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RESEARCH ARTICLE

GENETIC INTERACTION BETWEEN METHYLENETETRAHYDROFOLATE REDUCTASE C677T GENE POLYMORPHISMS AND FRAGILE SITES ASSOCIATED "RISK FACTOR" IN APLASTIC ANEMIA PATIENTS

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ABSTRACT

Aplastic anemia (AA) including Fanconi is a unique disease shows genetic compatibility between gene and environment. The role of MTHFR C677T gene polymorphism and chromosomal break points in aplastic anemia (Fanconi) has not been documented earlier in Indian literature but chromosomal fragility and MTHFR polymorphism modulate folate metabolism leads to increase "risk" of malignancy. Hence, the study of aplastic anemia becomes imperative to with the aim to evaluate the frequency of C/T genotypes and chromosome break points (fragile site).Present study statistically shows significant differences (P <0.001) between homozygous (CC) 24.57% and heterozygous (CT) 33.33% condition with increase four time calculated value of odd ratio at 95% confidence interval might have increase "risk factor". Interestingly, cytogenetic study reveals two spontaneous fragile sites-2q21 (3.1%) and 9p22-23 (2.8%) with high frequency of chromosome "bridge" formation (25.0%), suggest that these break points and genotypic variation of MTHFR together confirms gene – gene interaction in the patients of aplastic anemia. However, present study is reported first time to the best of our knowledge even in non- identical twins has not been reported earlier in Indian population.

INTRODUCTION

Aplastic anemia (AA) is characterized by an empty bone-marrow and panytopenia in peripheral blood. Epidemiological study suggest that incidence of aplastic anemia in Europe and USA varies from 2.0 to 11.0 per million/ year. However, immunosuppressive therapies give favorable response to the cases of AA. The interaction between gene and environment are more prominent for individual susceptibility (Strob, 1997; Salagovic, 1998). Fanconi anemia (FA), an autosomal disorder leads to appear tremendous multiple breaks & reunion to form chromosomal bridge formation, sharing an impairment of DNA metabolism proneness to malignancy towards mutagenic agents (Schroeder, 1982). Fragile sites appear under special culture conditions in metaphase chromosomes when cells are exposed to a perturbation of DNA replication (Sutherland, 1988). Chromosomal fragility disrupt gene expression due to bone-marrow failure with multiple congenital anomalies such as skin hypo or hyper pigmentation, combined radial & thumb deformities, cardiac, renal abnormalities and with or without microcephaly. (John, 2001). Chromosomes from affected individuals exhibit structural liability and there is a strong predisposition to the development of neoplasia (Porfirio, 1989). Folate, an essential component of DNA synthesis required for cellular proliferation and their deficiency induces chromosomal damage, formation of fragile sites resulting tumourigenesis. The change in folate level in cell influences nucleic acid synthesis, DNA repair and methylation. Although, large number of toxic chemicals (drugs), infections and inherited genetic factors has been postulated to contribute in etiology of aplastic anemia (Salagovic, 1998). The 5,10-methylenetertahydrofolate reductase **(**MTHFR) is a major circulating form of folate in blood.

MTHFR (methylenetetrahydrofolate reductase) regulate folic acid metabolism through conversion of 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate. MTHFR, a thermolabile gene mutation consist of a cytosine (C) to thymidine (T) substitute at nucleotide position 677, leads to the exchange highly conserved alanine to valine resulting decline folate supply. The genotypic variants of MTHFR C →T lead to increase homocysteine and DNA hypomethylation are associated to cause several malignancies (Bloom,*et al*., 1966; German & Crippa, 1966; Swift, 1971; German, 1972; Sutherland, 1998; Schwartz *et al*., 2005). It is still unknown that how MTHFR C677T genotypes and specific break points (fragile sites) are involved in maintaining the integrity of genomic DNA either independently or together, hence, the present study becomes imperative to find out the etiology of aplastic anemia by evaluating the prevalence of "fragile sites" and MTHFR polymorphism.

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MATERIALS AND METHODS

Collection of blood sample

Blood sample (0.5ml) were collected of clinically diagnosed Aplastic anemia (n=21) including three cases of Fanconi with inclusion criteria the appearance of congenital malformation such as skin hypo or hyper pigmentation, combined radial & thumb deformities, cardiac , renal abnormalities and with or without microcephaly (John, 2001). In the present study those cases are excluded who are under treatment with any chemotherapy/ radiotherapies with their respective controls (n=30) attending O.P.D of S.S. Hospital referred to Cytogenetic and Molecular Genetics Laboratory of Centre of Experimental Medicine and Surgery., I.M.S, B.H.U., Varanasi for cytogenetic and molecular studies. This study was duly approved by the ethical committee of the institute. Family history was recorded on predesigned performas for epidemiological study, after taken a informed consent from the parents or attendants of patients in case of minors.

