Genetic screening of spinal muscular atrophy using a real-time modified COP-PCR technique with dried blood-spot DNA

MAWADDAH AR ROCHMAH

(Degree)
博士（医学）

(Date of Degree)
2018-09-25

(Resource Type)
doctoral thesis

(Report Number)
甲第7281号

(URL)
https://hdl.handle.net/20.500.14094/D1007281

学位論文の内容要旨

Genetic screening of spinal muscular atrophy using a real-time modified COP-PCR technique with dried blood-spot DNA

リアルタイム-競合的オリゴ核酸プライミングPCR改変技術を用いた、濾紙血DNAによる脊髄性筋萎縮症遺伝子スクリーニング

神戸大学大学院医学研究科医科学専攻
疫学
指導教員：上野 易弘 教授

MAWADDAH AR ROCHMAH

BACKGROUND

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the degeneration of lower motor neurons resulting in muscle weakness and progressive loss of movement. SMA is one of the most frequent genetic disorders with an incidence of 1/10,000 live births.

The survival of motor neuron gene (*SMN*) was identified as a candidate gene for SMA. *SMN* exists as two nearly identical copies, *SMN1* and *SMN2*. *SMN1* is recognized as an SMA-causing gene as it is homozygously deleted in more than 95% of SMA patients and is deleteriously mutated in the remaining patients. *SMN2* is considered to be an SMA-modifying gene as high copy numbers of *SMN2* may improve survival outcomes and maintenance of motor function in SMA patients.

SMN1 and *SMN2* differ by only five nucleotides. One of the five nucleotide differences is in the coding region at position +6 of exon 7; c.840C in *SMN1* and c.840T in *SMN2*. This translationally silent transition alters the mRNA splicing pattern. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% exon7-lacking (Δ7) *SMN2* transcripts and 10% FL-*SMN2* transcripts.

The most common problem for genetic diagnosis of SMA is the presence of *SMN2* hampers easy and quick detection of homozygous *SMN1* deletion. Here, we combined DNA extraction from DBS on filter paper (DBS-DNA), pre-amplification of target DNA using conventional PCR, and real-time mCOP-PCR to establish a simple but accurate *SMN1*-deletion detection system.

METHODS

Patient and control samples

A total of 88 individuals were enrolled in this study. The *SMN1* and *SMN2* gene profiles of all individuals had been previously analyzed by PCR-RFLP using DNA extracted from freshly collected blood.

DNA extraction from DBS on filter paper

Blood samples were collected and spotted onto filter paper FTA® Elute Cards. Storage periods of DBS samples on FTA® Elute Cards varied from 1 week to 5 years.

Genomic DNA was extracted from four 3-mm diameter circles of DBS with a TE buffer-boiling method. The concentration and absorbance ratio of the DNA were measured at 260/280 nm (OD 260/280 ratio) using a NanoDrop® ND-1000 spectrophotometer.

Targeted pre-amplification by conventional PCR

Targeted pre-amplification of the sequence containing *SMN1/2* exon 7 was performed by conventional PCR. The primers were: R111 (5′-AGA CTA TCA ACT TAA TTT CTG ATC A-3′) and 541C770 (5′- TAA GGA ATG TGA GCA CCT TCC TTC-3′).

Separation between *SMN1* and *SMN2* exon 7 by PCR-RFLP

To separate between *SMN1* and *SMN2*, we performed PCR-RFLP. The primers were: R111 and X7-Dra (5′ -CCT TCC TTC TTT TTG ATT TTG TTT- 3′). Then, the PCR products underwent overnight digestion at 37°C with DraI.

Gene-specific amplification of *SMN1* and *SMN2* exon 7 by real-time mCOP-PCR

SMN1/SMN2 specific amplification was performed by real-time PCR using the LightCycler® 96 system. The primer set for *SMN1*-specific amplification was: R111 and SMN1-COP (5′-TGT CTG AAA CC-3′). For *SMN2*-specific amplification, the primer set was R111 and SMN2-COP (5′-TTG TCT AAA ACC-3′).

