



METHYLENETETRAHYDROFOLATE REDUCTASE GENE (677C→T) AND TUMOR NECROSIS FACTOR ALPHA (TNF- α) ASSOCIATED RISK FOR THE DEVELOPMENT OF GESTATIONAL TROPHOBLASTIC NEOPLASIA

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Abstract- Gestational trophoblastic neoplasia is a rare disease of female reproductive system occurs due to aggressiveness of trophoblastic cells start of invade into endometrium or myometrium results profusely bleeding with pain, if fail to cure early the “risk” for mortality may enhanced. The etiology of gestational trophoblastic neoplasia is still unknown but believes to be the interaction between gene and environment. RFLP analysis of MTHFR (C677T) gene showing 28.00% in CT (heterozygous) genotype in patients or cases as comparison to controls (10.00%), suggesting increase “risk factor” in such patients. However, the TNF- α gene showing variable frequency mutation i.e. over expression (57.00%) and down regulation (28.00%) were observed in cases. The odd ratio was also calculated at 95% C.I. for TNF- α gene which reveals highly significant ($p=0.009$) difference between cases and controls, suggest confirmation of gene-gene interaction in the patients of gestational trophoblastic neoplasia reporting first time in India.

Keywords- Gestational trophoblastic neoplasia, MTHFR, TNF- α

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Introduction

Gestational trophoblastic neoplasia (GTN) is reported less than 1% of females being well characterized by aggressive invasion of endometrium and myometrium by trophoblastic cells. Several clinical conditions of GTN such as hydatidiform moles, invasive mole, gestational choriocarcinoma, placental site trophoblastic tumor and epithelioid trophoblastic tumor have been reported but the most common is hydatidiform moles [1,2]. The diagnosis of GTN is often delayed due to subtle signs and symptoms of disease in patients with high incidence of mortality 14%. The genetic susceptibility to gestational trophoblastic tumor has been poorly established and genes involved with endothelial dysfunction, oxidative stress, and angiogenesis are contemplation to be multifactorial in origin including environmental factors [3].

Folate, an essential component of DNA synthesis and low folate level has implications for cell replication, DNA excision & repair and DNA hypomethylation [4]. Folate deficiency is thought to increase the risk of cancer through impaired DNA repair synthesis, disruption of DNA methylation and chromosome breaks [5-7] results arterial & venous thrombosis [8] development of different type of neo-

plasia e.g. cervix cancer [9-10], colon cancer [11], acute leukemia [12], prostate cancer [13], lung cancer [14] and gastric cancer [15].

Tumor necrosis factor (TNF), act as an anti-tumor cytokine is another candidate gene with multifunctional activity in inflammation, immunity, cellular homeostasis and tumor progression [16] through NK cells, T cells, B cells and macrophages mediated killing of specific tumors (soft tissue sarcoma, melanoma) [17], gastric tumors [18]. TNF- α is able to initiate cellular apoptosis and deactivate tumor cells in solid cancer and to maintain microenvironment promotes cell migration and invasion [19-20]. The mechanism by which TNF- α gestational trophoblastic neoplasia facilitates these events remains mysterious [21]. However, apart from its biological implications and cancer risk factor associated gene mutation resulting polymorphic variation of alleles may raise the possibility of enhancement carcinogenesis during pregnancy hence, TNF- α & MTHFR could be use as non-invasive tool as a genetic marker for pre/postnatal diagnosis. The present study dealt with to evaluate the possible gene-gene interaction including MTHFR C677T and TNF- α gene mutation in the patients of gestational trophoblastic neoplasia to explore the etiology of tumorigenesis.

