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Research Article

MTHFR C677T and RFC1 G80A Polymorphisms Affect the Multiplication Rate of Human Periodontal Ligament Stem Cells in Culture

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Abstract

Objective: The folate cycle mediates one-carbon metabolism, which is essential for DNA replication and cell division. Compromised neural crest cell proliferation and migration contribute to the development of nonsyndromic cleft lip and/or palate (NSCL ± P), while folic acid periconceptional supplementation can prevent it. This study investigates if the multiplication of human periodontal ligament stem cells (hPDLSC) is affected by folate cycle gene polymorphisms (methylene tetrahydrofolate reductase, MTHFR C677T, and reduced folate carrier 1, RFC1 G80A) that belong to candidate genes for NSCL ± P. Can intracellular folate availability modify the proliferation of hPDLSC that developed from neural crest cells?

Material and Methods: hPDLSCs were isolated from extracted teeth of nine patients and cultured in alphaMEM without nucleosides with 10% fetal bovine serum (IRB approval 2021-80). Genotypes of MTHFR C677T (rs1801133) and RFC1 G80A (rs1051266) polymorphisms were identified using real-time PCR (Taqman kit, Thermo Fisher Scientific). The CyQuantTM LDH test (Thermo Fisher Scientific) was used to quantify cells in culture. Daily multiplication rates were calculated for nine possible genotype combinations.

Results: All combinations of MTHFR C677T and RFC1 G80A genotypes showed clear genotype-specific differences in hPDLSC multiplication rates. Double wild-type homozygotes (MTHFR 677CC/RFC1 80GG) demonstrated the highest multiplication rate (4.1-fold/day), while double-mutated-allele homozygotes (MTHFR 677TT/RFC1 80AA) showed the lowest rate (0.3-fold/day), representing only 7% of the wild-type rate. Heterozygous combinations showed intermediate multiplication rates.

Conclusion: Combined MTHFR C677T and RFC1 G80A polymorphisms differentially affected hPDLSC proliferation in a genotype-dependent manner. Our results suggest that intracellular availability of active folate may similarly alter neural crest cell proliferation and influence the probability of NSCL ± P development. This human primary cell culture model can be utilized in future studies on metabolic disturbances caused by folate deficiency. With a mechanistic understanding of specific genetic influences at the cellular level, we can move closer to personalized prevention for patients at risk of non-syndromic orofacial clefts.

Keywords: *Folate; Human Periodontal Ligament Stem Cells; MTHFR C677T; RFC1 G80A; Cell Multiplication*

Abbreviations

5mTHF: 5-Methyl-Tetrahydrofolate; CD: Cluster of Differentiation (Cell Surface Antigen); hPDLSC: Human Periodontal Ligament Stem Cells; MTHFR: Methyl-Tetrahydrofolate Reductase; Ncc: Neural Crest Cells; NSCL ± P: Nonsyndromic Cleft Lip with or without Cleft Palate; NTD: Neural Tube Defect; RFC1: Reduced Folate Carrier; SLC19A1: Solute Carrier Family 19 Member 1

Introduction

Folate-cycle mediated one-carbon metabolism is essential for DNA replication and cell division [1]. Periconceptional supplementation with folic acid can prevent the development of non-syndromic cleft lip with or without cleft palate (NSCL ± P) and neural tube defects (NTD) [2-10]. In the embryo, cranial neural crest cells (NCC) form facial structures. Compromised proliferation and/or migration of NCC contribute to the embryonic development of NSCL ± P [8,11].

The etiology of NSCL ± P is multifactorial, involving interactions between gene variants (susceptibility genes) and environmental factors [4,8]. MTHFR (methylenetetrahydrofolate reductase) and RFC1 (reduced folate carrier 1), also known as SLC19A1 (solute carrier family 19 member 1), are susceptibility gene variants for NSCL ± P [12-15]. The SLC19A1 gene encodes the RFC1 protein, which transports folate into the cytosol. The MTHFR gene encodes an enzyme that produces active folate, a methyl-group donor for DNA and other substrates [16].

