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173

Sirolimus-mediated induction of fetal hemoglobin in beta-thalassemia: Impact of the XmnI rs74482144 polymorphism

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The *in vivo* effects of sirolimus on the induction of fetal hemoglobin (HbF) is of key importance for therapeutic protocols in a variety of hemoglobinopathies, including β-thalassemia and sickle-cell disease (SCD). We have previously reported the strong inducing effect of the mTOR inhibitors rapamycin (sirolimus) and everolimus on HbF production by erythroid precursors (ErPCs) from β-thalassemia patients. We took advantage from the availability of a β-thalassemia cellular bio-bank allowing stratification of the patients with respect to fetal hemoglobin production and response to HbF inducers. The results obtained by HPLC analysis of the ErPCs cultures from 38 patients led to the following conclusions: (a) sirolimus increases HbF in cultures from β-thalassemia patients with different basal HbF levels (the cultures from 51.4% of the patients were responsive to sirolimus treatment); (b) the cultures from 37.8% of the patients were not responsive to sirolimus or hydroxyurea (HU); (c) sirolimus was able to induce HbF in 46.15% of the cultures not responsive to HU; (d) sirolimus displayed higher efficiency than HU in 57.14% of the cultures responsive to both sirolimus and HU; (e) 42.86% of HU-treated cultures displayed HbF induction higher than sirolimus. In order to study a possible association between DNA polymorphisms and sirolimus-mediated HbF induction, the γ-globin *Xmn*I (rs74482144), two BCL11A (rs1427407 and rs10189857) and the HS1L-cMYB rs9399137 polymorphisms were analyzed. Both homozygous *Xmn*I (T/T,+/+) and heterozygous *Xmn*I (T/C,+/-) cultures (from 11 patients, 29.9% of the 38 patients analyzed) had high sirolimus-mediated HbF induction. The BCL11A rs1427407 G>T polymorphism was not associated with high HbF induction. Concerning the HS1L-cMYB T>C rs9399137 and the BCL11A A>G rs10189857 polymorphisms, an association with sirolimus-mediated HbF induction was found, but only in the homozygous patients (3/38 and 7/38, respectively). These results indicate that the γ-globin *Xmn*I rs74482144 should be considered a very useful polymorphism for recruitment of β-thalassemia patients in sirolimus-based clinical trials.

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174

Non-invasive prenatal detection of beta-thalassemia mutations in maternal plasma using Droplet Digital PCR

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Non-invasive prenatal testing (NIPT) is based on fetal DNA analysis with the aim to identify genetic abnormalities from the maternal plasma during pregnancy[(1)]. Actually, commercial NIPT kits can detect only aneuploidies, small deletions or insertions but not single point mutations causing genetic diseases. In this study, we have developed two genotyping assays, based on innovative and sensitive droplet digital PCR (ddPCR) technology[(2)] to identify the two most common thalassemia mutations in the Mediterranean population ($\beta^0$39 and β^+IVSI-110) maternally and/or paternally inherited on fetal DNA. First, the two genotyping assays were optimized and validated, in terms of amplification efficiency and hybridization specificity, using mixtures of two genomic DNAs carrying different genotypes and percentages to simulate fetal and maternal circulating cell-free DNA (ccfDNA) at different gestational weeks. Then the two ddPCR assays were applied to determine the fetal genotype from 36 maternal blood samples at different gestational ages. The diagnostic outcomes were confirmed for all the samples carrying paternally inherited mutation by DNA sequencing. In the case of maternally or both parents inheriting the mutation a precise dosage of normal and mutated alleles was required to determine the fetal genotype. In particular, we identified two diagnostic ranges for allelic ratio values that were statistically distinct and not overlapping, allowing the corrected fetal genotype determination in all the samples analyzed. In conclusion, we have developed a simple and sensitive diagnostic approach, based on ddPCR, for non-invasive pre-natal determination of β^+IVSI-110 and $\beta^0$39 mutations paternally and maternally inherited suggesting its application also for other single point mutations causing monogenic diseases.