Materials and Methods

Blood samples of clinically diagnosed cases of gestational trophoblastic neoplasia cases (n=7) of age group (20-32years) were collected from S.S. Hospital, of B.H.U., with their respective controls (n=10) for PCR based DNA analysis. The study was dually approved by ethical committee of the Institute of Medical Sciences and samples were collected after written consent either from the patients or their attendant. Genomic DNA was isolated from peripheral blood, as described previously by Miller, et al. [22]. The mutation in the MTHFR (C677T) and TNF-α gene was analyzed by polymerase chain reaction (PCR) of genomic DNA by using specific amplicons. The primers were selected for MTHFR C677T gene :forward 5'-TGA AGG AGA AGG TGT CTG CGG GA-3' and reverse -TGA GAG TGG GGT GCA GGG AGC TT-3' [23] and TNF alpha forward = 5'-AAC ATC CAA CCT TCC CAA ACG CCT-3' and reverse = 5'-CCA GGT TTC GAA GTG GTG GT-3' [24]. We have standardized polymerase chain reaction (PCR) specific strategies using forward and reverse primers in 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 MgCl₂ and 3 unit of Taq polymerase (New England Biolab). Cycling conditions were 4 min at 94°C for initial denaturation, 58 °C/1min and 61 °C/45sec for annealing followed by 35 cycles and 72 °C/7min and 72 °C/5min for final extension of MTHFR C677T and TNF alpha respectively. RFLP analysis was carried out for the polymorphism analysis of MTHFR C677T allele. PCR product (6 µl) were digested at 37°C 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. The bands were further characterized and visualized on Gel Doc system (SR Biosystem).

Results

Table-1 [Table-1] documented the details findings of distribution of genotypes frequency between cases and compare with controls. The polymorphic variation of MTHFR gene is consistently observed the CC genotypes (wild type) frequency (71.00 %) in GTN cases and 90.00 % in control. Fig. 1a. showing a high prevalence of homozygosity for C677T in gestational neoplasia with transmission of C→T at position 677. The frequency in heterozygosity ([Fig-1a], lane-1) condition is more than controls i.e. C→T genotypes in case 28.00% are increased than controls (10%). Interestingly, we have also evaluate the mutation in TNF-α gene of 160 bp DNA fragment as one of the important candidate gene for regulating signal transduction mechanism in cancer development in GTN cases and findings are observed as over expression or down regulation and compared with normal intensity of band as shown Fig. 1b [Fig-1b]. The findings of TNF-α gene is highly variable i.e. over expression in 57.00% cases, while, down regulation is observed in 28.00 % in GTN patients when compared with normal (14.00%). Apparently, in Fig. 2 [Fig-2] showing significantly relation of mutation rate in term of up / down regulation of TNF-α in GTN cases with respect to controls but when analyzed significant difference showing significant p valu (< 0.005). The odd ratio is also calculated at 95% confidence interval to find out "risk factor" and found statistically highly significant differences (p = 0.009) between cases and controls as details are documented in table-2 [Table-2]. The present findings were repeated thrice to confirmation of the TNF-α gene mutation.

Statistical analysis were carried out between cases and controls using chi square (two tailed) probability test showing lack of significant (p<0.05) differences in MTHFR gene but significant difference reveals in the TNF-α.

Table 1- MTHFR C667T showing genotype variation and calculated odd ratio at 95 % C.I. between cases and controls.

| Genotype | Cases n=7 % frequency | Controls n=10 % frequency | O.R at 95% C.I Cases Vs. Controls | p-value |
|----------|--------------------------|------------------------------|--------------------------------------|---------|
| CC | 5(71%) | 9(90%) | 0.278(0.007-5.803) | 0.323 |
| CT | 2(28%) | 1(10%) | 3.6(0.17-134.48) | 0.323 |
| C | 0.06 | 0.095 | 0.278(0.007-5.803) | 0.532 |
| T | 0.01 | 0.005 | Not observed | 1 |

