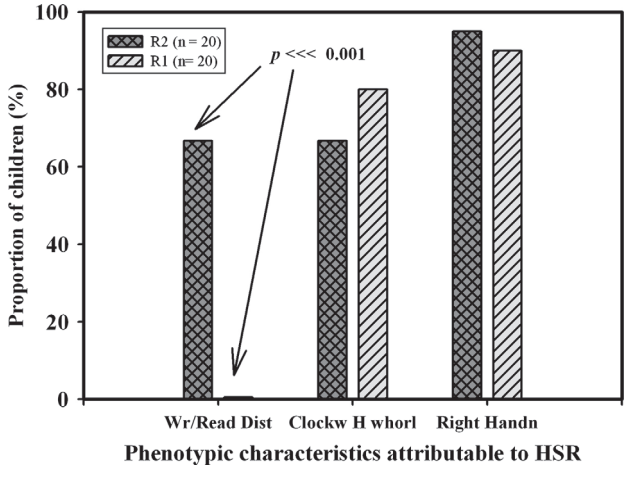

Fig. 1. Phenotypic characteristics attributable to the *HSR* gene measured in children from two geographical regions of La Rioja province (Argentine). W/Read Dist = Writing–reading disturbances; Clock H Whorl = Clockwise hair whorl direction; Right Handn = right handedness. R_1 and R_2, regions 1 and 2.

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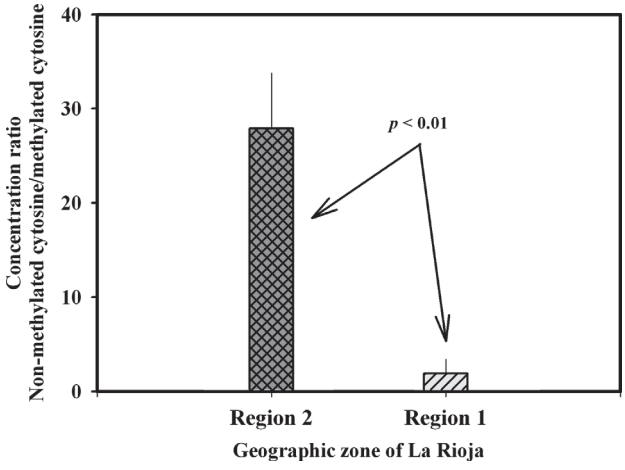

Fig. 2. Molecular ratio of non-methylated cytosine to methyl cytosine in DNA of children from two geographical regions of La Rioja province (Argentine). Results are expressed as the mean ± error of the mean.

phenotypic characteristics of children of Region 2, compared to those of Region 1.[29] In this communication, of the three expressions attributed to the HSR gene (Fig. 1) only the writing-reading capacity showed statistical significant differences in the present sample. Although a lower proportion for the clockwise hair whorl direction and a high proportion for right handedness were observed in children from Region 2 (Fig. 1), these numbers were not statistically significant. These results appear contradictory with previous findings for the complete sample,[29] where a significant decreased proportion for the clockwise hair whorl direction, and significant increase for the right handedness were found in children from Region 2, compared to those of Region 1. However, this contradiction may be only apparent. In the previous study, proportion of right hand use of children of Region 2 was 5.9% higher than controls.[29] This means that the estimated probability to find differences in subsequent sampling from these populations will be around 0.059 for this phenotypic characteristic. In this study, the proportion of increase of right hand use was found to be 5% over control (Fig. 1) in the sample of 20 children of Region 2; close to 5.9% but not statistically significant, due to the small size of the sample. A similar argument is valid for the clockwise hair whorl direction, the other phenotypic characteristic modulated by the HSR gene.

