SQU JOURNAL FOR SCIENTIFIC RESEARCH:
MEDICAL SCIENCES 2001, 1, 9–12

A polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) test to detect the common mutation (35delG) in the connexin-26 gene

*Mehmet Simsek[1], Nadia Al-Wardy[1] , Mazin Al-Khabory

إختبار سلسلة تفاعل البوليماريز مع الـ RFLP السائدة (فقد الـ G-٣٥) في جين الكونكسين – ٢٦

محمت سمسك، ناديا الوردي و مازن الخابوري

الملخص : الهدف : تطوير إختبار معتمد على سلسلة تفاعل البوليماريز لتحري طفرة الزيحان الهيكلي السائدة في جين الكونكسين – ٢٦ وإستقصاء حالة هذه الطفرة في عمان . **الطريقة :** تم تطوير إختبار سلسلة تفاعل البوليمارنر معتمدا على تولد المطفر المتجه للموقع بإستخدام مخفر مولد للطفرة وقد أدى هذا الى خلق موقع قابل للقطع بإستخدام ECONI في قطعة قصيرة من الحامض النووي والتي تم تكثيرها من جين الكونكسين – ٢٦ . وهذا الموقع القابل للقطع يتم خلقه فقط في حالة وجود الطفرة . ولهذا فإن تحليل الـ RFLP لقطعة الحامض النووي المتكاثرة بإستخدام الـ ١ECON أتاح لنا الفرصة لإستقصاء طفرة (فقد الـ ٣٥ – G) في جين الكونكاسين – ٢٦ . **النتيجة :** بعد فحص مشروعية الإختبار بإستخدام عينات للحامض النووي مضمونه الجودة والتى أحتوت على طفرة (فقد ٣٥ – G) في كلتي الحالتين :متجانس الزيجية أو متغاير الزيجية ، تم الكشف على ١٢٠ عينة مأخوذة من أشخاص أصحاء و ٣٥ عينة مأخوذة من مرضى عمانيين مصابين بالصمم الصبغي العادي غير المتلازم ذو الصفة المتنحية ولا يمتون لبعضهم بصلة القرابة . وقد تبين بعد الفحص عدم تواجد هذه الطفرة في أي شخص ممن تم عليه إجراء الإختبار . **الخلاصة :** أستطعنا تطوير إختبار جديد معتمد على سلسلة تفاعل البوليماريز مع الـ RFLP لتقصي الطفرة السائدة (فقد الـ ٣٥ – G) في جين الكونكسين – ٢٦ . وتدل نتائجنا الأولية من تطبيق هذا الإختبار على عدد محدود من المرضى العمانيين على أنه من الممكن أن هذه الطفرة لا تمت بصلة بالصمم الصبغي العادي غير المتلازم ذو الصفة المتنحية في عمان .

ABSTRACT. ***Objective:*** To develop a polymerase chain reaction (PCR) based test for the detection of a common frame-shift mutation (35delG) in the connexin-26 (GJB2) gene, and to investigate the status of this mutation in Oman. ***Method:*** A PCR test, based on site-directed mutagenesis, was developed for the 35delG mutation. A mutagenesis primer generated an EcoN I site in a short (87 bp) DNA fragment amplified from the connexin-26 gene. The EcoN I site is generated only if the 35delG mutation is present. Thus, a restriction fragment length polymorphism (RFLP) analysis of the amplified DNA fragment with EcoN I allowed us to detect the 35delG mutation in the connexin 26 gene. ***Result:*** After validating the test using quality control DNA samples, which contained the 35delG mutation in either homozygous or heterozygous form, 120 healthy subjects and 35 unrelated Omani patients with nosyndromic autosomal recessive deafness (NARD), were screened for 35delG mutation. The mutation was not present in any individual tested. ***Conclusion:*** We have been able to develop a new PCR-RFLP test for detecting the 35delG common mutation in the connexin 26 gene. Our preliminary results from application of this test on a limited number of Omani patients indicate that the 35delG mutation may not be associated with NARD in Oman.

Key Words: PCR-RFLP, connexin-26 gene, 35delG mutation

Congenital deafness occurs approximately 1 in 1000 live births, of which 50% are hereditary.[1] Recently, some of the mutations described in the connexin 26 gene (GJB2) were shown to be among the causes of non-syndromic autosomal recessive deafness (NARD).[2] A frame shift mutation, 35delG, was particularly reported to be responsible for more than 50 percent of all cases of childhood non-syndromic hearing loss in some populations.[3–5] Because of the high prevalence and clinical impact, early detection of congenital hearing impairment has become a public health problem.