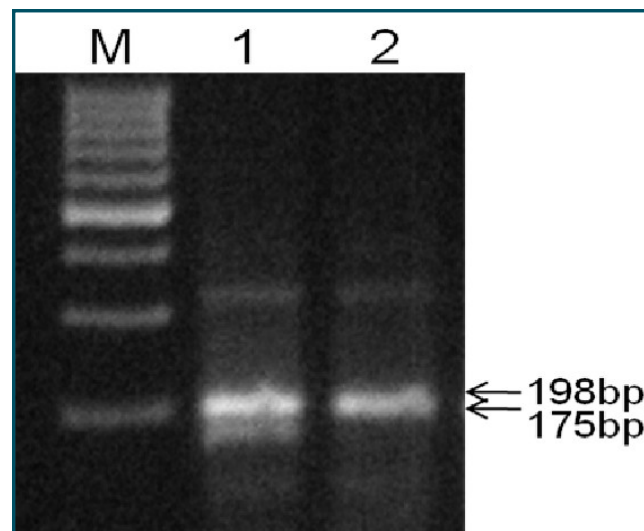


Fig. 1a- PCR based RFLP analysis of MTHFR C677T gene polymorphism after digestion of 198 bp digested with restriction enzyme (HinfI) showing 198bp & 175bp in heterozygous condition (lane-1) and 198bp homozygous (lane-2) in gestational trophoblastic neoplasia and lane -M showing 100bp ladder.

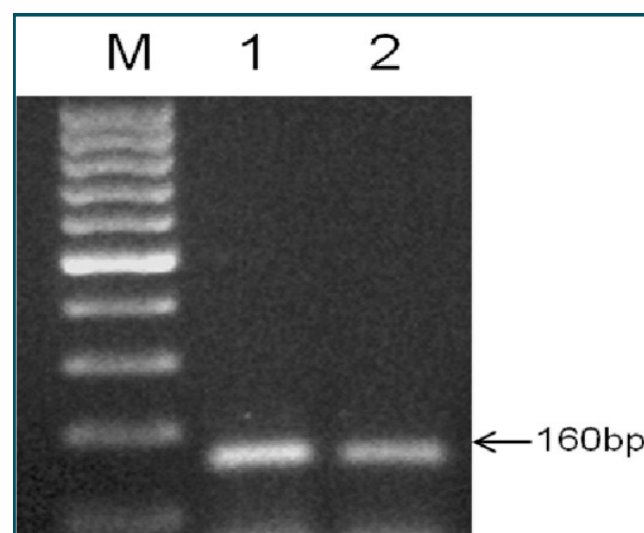


Fig. 1b- PCR based analysis of amplified product TNF-α gene mutation (160bp) showing in lane-1 up regulation (over expression), while lane-2 showing down regulation of amplified product in gestational trophoblastic neoplasia and lane M showing 100bp ladder.

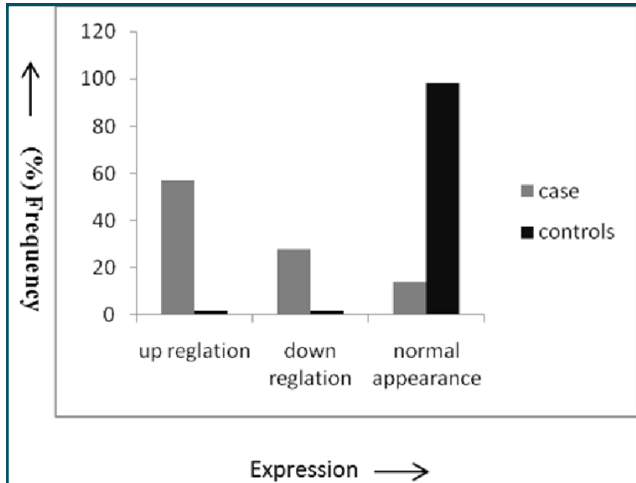


Fig. 2- Bar diagram showing the different % frequency of TNF- α gene mutation in term of up regulation (over expression), down regulation and normal appearance (null) of 160bp amplicon in gestational trophoblastic neoplasia with respect to controls.