Most molecular biology studies on the mechanisms of prevention of NSCL ± P and NTD by folate supplementation were conducted in mouse models [17]. Cell culture studies are rare [18]. Golja., *et al.* (2020) [19] studied the effect of folate on lymphoblastoid cell cultures. Herrmann., *et al.* (2007) [20] studied the effect of folate on cultures of human osteoblasts isolated from a long bone.

Cell culture models involving human cells derived from the neural crest are lacking. Dental pulp and periodontium developed from the neural crest in the embryo. Human periodontal ligament stem cells (hPDLSCs) can be isolated from extracted teeth and cultured [21,22].

Aim of the Study

The aim of this study was to investigate whether intracellular folate availability would modify the proliferation of cultured human periodontal ligament stem cells (hPDLSC) in relation to identified genotypes of RFC1 G80A and MTHFR C677T polymorphisms.

Materials and Methods

Preparation of primary cultures

hPDLSCs were isolated from extracted permanent teeth of 9 patients without NSCL ± P (4 females, 5 males, aged 15-24 years). Teeth were collected as part of routine dental and orthodontic treatment procedures, and informed consent was obtained from all participants or their guardians (IRB#2021-80).

Periodontal ligament tissue was scraped from the root surface and cultured in growth medium consisting of alpha MEM without nucleosides, 10% fetal bovine serum, 100 U/mL penicillin, 100 *µg/mL* streptomycin, and 0.29 *µg/mL L-glutamine at* 37.0°C, 5.0% CO_2, 98% humidity. Cells that migrated from tissue pieces were sub-cultured, frozen at passage 2, and stored at -80°C in growth medium containing 10% dimethyl sulfoxide until used in experiments. They were positive for CD29, CD90, and CD105 (> 95%) and negative for CD34 and CD45 (< 1%) by flow- cytometric analysis [23,22]. Passages 3-5 were used for experiments.

Genotyping

DNA was isolated from saliva samples (DNA/RNA Shield Saliva Collection kit, Zymo Research) using Quick-DNA™ Miniprep Plus kit (Zymo Research) according to the manufacturer's instructions. DNA concentrations were determined using Nanodrop One (Thermo

Fisher Scientific). Genotyping for MTHFR C677T (rs1801133) and RFC1 G80A (rs1051266) polymorphisms was conducted using specific TaqMan SNP genotyping assays (ThermoFisher Scientific) on the StepOne Plus Real-Time PCR System (Applied Biosystems) according to the manufacturer's instructions. Both genotypes were determined for each patient.

Multiplication rate assessment

Six replicate 60-mm plastic dishes (Corning) were seeded with 300,000 cells from each patient's hPDLSC. Cells were quantified by CyQuant™ LDH (Lactate Dehydrogenase) Assay Kit according to the manufacturer's instructions at the start (three dishes) and after four days of cultivation (three dishes). Multiplication rates per day were calculated as the ratio of the means of the triplicates at the start and end of the experiment, divided by the number of days.

Statistical analysis

GraphPad Prism (version 10) was used for descriptive statistics and a two-tailed t-test.

Results

We determined the multiplication rates of hPDLSC isolated from nine patients with known combinations of MTHFR C677T and RFC1 G80A genes in their genomes. A greater number of patients had been genotyped before we gathered a full set of nine combinations. We isolated and cultured hPDLSC from their extracted teeth. The measured multiplication rate was a functional outcome determined by the combination of MTHFR C677T and RFC1 G80A genotypes. Therefore, the effect of various genotype combinations could be quantified.

Corresponding daily multiplication rates are shown in table 1 and graphically in figure 1-3.

Combination table of genotypes

All possible genotype combinations of RFC1 G80A and MTHFR C677T polymorphisms with corresponding hPDLSC multiplication rates per day are shown in table 1.

