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G/A polymorphism in intronic sequence affects the processing of MAO-B gene in patients with Parkinson disease

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ABSTRACT

Monoamine oxidase B (MAO-B) plays an important role in the metabolism of neuroactive and vasoactive amines in the central nervous system and peripheral tissues. Increased levels of MAO-B mRNA and enzymatic activity have been reported in platelets from patients with Parkinson's and Alzheimer's diseases, however the triggers of enhanced mRNA levels are unknown. Our results demonstrate for the first time that G/A dimorphism in intron 13 sequence creates splicing enhancer thus stimulating intron 13 removal efficiency. The increased MAO-B protein levels might serve as a surrogate marker for – Parkinson disease.

1. Introduction

Parkinson's disease, the second most common neurodegenerative disease, is associated with selective degeneration of nigrostriatal dopamine neurons, which results in a symptom complex consisting of tremor, rigidity and slowed movements. About 10% of Parkinson's disease cases are caused by a mutation in one of several different genes, most are thought to result from a combination of variable environmental exposures superimposed on an individual's composite genetic susceptibility [1]. It is well established that MAO-B inhibitors delay progression of Alzheimer's and Parkinson's diseases [2,3].

Monoamine oxidase (MAO) exists in two functional forms: MAO-A and MAO-B. The genes of both protein isoforms possess identical exon–intron organization, but have different substrate and inhibitor specificities and are encoded by separate genes located tail-to-tail on the X chromosome. While MAO-A preferentially degrades serotonine, norepinephrine and is selectively inhibited by clorgyline, the MAO-B is more efficient in metabolizing phenylethylamine and benzylamine and is selectively inhibited by deprenyl [4]. Both enzymes are present in various tissues throughout the body with the highest levels of expression in liver and brain. However, some cell types predominantly express one type of MAO: placenta cells express mainly MAO-A, while platelets and lymphocytes cells posses only MAO-B activity [4,5]. The ability of monoamine oxidases to metabolize neurotransmitters suggests their role in the etiology of human behavioral traits and neuropsychiatric disorders.

Several DNA polymorphisms in the MAO-B gene have been described. Seven out of ten single-nucleotide polymorphisms (SNP) in the MAO-B gene have been identified in African-American or/and Native-American populations but were not detected in Caucasians and Asians. The only SNP found in all human populations, that is the G/A dimorphism in intron 13 sequence, located 36 b.p. upstream from the intron 13–exon 14 boundary. The "A" allele was previously associated with an approximately two fold risk of Parkinson disease [6–8].

In the present study we have examined the impact of A/G dimorphism in MAO-B gene intron 13 sequence for pre-mRNA splicing. Our studies revealed that A/G dimorphism in a non-coding sequence of MAO-B gene in platelets regulates MAO-B mRNA formation and MAO-B protein expression levels and could serve as disease marker.

2. Materials and methods

2.1. Pre-mRNA substrates

DNA constructs were made from the MAO-B gene (spanning exon 13-intron 13-exon 14) by PCR using appropriate primer pairs:

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P1–P2 – constructs 5′ fragment (P1: 5′-d(AAGGAGAGCTCGGATTTA CTTTGCAGGCACC)-3′; P2: 5′-d(TTCCTAAGCT TCTGGAGAGTTGGTCT CCAGG)-3′) and P3–P4 – constructs 3′ fragment (P3: 5′-d(TTCC TCTCGAGCAGACTCTGGTTCTGACTGC)-3′ P4: 5′-d(AAGGAAAGCTTT TATACCA CAGGAGAAAGACC)-3′. PCR fragments ($MaoB_a$ and $MaoB_g$) through *Sac*I/*Hind*III and *Hind*III/*Xho*I restriction endonuclease sites respectively were cloned into pBluescript II KS (+) plasmid DNA (Thermo Fisher scientific).

2.2. T7 transcription and pre-mRNA splicing in vitro

Labeled pre-mRNAs from the plasmids were generated in an in vitro transcription reaction using T7 RNA polymerase and [^{32}P]CTP. Transcription reaction products were purified. crossBriefly, splicing reactions (25 μl) contained: ≈10 fmol in vitro transcribed, capped and [$_{32}$P]-labeled pre-mRNA, 20% nuclear extract (CilBiotech), 2.6% (w/v) polyvinyl alcohol, 2.8 mM $MgCl_2$, 2 mM ATP, 20 mM phosphocreatine and buffer D (20% (w/v) glycerol, 20 mM HEPE pH 7.9, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.3 mM PMSF). Reactions were incubated 0.5–2 h at 30 °C followed by proteinase K digestion (40 μg) (Thermo Fisher scientific) for 30 min at 37 °C, extracted with phenol–chloroform and ethanol precipitated. Pre-mRNA splicing reaction products were resolved on 8% denaturing polyacrylamide gels.

2.3. Splicesome assembly

Splicesome assembly performed under splicing conditions as described above. Reactions were incubated at 30 °C either for 0 or 20 min. Complexes were separated on 1,2 % low melting agarose gels [9].