RESULTS

DNA extraction from DBS

A total of 88 DBS samples were analyzed: 35 controls carrying *SMN1* and *SMN2*, 12 carriers carrying *SMN1* and *SMN2*, 4 controls carrying only *SMN1*, and 37 SMA patients carrying only *SMN2*. The storage periods of the DBS samples ranged from 1 week to 5 years. The DNA amount of each DBS was 23.0 ± 6.41 μg (mean ± SD), with an OD A260/280 ratio from 1.86 to 0.12 (mean ± SD). There was a significant relationship between the DNA amount and the storage periods (one-way ANOVA, $p<0.01$). The DNA of DBS stored for 5 years showed the lowest abundance.

Targeted pre-amplification by conventional PCR followed by PCR-RFLP

To ensure that the quantity and quality of DBS-DNA were not limiting factors for this technique, we pre-amplified the sequence region of *SMN1/2* containing intron 6 and exon 7 using conventional PCR, and used the pre-amplified product as template in the real-time mCOP-PCR. To test whether the pre-amplification affect the presence or absence of *SMN1*, we separated between *SMN1* and *SMN2* exon 7 by PCR-RFLP. Our findings indicated that the targeted pre-amplification did not affect the presence or absence of *SMN1* and *SMN2*.

Gene-specific amplification of *SMN1* and *SMN2* exon 7 by real-time mCOP-PCR

SMN1/SMN2 specific amplification was performed by real-time PCR using the LightCycler® 96 system. Our mCOP-PCR technology using real-time PCR accurately detected the presence or absence of *SMN1* and *SMN2*. The amplification was assessed from quantification cycle (Cq) values: a Cq value of less than 12 was the judged to indicate the presence of *SMN1* or *SMN2*. The results with the real-time mCOP-PCR were fully consistent with those of PCR-RFLP. Moreover, the real-time mCOP-PCR required shorter time to obtain the results in comparison to PCR-RFLP.

Real-time mCOP-PCR with DBS-DNA vs. PCR-RFLP with DNA from freshly collected blood

Compared with PCR-RFLP using DNA from freshly collected blood, results from real-time mCOP-PCR using DBS-DNA for detection of *SMN1* exon 7 deletion showed a sensitivity of 1.00 (CI [0.88, 1.00])] and specificity of 1.00 (CI [0.91, 1.00]), respectively. The sensitivity and specificity of real-time mCOP-PCR using DBS-DNA for detection of *SMN2* exon 7 deletion were also 1.00 (CI [0.40, 1.00])] and 1.00 (CI [0.95, 1.00]), respectively.

The results of Real-time mCOP-PCR using DBS-DNA completely matched those of PCR-RFLP with DBS-DNA and from freshly collected blood. These findings suggest that mCOP-PCR using DBS-DNA can be applied for accurate diagnostic screening of SMA patients.

DISCUSSION

We have established a genetic screening system of spinal muscular atrophy. Three characteristic features of our system are DNA extraction from DBS, pre-

amplification of the target sequences and mCOP-PCR technology. Our method of DNA extraction from DBS was very simple; boiling procedure eluted DNA from the card into the TE buffer.

Pre-amplification of the target sequences including *SMN1* / *SMN2* gene-specific nucleotides may overcome the qualitative and/or quantitative problem of DBS-DNA, eliminate PCR-inhibiting components from filter paper on which blood is spotted, and facilitate gene-specific annealing of mCOP-PCR primers.

In mCOP-PCR in our system, *SMN1*-specific oligonucleotides predominantly bind to *SMN1* sequence and *SMN2*-specific oligonucleotides predominantly bind to *SMN2* sequence. Here, we used the threshold (cut-off) Cq values of 12. The cycle number of 12 was enough to confirm the specific amplification with matched primers, and also enough to avoid non-specific amplification with mismatched primers. However, the threshold (cut-off) Cq value would be changed with different PCR conditions including different machines, different primer concentrations, etc.