Isolation of Genomic DNA and PCR based RFLP analysis

DNA was isolated from the whole blood using Bioner Kit (Korea) and samples were kept at -20ºC till further analysis. In the present study PCR technique for MTHFR C677T was developed using specific primers (forward =5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and reverse =5'-TGA GAG TGG GGT GCA GGG AGC TT-3') by the method of Frosst *et al*.,1995. The reaction mixture contain total volume of 25 µl contain 50-100 ng of DNA , 20 pmole of each primer, 200µM of each dNTPs mix with Taq buffer (10mM Tris HCl pH 8.3, 50mM KCl), 3.0mM $MgCl_2$ and 3 unit of Taq polymerase (New England Biolab). Cycling conditions were 4 min at 94ºC for initial denaturation, 58 ºC/1min for annealing followed by 35 cycles and 72 ºC/7min for final extension. RFLP analysis was carried out for the study of MTHFR genotype variants. PCR product (6 µl) were digested at 37ºC for 3hr. in reaction volume of 25 µl containing 1U of *Hinf-I* restriction enzyme (New England, Biolabs) and NEB buffer (2.5 µl). The digested product of RFLP was separated on 3% agarose gel, stained with Et.Br, bands were visualized and characterize on Gel Doc system (SR Biosystem).

Cytogenetics Study

Blood samples (2.5 ml) were collected for cytogenetic analysis including three families of Fanconi under sterile condition. Lymphocytes were cultured in RPMI-1640 media, 5% FCS, stimulated with phyto-M for 72hrs at 37ºC with and without folic acid supplementation to analyzed specifics break points (fragile sites). The cultures were harvested after addition of cholchicine (0.01ug/ml) and chromosomal preparations were made on pre chilled slides using air dried method for observations as follow laboratory protocol. The slides were used for GTG banding and stained with Giemsa (5%). For each data point, chromosomal analysis were carried out in at least 25 well spread metaphase plates/case and karyotyped according to ISCN (1995) for structural and numerical variation (Karger, 1995).

Statistical analysis

The data was processed by the method of Porfirio (1989) and calculations were performed on the assumption that breakage occurred randomly including chromosome & chromatid and compared with controls. Statistical analysis was carried out to compare the expected value with observed by using chi square test. The Hardy - Weinberg equilibrium equation was used to determine the allele frequency.

RESULTS

Hematological examination showed mean hemoglobin (gm/dl) level was 3.9 ± 1.86, platelet count (mean) 19.17± 1.56 ($x10^9$ / L) and WBC count 2.8 $x10^9$ / L with absolute neutrophils count (mean) 0.69± 0.44 $x10^9$ / L. Partial pedigree analysis reveals the mode of inheritance in twins family of Fanconi either follows law of segregation of alleles or incomplete mode of dominance and peniterance of gene as documented in figure-1. The variation in the frequency of chromosomal aberrations such as "fragile" site expression, chromatid breaks, and chromosomal bridge formation were observed under folic acid deficient cultured lymphocytes in FA patients as represented in figure-2 (partial metaphases) with spontaneously observed break points 2q21 (3.1%) and 9p22-23 (2.8%). Besides this most interesting findings were the appearance of chromosomal bridge formation (Fig.2D) with high incidence (25.0%) with the appearance of acentric fragments (8.3%). Simultaneously, in controls sample showing lack of cytogenetic anomalies.. The statistical analysis were carried out showed highly significant ($p<0.001$) differences with respect to controls using chi square test.

Table-1 depicted the details findings of RFLP analysis of MTHFRC677T gene polymorphism in aplastic anemia cases and their respected controls. The present study shows variable frequency of genotypic variants i.e. CC genotype in (24.57%) (wild type) and CT genotype in (33.33) heterozygote condition, where as in controls the genotype frequency varies between 73.30 (CC) – 10.00 in (CT). The homozygous mutant TT (rare type) genotype was unable to observed in both cases and their respective controls. The frequency of heterozygote CT genotype increase significantly ($p<0.0039$) as compared to controls when analyzed statistically using chi square test. We have also calculated odd ratio (O.R) at 95 % C.I.(confidence interval) to evaluate the "risk factor" in heterozygote condition (O.R. 4.5) was at 95% C.I (0.84-26.3) between cases and controls. Although, the individual allele frequency (C/T) was also calculated using Hardy Weinberg equilibrium between cases and controls.