Multiplication rates of double-genotyped hPDLSC				
(Mutated alleles in red font)				
		RFC1		
		GG	**GA**	**AA**
MTHFR	CC	4.145	0.623	0.929
	CT	1.054	0.739	0.768
	TT	1.025	0.940	0.295

Table 1: *Nine combinations of double genotypes of hPDLSC. MTHFR C677T: wild-type allele is C, mutated allele is T. RFC1 G80A: wild-type allele is G, mutated allele is A. Double wild-type homozygotes (MTHFR CC/RFC1 GG) had the highest multiplication rate per day (4.145-fold), while double mutated-allele homozygotes (MTHFR TT/RFC1 AA) showed the lowest rate (0.295-fold). (Multiplication rate 4.145 means 414.5% increase, multiplication rate 0.295 means 29.5% increase with respect to no increase).*

For example, RFC1 80GG, compared with MTHFR 677 CC, CT, and TT, shows a decreasing trend in functional outcome. Comparisons of RFC1 80AA to MTHFR 677 CC, CT, and TT show a similar decreasing trend but at lower multiplication rates per day.

Combined homozygous genotypes

We estimated the proportional changes in the multiplication rate caused by RFC1 and MTHFR individually by comparing combinations of RFC1 G80A and MTHFR C677T homozygotes (Figure 1). The highest multiplication rate (4.1-fold) was observed for the combination of RFC1 80GG and MTHFR 677CC; this combination is a pair of wild-type homozygotes. The lowest multiplication rate (0.295-fold) was observed in the combination of RFC1 80AA and MTHFR 677TT; this is a pair of mutant-allele homozygotes. It represented only 7% of the wild-type multiplication rate per day.

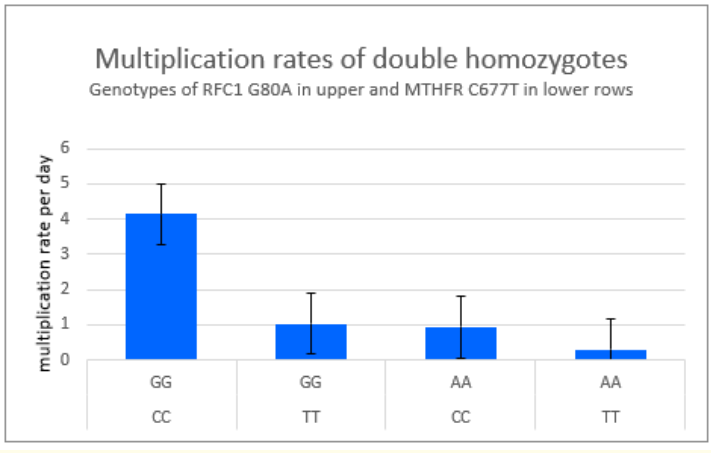

Figure 1: *Multiplication rates per day of double homozygotes of MTHFR C677T and RFC1 G80A polymorphisms. Left side: RFC1 80GG compared to MTHFR 677 CC and TT. Right side: RFC1 80AA compared to MTHFR 677 CC and TT. Means ± SE for triplicate cultures are shown.*

Combined heterozygous genotypes

Interestingly, all combinations of heterozygotes showed low multiplication rates. Just one mutated allele of a heterozygous gene combined with a wild-type homozygous gene in a pair had a dominant lowering effect on the multiplication rate of hPDLSC, like in combination of MTHFR 677CC (wild-type homozygote) with RFC1 G80A (heterozygote) or in combination of MTHFR C677T (heterozygote) with RFC1 80GG (wild-type homozygote) (Figure 2).

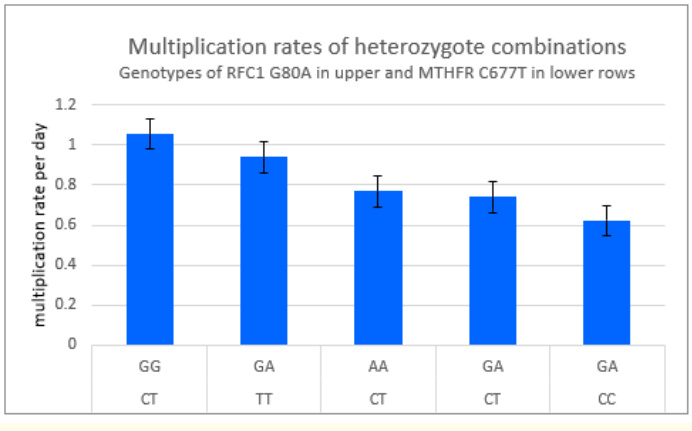

Figure 2: *Combinations of all heterozygote genotypes of RFC1 G80A and MTHFR C677T polymorphisms showing corresponding hPDLSC multiplication rates per day. Means ± SE for triplicate cultures are shown.*