SMA has been thought to be an incurable disease. However, in 2016, clinical trial results of intrathecal administration of an antisense oligonucleotide drug, nusinersen (SPINRAZA™, Biogen and Ionis Pharmaceuticals), demonstrated acceptable safety and encouraging clinical efficacy. The development of a successful treatment for SMA leads to the social requirements of early diagnosis of SMA. A newborn screening system for SMA may be essential for early diagnosis of the disease.

CONCLUSION

We combined DNA extraction from DBS on filter paper, pre-amplification of target DNA, and real-time mCOP-PCR to specifically detect *SMN1* and *SMN2* genes, thereby establishing a rapid, accurate, and high-throughput system for detecting *SMN1*-deletion with practical applications for newborn screening.

神戸大学大学院医学(系)研究科（博士課程）

論文審査の結果の要旨			
受付番号	甲 第2803号	氏　名	MAWADDAH AR ROCHMAH
論文題目 Title of Dissertation	Genetic screening of spinal muscular atrophy using a real-time modified COP-PCR technique with dried blood-spot DNA リアルタイム-競合的オリゴ核酸プライミング PCR 改変技術を用いた、濾紙血 DNA による脊髄性筋萎縮症遺伝子スクリーニング		
審査委員 Examiner	主　査 Chief Examiner 林 祥剛 副　査 Vice-examiner 辛田 建一 副　査 Vice-examiner 河野 誠司		

（要旨は1，０００字～2，０００字程度）

要旨

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the degeneration of lower motor neurons resulting in muscle weakness and progressive loss of movement. SMA is one of the most frequent genetic disorders with an incidence of 1/10,000 live births. The survival of motor neuron gene (*SMN*) was identified as a gene responsible for SMA. *SMN* exists as two nearly identical copies, *SMN1* and *SMN2*. *SMN1* is recognized as an SMA-causing gene as it is homozygously deleted in more than 95% of SMA patients and is deleteriously mutated in the remaining patients. *SMN2* is considered to be an SMA-modifying gene as high copy numbers of *SMN2* may improve survival outcomes and maintenance of motor function in SMA patients. *SMN1* and *SMN2* differ by only five nucleotides. One of the five nucleotide differences is in the coding region at position +6 of exon 7; c.840C in *SMN1* and c.840T in *SMN2*. This translationally silent transition alters the mRNA splicing pattern. *SMN1* exclusively produces full-length (FL) *SMN1* transcripts, while *SMN2* produces ~90% exon7-lacking (Δ7) *SMN2* transcripts and 10% FL-*SMN2* transcripts. The most common problem for genetic diagnosis of SMA is the presence of *SMN2* hampers easy and quick detection of homozygous *SMN1* deletion. Here, the candiate combined DNA extraction from DBS on filter paper (DBS-DNA), pre-amplification of target DNA using conventional PCR, and real-time mCOP-PCR to establish a simple but accurate *SMN1*-deletion detection system.

In this study, a total of 88 DBS samples were analyzed: 35 controls carrying *SMN1* and *SMN2*, 12 carriers carrying *SMN1* and *SMN2*, 4 controls carrying only *SMN1*, and 37 SMA patients carrying only *SMN2*. The storage periods of the DBS samples ranged from 1 week to 5 years. The DNA amount of each DBS was 23.0 ± 6.41 μg (mean ± SD), with an OD A260/280 ratio from 1.86 to 0.12 (mean ± SD). There was a significant relationship between the DNA amount and the storage periods (one-way ANOVA, $p<0.01$). The DNA of DBS stored for 5 years showed the lowest