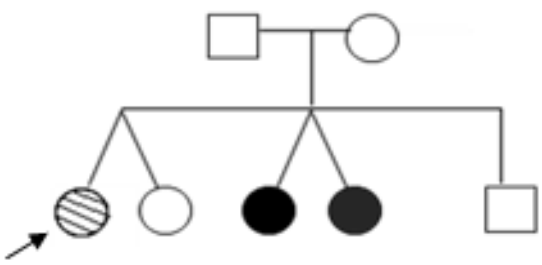

Figure 1. Partial Pedigree as representative of non-identical twins family of Fanconi including proband (arrow) showing peniterance of gene

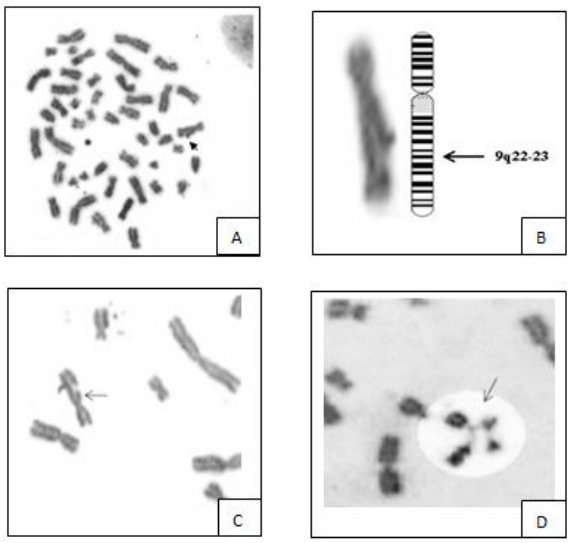

Figure 2. Metaphase showing spontaneous fragile site 9q22-23 (fig. A) and its enlarge view with ideogram as shown in figure-.B. Partial metaphase showing chromatid break (fig. C) and "chromosomal bridge" formation (fig. D arrow head.)

Table 1: MTHFR C677T gene showing variable frequency of genotypes between homozygous and heterozygous condition and their individual's allele frequency in aplastic anemia cases with their respective controls

Genotype	Cases	Controls	O.R at 95% C.I cases vs. controls	p-value
	% frequency			
CC	24.57	73.30	0.145(0.03-0.58)	0.002*
CT	33.33	10.00	4.5(0.84-26.5)	0.039*
C	0.17	0.23	0.11(0.01-0.73)	0.005*
T	0.035	0.01	NaN	1.000

* Significant difference ($p<0.001$) were observed using chi square test between cases and controls.
NaN= Not Observed

DISCUSSION

Genomic instability is a characteristic feature of human cells, if there is a variation in physiological conditions might be responsible for changes in the anatomy of chromosomes like deletions and amplifications might have increase mutational spectra. This hypothesis has limitations to explain variation between ethnic group differences in the prevalence of aplastic anemia because of association with chromosomal instability. The exact mechanism of drug - induced aplastic anemia is unknown and may associated with specific metabolic pathway. Over the past several years scientist have documented many areas of unusual genomic DNA instability as being involved in a wide variety of neoplastic conditions ranging from neuroblastoma to leukemia (Swift, 1971; Sutherland, 1998). These genomic alterations are responsible for the production of "fragile sites". Because of the high frequency of chromosomal abnormalities, predominantly breaks has been suggested that an error in DNA repair exists in Fanconi's anemia. The FANCA, FANCC, FANCE, FANCF, and FANCG genes have been cloned and mapped on chromosomes 16q24.3, 9q22.3, 6p21.2–21.3, 11p15, and 9p13, respectively. Our results demonstrate higher prevalence of chromosomal breakage (25.0%) and fragile site expression (2q21& 9p22-23) perhaps due to increase of intracellular folate level during folate deficient culture conditions has been strikingly observed probably either due to failure of non-disjunction event during cell -division or low mitotic index. Two of these fragile sites (2q21& 9p22-23) are frequently observed in tumor patients with variable frequency either due to different culture conditions or unknown environmental factors (Yunis, 1984). Such genetic variation occurs due to a mixture of factors susceptible towards cancer and their management such interesting pattern emerged with high prevalence of "chromosomal bridge formation" arises after multiple breakage and reunion (Figure-2D), warrant the condition of pre leukemic patients either in low folate or thymidylate induced stress in lymphocyte cultures. Since it has been realized that fragile sites study are very difficult task in FA cases because of following reasons-1. low mitotic index due to failure of bone - marrow in such cases, 2. In normal population, frequency of spontaneous fragile sites is very low, 3. These are distributed non- randomly on human chromosomes, the definition follow the null hypothesis dealing with such crucial subject.