Table 2- TNF- α showing the calculated odd ratio at 95 % C.I. between cases and controls.

| Expression | Case (n=7) | Controls (n=9) | OR at 95% (C.I.) | p-value |
|-------------------|------------|----------------|------------------|---------|
| Up regulation | 57% | 0 | Inf(1.088-inf) | 0.009* |
| Down regulation | 28% | 0 | Inf(0.300-inf) | 0.086* |
| Normal appearance | 14% | 100 | 0.00(0.00-0.315) | 0.00* |

*Highly significant ($p < 0.001$).

Discussion

Epidemiological studies shows that an association between low folate intake and an increased risk for the development of cancer. In this manuscript, we have undertaken a case-control study to investigate the role of MTHFR (C677T) gene polymorphisms and their correlation with tumor necrosis factor alpha (TNF- α) gene and their susceptibility in gestational trophoblastic neoplasia. MTHFR, an enzymes that play a crucial role in the folate metabolic pathway due to involvement in both DNA synthesis and DNA methylation. Interestingly, in the recent year we have observed the genetic susceptibility in various types of tumors leads to a growing attention to the MTHFR C677T gene polymorphisms associated risk in tumorigenesis.

Our study showing high prevalence of homozygosity of CC genotype (wild type) in (71.00%) cases, while unable to observed TT genotype (rare type) may be either due to small sample size or unknown environmental factors such variation of gestational age or ethnicity. Although, both cases and controls belonged to the same ethnic background and shared a common geographic origin in eastern part of the India. Interestingly, CT genotype (28.00%) in heterozygous condition was quite high when compare with controls suggesting increases "risk factor" in gestational trophoblastic neoplasia cases. However, our findings lack of support to the finding of Lasecka, et al. in gestational trophoblastic cancer [3], while supporting to the earlier findings of Suzuki, et al [14] and Marchal, et al [25] in different type tumors. A common C to T transition at nucleotide 677 (C \rightarrow T) associated as a risk for various clinical lesions including cancer has also been reported [3-6].

During embryonic development of MTHFR 677TT genotype (homozygous) in pregnant female (third trimester) lack of the enzyme 5,10-methylenetetrahydrofolate reductase increased risk of mortality during postnatal life [14]. However, in prostate cancer MTHFR 677TT genotype also reduced the risk by inadequate synthesis of folate and may also provoke to decrease in DNA methylation and favoring of the CpG promoter sequences of the tumors suppressor genes during tumorigenesis [26].

Tumor necrosis factor α and another relevant genetic marker making close link between inflammation and cancer [19]. The present study reveals mutation of TNF- α gene (either complete deletion/ over expression / under expression) may failed to contributes the significant development of the tissue architecture required necessary for tumor growth & metastasis [18]. These tumor-promoting activities suggest that inhibition of TNF- α is an effective strategy for cancer therapy [21] thus TNF- α gene mutation are involved in the complications during pregnancy and extend their effects to increase "risk factor" for development of cancer. However, we have observed first time that over expression / up regulation are seems to be highly associated to gestational trophoblastic neoplasia GTN as compared their down regulation may be due to the multifunctional properties of TNF- α gene used to treat various types of tumors [20].

Conclusion

In the present study we concluded to assess first time association between the polymorphisms of MTHFR C677T and TNF- α genes in gestational trophoblastic neoplasia. There are four possible reasons associated with the disease-1. MTHFR 677 C \rightarrow T genes in heterozygous condition increased risk as an independent factor for GTN, 2. Gestational age is equally relevant for developing complication during early embryonic life, 3. TNF- α genes mutation also confirms risk either as an independent or with association to MTHFR, 4. Increased risk in consanguineous manner may due to extra copy of alleles implicated high risk factor to severity of such disease. However, our findings are small but promising needs further evaluation to determine allele frequency by collecting large sample size to confirm gene-gene interaction in gestational trophoblastic neoplasia patients.

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