2.4. UV crosslinking and "fishing out" bound proteins

UV crosslinking was performed as earlier described [10]. HeLa-NE (100 μg proteins) were incubated with 25–40 fmol [$_{32}$P] and biotin double labeled RNA in splicing buffer lacking creatinine phosphate (25 μl total volume), exposed to 254 nm for 15 min on ice. Bound to pre-mRNA proteins separated from unbound proteins using streptavidin beads (Invitrogen), digested with 10 μg RNase A (Thermo Fisher scientific) at 30 °C for 30 min., separated on a 10% SDS–polyacrylamide gel and transferred to the membrane. Membrane incubated with anti-SR antibodies (Santa Cruz) washed, and incubated with secondary antibody (Dako). Membrane developed using ECL reagent (GE Healthcare).

2.5. Preparation and analysis of the blood samples

Blood samples from healthy control individuals and patients with Parkinson's disease were fractionated using Ficoll gradient centrifugation. The upper (platelet) fraction was collected. The proteins (20 μg/per lane) were separated on a 10% SDS–polyacrylamide gel, transferred to the nitrocellulose membrane and detected using anti-Mao-B antibody (Santa Cruz). DNA from platelet fraction was isolated as described [9]. For MAO-B intron 13 sequence amplification P3–P4 primer pair was used.

3. Results

3.1. MAO-B protein levels are enhanced in platelet fraction of Parkinsonian patients

Earlier it has been demonstrated that enzymatic activity of MAO-B is increased in platelet fraction of parkinsonian patients [11,12], but the occasion, until this time, of this increase is not understood. We have compared MAO-B protein expression levels in the platelet fraction, obtained from blood of healthy persons and from patients with Parkinson disease. Data presented in Fig. 1A,B show elevated levels of MAO-B protein expression in PD patient platelets fraction when compared to healthy individuals. The elevated MAO-B protein expression levels were detected in all eight tested PD patient blood samples. This indicates that not only the enzymatic activity and mRNA levels of MAO-B is increased in the platelet fraction of Parkinsonian patients but that MAO-B protein levels are also significantly upregulated. The isolated platelet DNA sequencing data from the same patients showed the association of A-allele with the enhanced MAO-B expression levels (supplemental Fig. 1).

3.2. SNP in MAO-B gene intron 13 sequence influences intron removal efficiency

Dimorphism of MAO-B gene in intron 13 sequence does not cause changes in the coding sequence, since it is located within an intron, and does not affect the consensus acceptor and donor sites [13]. In order to investigate the influence of G/A dimorphism in MAO-B gene intron 13 for pre-mRNA splicing, we have created constructs containing either "G" allele ($MaoB_g$) or "A" allele ($MaoB_a$) intronic sequences (Fig. 1C). From these constructs, the transcription reactions in vitro were performed and the transcribed pre-mRNAs were tested in the in vitro splicing assays using nuclear extracts prepared from HeLa cells (HeLa-NE). Splicing assays revealed the enhanced efficiency of $MaoB_a$ pre-mRNA splicing when compared to $MaoB_g$ construct splicing. The intron 13 containing the "A" allele is removed more efficiently than "G" allele (Fig. 1D and E).

To elucidate whether one nucleotide change in the intronic sequence, close to 3′ splice site is sufficient to influence pre-mRNA splicing efficiency, we have constructed chimeric β globin constructs where intronic 3′ splice site sequence (69 nt.) of β-globin pre-mRNA substituted with MAO-B intron 13 sequences, containing G or A alleles (β-gl/$MaoB_g$ and β-gl/$MaoB_a$ constructs, respectively) (Fig. 2A). These chimeric constructs were tested in the in vitro splicing assays. Splicing results illustrate that β-gl/$MaoB_a$ construct was also spliced more efficiently than β-gl/$MaoB_g$ construct (Fig. 2B and C). Collectively, the obtained results indicate that a single nucleotide dimorphism in a non-coding sequence may greatly influence intron removal efficiency.

3.3. G/A dimorphism in intron 13 sequence does not affect branch point site

It has been previously shown that changes from G nucleotide to A in the intronic sequence close to 3′ splice site can influence branch point location during splicesome formation [14]. To establish whether this dimorphism affects branching point location during splicesome formation, we used bioinformatics analysis as described in [15]. The results of this analysis revealed that the G/A dimorphism in intron 13 sequence does not affect branch point site as shown in the supplemental material Fig. S2.

3.4. "A" allele in intron 13 sequence increases the efficiency of early-stage splicesomal complex assembly

To study at which stage of the splicesome formation the efficiency of intron removal is affected, we used a splicesome assembly assay by performing SDS–PAGE electrophoresis under non-denaturing conditions. Our results demonstrate that splicesome complex formation on $MaoB_a$ pre-mRNA transcript was more efficient as compared to the complex formation on the $MaoB_g$ transcript (Fig. 3A). Since under the tested conditions only the early-stage splicesome complexes can be detected, this finding indicates