Overview of all combinations of genotypes

All multiplication rates were arranged in decreasing order in figure 3. We found the maximal multiplication rate in the combination of wild-type homozygous genes (4.1-fold). Multiplication rates were lowered to 1.0 or less if one or two heterozygous genes were combined in a pair of RFC1 G80A and MTHFR C677T gene polymorphisms. Minimal multiplication rate was found in the combination of mutated-allele homozygotes (0.295-fold). Genotype combinations of the RFC1 G80A and MTHFR C677T polymorphisms tightly controlled the multiplication rate of hPDLSCs.

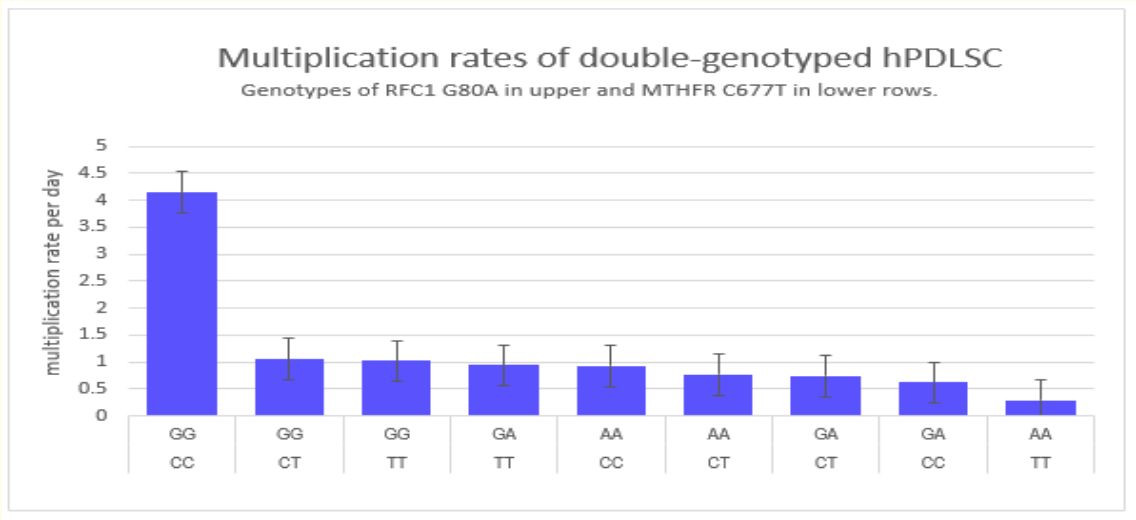

Figure 3: *Comparison of all hPDLSC multiplication rates per day with respect to their RFC1 G80A and MTHFR C677T specific combinations of double genotypes. Means ± SE for triplicate cultures are shown. The difference between the RFC1 80GG/MTHFR 677CC and all other genotype combinations was highly significant ($p < 0.05$).*

Discussion

The MTHFR C677T and RFC1 G80A polymorphisms differentially affected the multiplication rate of hPDLSCs in a genotype-dependent manner. As our studies continue, the number of patients will be increased to quantify individual variations in proliferative response across a larger cohort.

MTHFR 677TT homozygotes with spina bifida had MTHFR enzyme activity decreased by 60% below normal [24]. Golja., *et al*. (2020) [19] measured intracellular concentration of 5-methyl-tetrahydrofolate (5mTHF, active folate) in a lymphoblastoid cell line. Folate supplementation increased the concentration of 5mTHF in cells with normal MTHFR activity, but there was no increase in 5mTHF in cells with low MTHFR activity. The RFC1 80AA genotype was linked to a decrease of folate uptake by about 30% [25]. These data, taken together, correspond well with the reduction in the multiplication rate of hPDLSC from 100% of the MTHFR 677CC/RFC1 80GG wild-type rate to 7% of the double-mutant genotype, MTHFR 677TT/RFC1 80AA.