The most fundamental finding of the molecular analysis of DNA from children from both regions was the difference in the ratio of non-methylated to methylated cytosine concentrations (R_{NMM}), shown in Figure 2. There is a greater relative proportion of non-methylated cytosine in the DNA of children from Region 2 than that observed in DNA from control children, suggesting that in these children DNA are less suppressed. Since in the present experimental conditions we worked with whole DNA, it is not possible to attribute this decreased methylation to some specific genes. Nevertheless, this result is in agreement with a previous preliminary study of our laboratory using HpaI and HpII restriction enzymes that discriminate the methylated sequence 5′-CCGG-3′ in DNA, showing a difference in methylated cytosine between the two groups.[28] This evidence is highly suggesting that whatever the genes involved in the expression of the phenotypic characteristics so far analyzed it appears that an epigenetic mechanism might be participating.[16, 19, 20] In the most studied epigenetic processes such as imprinting, the expression of certain imprinted genes changes along the maturation period of subjects, as observed in the expression of IGF2 gene in the human liver tissue.[5] At 6 months of age the IGF2 expression is monoallelic.[5] However, from the 6th to the 8th month of life, relaxation of imprinting occurs giving expression of the IGF2 clearly biallelic by 2 years of age.[5]

In some other occasions, lost of normal imprinting, or relaxation of imprinting have been linked to some neurologic diseases such as the Rett syndrome (OMIM 312750) with mental retardation linked to the X chromosome (OMIM 300624, 300419), the Prader-Willi-Angelman syndrome (OMIM 176270), and some neoplassic diseases.[15] On the other hand, severe loss of DNA methylation appears to be a common mechanism for the development of adenoma and colorectal cancers in humans.[9] In addition, hypomethylation in the DNA of TCD4+ lymphocyte is able to induce the generation of antibodies to DNA which is the base of the autoimmune diseases, such as the case of the lupus disease.[31] At present it is not clear how epigenetic processes can be influenced by factors from endogenous or exogenous cell origin. In recent studies, the administration of L-methionine in the diet of mice was crucial to maintain the epigenetic state of the brain tissue, since this amino-acid is the physiological substrate for the DNA-methyltransferase enzyme.[25] Also apparently subtle factors, such as maternal behaviour can also influence the epigenetic state of the descendant mice.[25] Thus, it is possible that these complex molecular mechanisms affecting the DNA expression can be influenced by specific external signals.

Considering that children from Region 2 are different from control children regarding the phenotypic characteristics attributable to the HSR gene, and in addition supporting this phenotypic change hypomethylation of DNA was found suggesting an epigenetic adaptation, the cause for this change cannot be evident from the present experiments. As discussed previously, the main known factors that could modify the phenotypic expression of the HSR gene (nutritional, behavioural and genetics) were discarded.[29] Thus, taking into account the only quite evident difference between Region 2 and 1, i.e., the geographic characteristics, it is not unreasonable to consider that the trace elements present in abnormal amounts in Region 2 [10] can be an exogenous factor influencing the regulation of the epigenetic processes of genes. In spite than little is known about the possible interaction of

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trace elements with DNA, it is interesting to note that it has been reported that alkaline metals appeared to concentrate in some determined repetitive DNA sequences of nucleotides.[1] Using the magnetic dispersion relaxation technique, it was found that some monovalent cations such as sodium interacts directly with DNA in those regions rich in AT sequences.[1] In addition, Mg^{+2} can act as bridge between intracellular enzymes and nucleotides giving stabilization of the secondary and tertiary structure of DNA.[1] Increased concentration of Mg^{+2} are able to produce changes in the molecular conformation of DNA which can be modified by some other divalent cations such as Ca^{+2}, Zn^{+2}, Co^{+2}, Ba^{+2}, Mn^{+2} and Cd^{+2}.[1] Thus, it appears that monovalent cations interact with those molecular regions rich in AT content and forming the minor curvature of DNA; while divalent cations interact with that region rich in CG content forming the mayor curvature of DNA.[1] All this evidence suggests that trace elements can be candidates for external regulators of epigenetic processes in man and might be involved in the phenotypic and molecular changes found in children from Region 2 of La Rioja. Our laboratory is currently evaluating this possibility in an experimental animal model of HSR-like phenotypic characteristics.

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