So far, only limited methods have been available for the detection of the 35delG mutation. Rabionet and Estivil described an allele-specific oligonucleotide (ASO) hybridisation method, but it required the use of radiophosphorous (^{32}P) probes.[6] Recently, Wilcox et al described a PCR test based on site-directed mutagenesis and restriction fragment length polymorphism (RFLP) analysis.[7] In most studies, however, a direct sequencing of PCR amplified DNA was used, because the whole protein-coding sequence of the GJB2 gene is located in one exon, which makes it relatively easy to screen for mutations in this gene.[2,4,5] Considering

[1]Department of Biochemistry, College of Medicine Sultan Qaboos University, P.O. Box 35, Al Khod 123 Muscat, Sultanate of Oman. [2]Department of Otolaryngology and Head and Neck Surgery Al-Nahda Hospital, P.O. Box 937, Muscat-112 Sultanate of Oman

*To whom correspondence should be addressed. E-mail: mssimsek@omantel.net.om

that DNA sequencing facilities may not be available in all centres, we present a new polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) method for analysing the 35delG mutation. The method is based on the amplification of a short (87 bp) DNA fragment of the GJB2 gene using a semi-nested PCR. If the 35delG mutation is present, an EcoN I site is generated in the amplified DNA. Hence, a subsequent RFLP analysis with EcoN I easily distinguishes different genotypes at the 35delG mutation site of the GJB2 gene.

METHOD

Genomic DNA was extracted from whole blood of patients and healthy subjects using a kit (Nucleon II, Scotlab Inc). Two quality control genomic DNA samples containing the 35delG mutation were kindly provided by Dr Wilcox of Murdoch Institute, Melbourne, Australia. Oligonucleotide primers were synthesized on a Pharmacia DNA synthesizer and used in a semi-nested PCR using two rounds of DNA amplifications. A 285 bp DNA fragment was amplified in the first round using the primer pair (167F and 452R) and the PCR conditions described by Kelsell et al.[5] In the second round, an 87 bp DNA fragment was amplified using a new mutagenesis primer (35DG: 5'–CTG GTG GAG TGT TTG TTC c C t C–), where the lower case letters represent the two nucleotides that would produce mismatches with the template DNA. The reaction mixture (50 µl) in the semi-nested PCR contained: 50 mM Tris.Cl pH 8.3, 2 mM magnesium chloride ($MgCl_2$), 200 µM each dNTP, 1 µM each of the primers (35DG and 167F), 0.01% gelatin, and 2µl of the previously amplified 285 bp PCR product, but diluted 1000-fold before use. The PCR cycling conditions were: 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min, for 35 cycles in a thermal cycler (Perkin Elmers 480). There was a 5 min pre-incubation at 95°C before starting the cycles, and 5 min at 72°C after the completion of the cycles. A small aliquot (10 µl) of the reaction mixture was treated with 3 units of EcoN I (Biolabs) at 37°C for 12 hours, and subsequently analysed on a 10% polyacrylamide gel.

RESULTS

Using the above method, we were able to develop a new PCR-RFLP test for the detection of 35delG mutation in the connexin 26 gene. Our strategy was to amplify a short (87 bp) fragment using a mutagenesis primer that generated an EcoN I site if the 35delG mutation was present. Figure 1 shows the partial sequence of the connexin 26 gene around the mutation site, and the nature of two mismatches introduced into the mutagenesis primer (35DG) at its (–1) and (–3) positions. These mismatches (T/T and T/C) with the template DNA were essential to generate an EcoN I site (5'–CCTN5AGG) in the amplified DNA.

First, we amplified a 285 bp product using the primer pair (167F and 452R) described previously.[5] The amplified DNA fragment was used as a template, after serial dilutions,

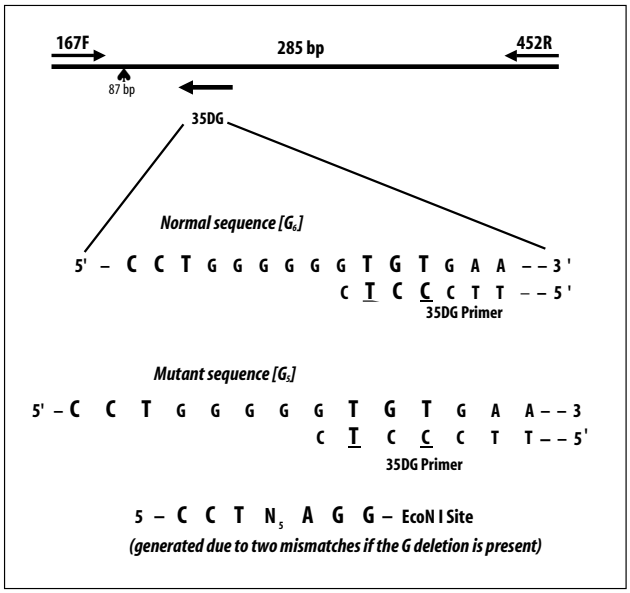

Figure 1. *Principles of the semi-nested PCR to amplify 285 and 87 bp DNA fragments from connexin 26 gene.*