When both RFC1 G80A and MTHFR C677T genes had only wild-type alleles, the multiplication rate was maximal (4.1-fold). When one gene with both wild-type alleles was combined with another gene with both mutated alleles, the multiplication rate was significantly lowered. Even one mutant allele in a constellation of all other wild-type alleles in combined genotypes resulted in the low multiplication

rate of hPDLSC (1.0 or less). When both genes had only mutated alleles, the multiplication rate was minimal (0.3). Active folate (5mTHF) generated by MTHFR is essential for the multiplication of cells [1]. In folate cycle metabolism, RFC1 controls the cellular uptake of folate, and MTHFR controls the production of intracellular active folate. If any of those two were compromised, the active folate level declined, and the multiplication rate declined as well. Combined MTHFRC677T/RFC1 G80A polymorphisms had a discriminative effect on the multiplication rate of hPDLSC even when external folic acid was sufficiently supplied by alpha MEM medium. It means that intracellular active folate levels were tightly controlled by RFC1 G80A and MTHFR C677T genotypes, and that hPDLSC multiplication rates corresponded to intracellular active folate levels.

Adequate cell proliferation is essential for the migration of sufficient neural crest cells for normal facial development during embryogenesis [8,11]. It is intriguing that the risk of NSCL ± P depends on MTHFR 677CT genotypes in the reverse order of our hPDLSC multiplication rates: the lowest-to-highest risk for NSCL ± P is CC < CT < TT MTHFR allele combinations [8], whereas our results showed the highest-to-lowest multiplication rates for CC > CT > TT MTHFR allele combinations. For example, for MTHFR 677TT, the highest risk of NSCL ± P would correspond to the lowest hPDLSC multiplication rate. A sufficient rate of growth of facial processes is needed to reach each other within the limited time of a developmental window; otherwise, a cleft would arise. For prevention through periconceptional folate supplementation, it is important to diagnose the MTHFR C677T and RFC1 G80A genotypes in the mother and child.

Patients who donated their teeth did not have NSCL ± P; however, the studied polymorphisms were associated with different proliferation rates in hPDLSCs. The etiology of NSCL ± P is multifactorial, with both genetic and environmental factors contributing in complex ways [26]. The environmental factors contributing to the etiology of NSCL ± P were missing in our hPDLSC cell culture system. Our results on hPDLSC multiplication rates indicate a genetic risk for NSCL ± P associated with the RFC1 G80A and MTHFR C677T genotypes.

In summary, intracellular folate availability was the predictor variable, and the proliferation rate of hPDLSCs was the outcome variable in our cell culture model. The availability of intracellular folate was controlled by the combined genotypes of the MTHFR C677T and RFC1 G80A polymorphisms. We measured the multiplication rate of hPDLSC as their physiological response. This system is natural; no gene manipulation was needed. We used primary human stem cells that are developmentally related to neural crest cells. Future studies will focus on the role of folate metabolism in the pathogenesis of NSCL ± P and other disorders caused by a deficiency of intracellular active folate.

Conclusion

We have introduced primary human periodontal ligament stem cells (hPDLSCs) as a cell culture model to study the effects of folate on their cell proliferation and other physiological functions.

Combined MTHFR C677T and RFC1 G80A polymorphisms differentially affected hPDLSC proliferation in

genotype-dependent manner.

This human primary cell culture model can be utilized in future studies on metabolic disturbances caused by folate deficiency. With a mechanistic understanding of specific genetic influences at the cellular level, we can move closer to personalized prevention for patients at risk of NSCL ± P and other disorders related to intracellular active-folate deficiency.

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Conflict of Interest

The authors declare that no financial interests or conflicts of interest exist.

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