Because MTHFR play a key role in folic acid metabolism and changes in its activity associated with polymorphism in MTHFR gene could modify the susceptibility of cancer, either inhibit DNA replication or fail to facilitate DNA repair mechanism However, the genetic interaction remains unclear until large number of cases /patients are required to maintain homogeneity in population . Although, TT genotype (rare type) was unable to observed in the present case controls study. The allele frequencies of C677T are quit variable in Canadian population (Frosst *et al*.,1995). However, similar observation were also observed in our study probably either due to heterogeneous group of population belongs to different ethnic background or unknown environmental (dietary) factors. About half the general population carry at least one mutated allele in homozygous (TT) condition varying from 1 to 20 % (Botto & Yang, 2000). The statistical analysis showing the significant interaction by calculating odd ratio varies from 0.14 to 4.5 at 95% confidence interval suggesting increase risk for heterozygous condition. However, it is evident that large number cases are required to fully elucidate the mechanism of aplastic anemia included Fanconi.

In conclusion

Present case study is quite fascinating because of non - random chromosomal breakages in FA has not been reported earlier including even non identical twins in Indian population. The mode of inheritance is not yet clear from the above pedigree because lack of family data but authors hypothesize that may either due to segregation of alleles or incomplete dominance with peniterance of gene in proband. Such study has an important implications on the assessment of potential "risk factor" either due to folate deficient diet (nutritional factor) or unknown environmental factor.

REFERENCES

Bloom G, Warner S, Gerald P, Diamond L. 1966. Chromosome abnormalities in constitutional aplastic anemia. N Engl J Med., 274,8-14.

Botto L D, Yang 2000 Q5,10- methyl enetetrahy drofolareductase gene variants and congenitalanomalies: a HuGE review. Am J Epidemiol: 151: 862-877.

Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG *et al.*, 1995.A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. Nat Genet., 10, 111–3.

German, J. & Crippa, L. P. 1966 .Chromosomal breakage in diploid cell lines from Bloom's syndrome and Fanconi's anemia. Ann. Genet., 9, 143-154.

German, J. 1972. Genes which increase chromosomal instability in somatic cells and predispose to cancer. In Steinberg AG, Bearn AG, eds. Progr. in Med. Genet., 8,61-101.

John J. Gregory, Jr., John E. Wagner, Peter C. Verlander *et al.*, 2001. Somatic mosaicism in Fanconi anemia: Evidence of genotypic reversion in lymphohematopoietic stem cells. PNAS., 98, 2532–2537.

Porfirio B, Tedeschi B, Vernole P, Caporossi D, Nicoletti B. 1989. The distribution of MspI- induced breaks in human lymphocyte chromosomes and its relationship to common fragile sites. Mutat Res., 213,117-124.

Salagovic J, kalina I, Stubna J *et al*., 1998. Genetic Polymorphism of glutathione-S- transferase M1 and T1 as a risk factor in lung and bladder cancers. Neoplasma., 45:312-317.

Schroeder TM. 1982. Genetically determined chromosome instability syndromes. Cytogenet Cell Genet., 33,119-132.

Schwartz M, Zlotorynski E, Goldberg M, Ozeri E, Rahat A, le Sage C *et al*., 2005. Homologous recombination and nonhomologous end-joining repair pathways regulate fragile site stability. Genes Dev., 19,2715–2726.

Strob R. 1997 .aplastic anemia. J Intraven Nure, 20:317-322.

Sutherland GR, Simmers RN. 1988 .No statistical association between common fragile sites and non random chromosome breakpoints in cancer cells. Cancer Genet Cytog., 31, 9-15.

Sutherland GR, Backer E, Richard RI., 1998 .Fragile sites are still breaking. Trends in Geneti., 14,501-506.

S. Karger Mitelman F. 1995 . ISCN An international system for Human cytogenetic nomenclature. editor.Basel.

Swift M. 1971 .Fanconi's anemia in the genetics of neoplasma. Nature., 230,370-373.

Yunis JJ., 1984. Fragile sites and predisposition to leukemia and lymphoma. Cancer Genet. Cytoge., 12 , 85-88.

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