Figure shows the position of three primers used with respect to the 35 delG mutation, and the partial sequence of the mutagenesis primer (35DG), which anneals to the sense strand of the connexin 26 gene around the mutation site. The primer pair (167F and 452R) were as described by Kelsell et al.[5] The mutagenesis primer (35DG) had two mismatches (T/T and T/C) at its 3'— end with the template DNA. An EcoN I recognition site is generated if the 35delG mutation is present in the connexin 26 gene.

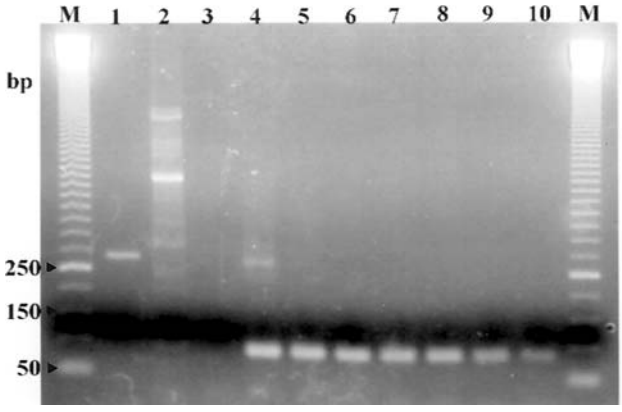

Figure 2. *Amplification and electrophoretic pattern of 285 and 87 bp DNA fragments on a 4 % agarose gel*

Lane 1 shows the amplification of a 285 bp DNA fragment using the primer pair 167F and 452R; Lane 2: trial amplification of 87 bp with the 167F and 35DG mutagenesis primers; Lane 3: negative PCR control without any added template DNA; Lanes 4 to 10: semi-nested PCR for 87 bp using serially diluted 285 bp DNA as template (10^0, 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 fold dilutions, respectively). M indicates 50 bp ladder (Pharmacia) as DNA size markers.

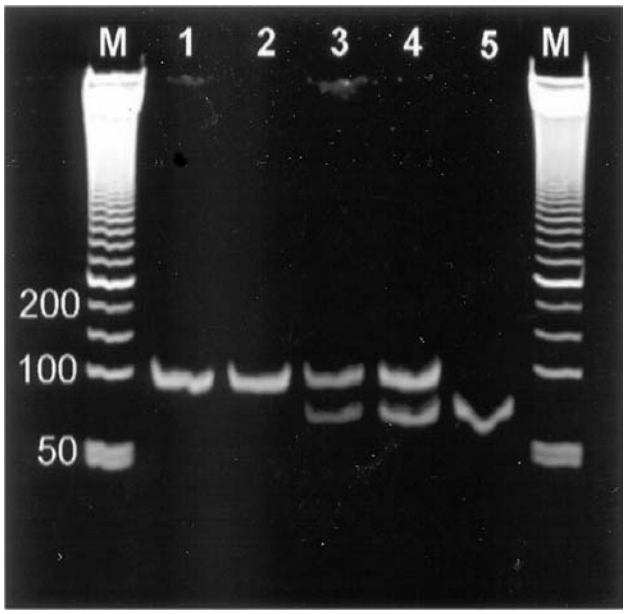

Figure 3. *Identification of 35delG mutation in the connexin 26 gene by RFLP analysis with EcoN I.*

An 87 bp fragment flanking the mutation site was produced using a semi-nested PCR as in Figure 2, and treated with EcoN I restriction endonuclease. The resulting fragments were separated on a 10 % polyacrylamide gel by electrophoresis at 100 volts for 80 minutes. Control genomic DNAs that contained the 35delG mutation were provided by Dr Wilcox. Lanes 1 and 2: normal genomic DNA; Lanes 3 and 4: heterozygous mutant DNAs; Lane 5: homozygous mutant DNA. Lanes M indicate the 50 bp ladder as DNA size markers.