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175

Identification of dysregulated miRNAs in liquid biopsies from colorectal cancer (CRC) patients: Impact on personalized miRNA therapeutics

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MicroRNAs (miRNAs) are small non-coding RNAs regulating gene expression by the sequence-selective targeting of mRNAs, leading to translational repression or mRNA degradation. In cancer, miRNAs are associated with tumor onset and progression. Liquid biopsy of cancer is based on the analysis of circulating tumor cells and/or cell-free nucleic acids (including miRNAs) in peripheral blood of cancer patients and it is considered one of the most advanced non-invasive diagnostic systems suitable for early diagnosis, staging, prognosis, prediction of therapy responses, therapy outcome, and follow-up during therapeutic intervention (1). We performed NGS of plasma isolated from 35 colorectal carcinoma (CRC) patients and identified a short-list of 12 dysregulated miRNAs, including miR-221, miR-222 and miR-141. These data were further validated by droplet digital RT-PCR (dd-RT-PCR). Despite the fact that patient-to-patient heterogeneity was found, this study provides a list of novel potential targets for the development of therapeutic protocols for CRC. The association between the miRNAs expressed in tumors and their plasma content was validated in experimental mice xenografted with tumor cell lines derived from CRC patients (2). For miRNA targeting, peptide-nucleic acids (PNAs), DNA analogues in which the sugar-phosphate backbone has been replaced by N-(2-aminoethyl)glycine units, are excellent tools. We developed novel delivery strategies for PNAs targeting miRNAs, based on i) the use of PNAs linked to a poly-arginine R8 peptide tail; ii) nanoparticles; and iii) novel molecules constituted by a macrocyclic multivalent tetraargininocalix[4]arene to be used as non-covalent vector for anti-miRNA PNAs (3). As far as the validation of PNA activity, we focused on PNAs targeting miR-221, miR-222 and miR-155 in glioblastoma cells. Increased pro-apoptotic effects were obtained with the co-administration of these PNAs. In addition, synergistic effects were found with the co-administration of corilagin and a PNA targeting miR-221. Finally, the combined treatment of glioma U251 cells with the pro-apoptotic pre-miR-124 and the PNA targeting miR-221, led to the induction of apoptosis at very high levels. In conclusion, the liquid biopsy approach may be considered the basis for a personalized miRNA based therapy of cancer, and PNAs may be a relevant therapeutic tool for the inhibition of oncomiRNAs, also in combination with miRNA replacement molecules mimicking tumor-suppressor miRNAs.

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176

Downregulation of LYAR is associated with induction of fetal hemoglobin in mithramycin-treated erythroid cells

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LYAR (Ly-1 antibody reactive clone) protein is a recently identified repressor of Aγ-globin gene transcription in erythroid cells and binds to the 5'-GGTTAT-3' sequence of the 5'-region of the gene. The rs368698783 G>A polymorphism of this site is present in β-thalassemia patients and is associated with a high production of fetal hemoglobin (HbF). The finding that LYAR binds less efficiently to the G>A mutated 5'-GGTTAT-3' binding site might explain the increased basal and induced levels of HbF in erythroid cells. The present study was undertaken to verify the effects of the HbF inducer mithramycin (MTH) on LYAR. We first determined whether MTH was able to inhibit the LYAR/DNA interactions using both nuclear factors from K562 cells and recombinant LYAR protein. Electrophoretic mobility shift assay demonstrated that MTH strongly interfered with the binding of LYAR to the double-stranded oligonucleotides containing the Aγ-globin LYAR binding site. We also performed RT-qPCR and western blot analysis of MTH-treated cells demonstrating an association between LYAR mRNA and Aγ-globin gene expression. LYAR expression (analyzed at mRNA and at the protein level) was downregulated in association with the upregulation of γ-globin gene expression and HbF production. LYAR downregulation was confirmed in MTH-treated K562 cells, as well as in erythroid precursor cells (ErPCs) from β-thalassemia patients. In the analyzed ErPCs those which were found as non-responders to MTH demonstrated unchanged LYAR content. In order to verify possible MTH-dependent effects on LYAR transcription, the LYAR promoter was studied and at least five Sp1 CG-rich binding sites identified. EMSA was performed using double-stranded oligonucleotides mimicking these binding sites and the effects of MTH addition were determined, demonstrating that MTH is able to inhibit the interactions between Sp1 and the Sp1 binding sites present within the LYAR promoter. In conclusion, downregulation of LYAR expression and functions might strongly contribute to induction of Aγ-globin gene expression and HbF production in erythroid cells.

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