abundance. To ensure that the quantity and quality of DBS-DNA were not limiting factors for this technique, the candidate and her collaborative researchers pre-amplified the sequence region of *SMN1/2* containing intron 6 and exon 7 using conventional PCR, and used the pre-amplified product as template in the real-time mCOP-PCR. To test whether the pre-amplification affect the presence or absence of *SMN1*, they separated between *SMN1* and *SMN2* exon 7 by PCR-RFLP. their findings indicated that the targeted pre-amplification did not affect the presence or absence of *SMN1* and *SMN2*. *SMN1/SMN2* specific amplification was performed by real-time PCR using the LightCycler® 96 system. Their mCOP-PCR technology using real-time PCR accurately detected the presence or absence of *SMN1* and *SMN*2. The amplification was assessed from quantification cycle (Cq) values: a Cq value of less than 12 was the judged to indicate the presence of *SMN1* or *SMN2*. The results with the real-time mCOP-PCR were fully consistent with those of PCR-RFLP. Moreover, the real-time mCOP-PCR required shorter time to obtain the results in comparison to PCR-RFLP. Compared with PCR-RFLP using DNA from freshly collected blood, results from real-time mCOP-PCR using DBS-DNA for detection of *SMN1* exon 7 deletion showed a sensitivity of 1.00 (CI [0.88, 1.00])] and specificity of 1.00 (CI [0.91, 1.00]), respectively. The sensitivity and specificity of real-time mCOP-PCR using DBS-DNA for detection of *SMN2* exon 7 deletion were also 1.00 (CI [0.40, 1.00])] and 1.00 (CI [0.95, 1.00]), respectively. The results of Real-time mCOP-PCR using DBS-DNA completely matched those of PCR-RFLP with DBS-DNA and from freshly collected blood. These findings suggest that mCOP-PCR using DBS-DNA can be applied for accurate diagnostic screening of SMA patients. They established a genetic screening system of spinal muscular atrophy. Three characteristic features of our system are DNA extraction from DBS, pre-amplification of the target sequences and mCOP-PCR technology. Their method of DNA extraction from DBS was very simple; boiling procedure eluted DNA from the card into the TE buffer. Pre-amplification of the target sequences including *SMN1 / SMN2* gene-specific nucleotides may overcome the qualitative and/or quantitative problem of DBS-DNA, eliminate PCR-inhibiting components from filter paper on which blood is spotted, and facilitate gene-specific annealing of mCOP-PCR primers. In mCOP-PCR in our system, *SMN1*-specific oligonucleotides predominantly bind to *SMN1* sequence and *SMN2*-specific oligonucleotides predominantly bind to *SMN2* sequence. Here, they used the threshold (cut-off) Cq values of 12. The cycle number of 12 was enough to confirm the specific amplification with matched primers, and also enough to avoid non-specific amplification with mismatched primers. However, the threshold (cut-off) Cq value would be changed with different PCR conditions including different machines, different primer concentrations, etc. SMA has been thought to be an incurable disease. However, in 2016, clinical trial results of intrathecal administration of an antisense oligonucleotide drug, nusinersen (SPINRAZA™, Biogen and Ionis Pharmaceuticals), demonstrated acceptable safety and encouraging clinical efficacy. The development of a successful treatment for SMA leads to the social requirements of early diagnosis of SMA. A newborn screening system for SMA may be essential for early diagnosis of the disease. They combined DNA extraction from DBS on filter paper, pre-amplification of target DNA, and real-time mCOP-PCR to specifically detect *SMN1* and *SMN2* genes, thereby establishing a rapid, accurate, and high-throughput system for detecting *SMN1*-deletion with practical applications for newborn screening.

The candidate, MAWADDAH AR ROCHMAH, having completed studies on molecular genetic analysis of spinal muscular atrophy (SMA), with a specialty in genetic diagnosis of *SMN1* deletion, and having established a new technique applied to newborn screening, is hereby recognized as having qualified for the degree of Ph.D. (Medicine).