in a subsequent semi-nested PCR [Figure 2, lanes 4 to 10]. The desired 87 bp product was amplified in good yield even after a 10^5-fold dilution of the template DNA. Figure 3 shows the results obtained upon treatment of the 87 bp DNA fragment with EcoN I, followed by electrophoresis on a 10% polyacrylamide gel. For normal DNA samples, there was no cleavage of the 87 bp DNA fragment as expected [Figure 3, lanes 1 & 2]. Two shorter fragments (62 and 25 bp) were produced from a homozygous mutant DNA [Figure 3, lane 5], but the short (25 bp) fragment was too small to be seen in the gel. Two heterozygous DNA samples [Figure 3, lanes 3 & 4] yielded three fragments (87, 62, and 25 bp respectively). Visualization of the 25 bp fragment was not necessary since the identification of the 35delG mutation was easily demonstrated by the inspection of the two larger fragments (62 and 87 bp) in different samples.

DISCUSSION

The 35delG mutation in the connexin 26 gene does not create or destroy a restriction endonuclease site, and hence a direct PCR-RFLP method cannot be used for its detection. In a recent paper it was argued that due to sequence complexity around the 35delG mutation site, a mutagenesis primer could not be used to create a restriction site for the detection of this mutation.[6] We designed a mutagenesis primer (35DG) by introducing two mismatches at its 3'-end, but the amplification of the desired 87 bp product was too weak when the mismatched primer was used directly in a PCR amplification [Figure 2, lane 2]. In addition, there were some non-specific PCR products of higher molecular weight. We tried various PCR conditions including different magnesium ion (Mg^{2+}) concentrations (1.0 to 3.0 mM) and annealing temperatures in the range from 50 to 65°C but all failed to produce the desired 87 bp fragment in good yield (data not shown). This problem was circumvented by using a semi-nested PCR, which resulted in better amplification of the desired 87 bp product. Subsequent treatment of this fragment with EcoN I allowed us to detect the 35delG mutation either in the homozygous or in the heterozygous state [Figure 3].

The principles involved in our PCR-RFLP test is very similar to the methods described by Wilcox,[7] and Storm[10] except for the use of a semi-nested PCR. A problem usually encountered in PCR-based tests is the absence or low yield amplification of target DNA in some samples. This makes the subsequent RFLP analysis extremely difficult. The use of a semi-nested PCR was advantageous here since the target DNA fragment (87 bp) was produced in good yield in all the samples tested, even after a dilution of the template DNA up to 100,000 fold [Figure 2]. Another advantage of the semi-nested PCR used in our procedure is that it allows simultaneous detection of the 35delG mutation together with another frame-shift mutation, known as 167delT in the connexin 26 gene. The T deletion was observed mainly in the Askhenazi Jewish population,[11] while in others, its prevalence was lower than the 35delG mutation. The 167delT mutation destroys an existing Pst I site in the 285 bp DNA fragment, which was obtained in the first round of semi-nested PCR [Figure 2, lane 1]. Thus, a Pst I treatment of this fragment followed by RFLP analysis would be sufficient for detecting the 167delT mutation.

We tested the validity of our method using quality control genomic DNAs [Figure 3], previously characterized by sequencing. Thereafter, we screened 120 healthy subjects and 35 unrelated Omani patients with hereditary sensory deafness. Surprisingly, none of these samples contained the 35delG mutation in the homozygous or heterozygous form, considering that it has been detected at a fairly high frequency (28 to 60%) in most populations studied.[3,4,8] However, recently Abe[12] reported the absence of the 35delG mutation in Japanese patients with prelingual hereditary deafness, a finding that parallels our results.

CONCLUSION

The detection of the 35delG mutation by a robust procedure, such as the PCR-RFLP method described in this

paper, would be a valuable complement to the clinical audiometric screens in identifying neonates with heritable hearing impairment. Early diagnosis of such infants becomes particularly important for treatment and management, because some of them may be candidates for a cochlear implantation, more successful when performed by 18–24 months of age.[9] DNA-based detection of the 35delG mutation in the GJB2 gene would also be useful to determine the prevalence of carriers in the general population to provide a better genetic counselling in future. Our preliminary studies in Oman with a limited number of patients indicate that the prevalence of 35delG may be extremely low or absent in the Omani deafness patients. Studies with a larger group of patients should reveal the exact status of the 35delG mutation in Oman.

ACKNOWLEDGEMENTS

The authors wish to thank to Dr Wilcox at the Murdoch Institute for Research into Birth Defects, Melbourne, Australia, for kindly providing us with characterized DNA samples, which contained the 35delG mutation in either homozygous or heterozygous form. We also thank Ms Hameeda Al-Barwani for her help in the preparation of the illustrations.

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