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Reactivation of *SNURF-SNRPN* gene by DNA Methyltransferase inhibitors in a Prader-Willi lymphoblastoid cell line

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INTRODUCTION

Epigenetic modifications, like the methylation of DNA, play an important role in the interpretation of genetic information. DNA methylation is a reversible process that regulates both gene expression and chromatin organization. There are several examples of different DNA methylation patterns in pathologic conditions such as imprinting disorders and cancer (1).

DNA methylation is an epigenetic event in which the addition of a methyl group to the fifth carbon position of a cytosine residue (m^5C) occurs frequently in CpG dinucleotides. This process is closely associated with modifications of chromatin structure at gene promoter regions and it plays an important role in regulating gene expression (2). In cancer cells, dysregulation DNA methylation may lead to hypermethylation of promoter CpG islands, inhibiting the transcriptional initiation of controlled genes (3). Methylation of CpG dinucleotides is known to be mediated by at least three DNMTs (DNA Methyltransferases), including DNMT3a, DNMT3b, and DNMT1. Two of these enzymes, DNMT3a and DNMT3b, are thought to be responsible for an initial setup of methylation patterns during the development. Because the process of establishing methylation patterns is critical during early development, these methyltransferases are highly expressed in embryonic cells but are present at lower levels also in adult cells. Instead, DNMT1 is constitutively expressed in proliferating cells and it functions as a maintenance enzyme to ensure that the methylation patterns are faithfully copied to daughter cells during DNA replication (4).

The possibility to reverse epigenetic modifications has generated considerable interest in the development of DNA methyltransferase inhibitors (5). The identification and the development of small molecules that block the active sites of human DNA methyltransferases represents a new class of epigenetic modifier compounds (3).

The inhibition of DNMT, especially DNMT1, would block the hypermethylation of the new synthesized DNA filament with the result of a reversion of the hypermethylation and possibly re-expression of silenced genes (3).

Generally, drugs used to achieve this aim can be divided into two subgroups: nucleoside and non-nucleoside inhibitors (6). The first group consists of Azacitidine (5-Azacytidine), Decitabine (5-Aza-2'-deoxycytidine) and Zebularine [1-(b-D-ribofuranosyl)-2(1H)-pyrimidinone]. They are established drugs whose function is that of suicide inhibitors after their incorporation into DNA. The second group contains EGCG [(-)-epigallocatechin-3-gallate], pro-EGCGs (Triolanalog and Diolanalog of EGCG) and RG108 [2-(1,3-dioxo-1,3-dihydro-2H-isoindol-2-yl)-3-(1H-indol-3-yl) propanoic acid] that are considered three compounds whose function is the inhibition of DNA by interfering with enzyme activity (6).

It is worth to note that Azacitidine and Decitabine have been approved by the U.S. Food and Drug Administration (FDA) for treatment of Mieloid Displastic Syndrome (MSD) (7,8).

Moreover, it had been showed in nude mice that Zebularine can be orally administered causing demethylation and reactivation of a silenced and hypermethylated *p16* gene in human bladder tumor cells (9). One of the major problem with the use of inhibitors of DNA methylation is the re-methylation and re-silencing of genes at the end of the treatment. That makes the clinical application of these drugs quite limited. To overcame these limits it could be ideal a drugs that could be administered continuously without toxic effects. Interestingly, in (9) Zebularine shown high stability *in vitro* and minimal cytotoxicity also during long time treatments.

These observation suggests possible therapeutic strategies and clinical benefits in the continuous application of Zebularine (9).

Probably, Prader-Willi Syndrome (PWS) represents the best example of genomic imprinting diseases in humans (10). At the present time all genetic and epigenetic modifications, which cause PWS lead to a loss of expression of the genes that are

usually expressed by paternal chromosome on 15q11.2-q13. The absence of the paternal copy of these genes or the failure in their expression can cause the total absence of these genes expression in affected individuals because genes on maternal chromosome are genetically programmed to be silenced by epigenetic factors (11).
This lack of expression occurs by three primary mechanisms: deletion of a 5–6 Mb region from the paternal chromosome 15 (found in 65–75% of affected individuals), maternal UniParental Disomy 15 (UPD) (found in 20–30%), and a defect in the genomic region that controls the imprinting process called Imprinting Center (IC) (1–5%) (11).

The major clinical manifestations of PWS include hypotonia with weak suck and poor feeding in infancy leading to failure-to-thrive, and later development of hyperfagia. Other clinical features include developmental delay, cognitive disability, and behavioral problems: stubborness, obsessive-compulsive behaviors, and skin picking. The major endocrine manifestation of PWS are: high level of hormone ghrelin, deficiency of growth hormone (GH), central adrenal insufficiency, hipogonadism, hypothyroidism, diabetes and altered glucose metabolism (12).

AIMS OF THE RESEARCH

The vast majority of Prader-Willi patients have a hypermethilated state of *SNURF-SNRPN* gene promoter, that seems to be the causative agent of the syndrome. We have hypothesize that DNA methiltransferases might be used to demethylate the promoter and reactivate the genes controlled by it.

RESULTS

To test this hypothesis, lymphoblastoid cell line (DMI) derived from EBV-immortalized lymphocytes from a Prader-Willi patient with defect of imprinting were used.

We tested their time and dose effect on DMI cells as demethylating agents. Specifically, to test their ability to reactivate the expression of genes under the control of the Imprinting Center, we tested the expression of *SNURF-SNRPN* gene. In detailes, we explored the effect of DNMT inhibitors on *SNURF-SNRPN* gene expression through end-point duplex PCR and subsequently by TaqMan Real Time assay. Our study demonstrates that silenced *SNURF-SNRPN* gene can be reactivated by DNMT inhibitors treatments.

Moreover we investigated the global demethylation effect of DNMT inhibitors noting an overall low demethylating effect. The expression of DMNT1 gene, evaluated by RT-PCR, was not different between controls and treated samples at different drugs concentrations and different time-points.

These results show that the use of those drugs may be useful to reverse aberrant DNA methylation, restoring critical gene functions, and thereby might be used to treat imprinting disorders such as Prader-Willi Syndrome.

These effects occur in a context of minimal cellular toxicity, low global genomic hypomethylation, while maintaining the expression of the DNMT1.

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