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Rancangan Karya Teknologi:

PETANDA OLIGONULEOTIDA (ACTTAATTGG GAGCCATATA) BERLABEL BIOTIN UNTUK DETEKSI DINI KELAINAN GENETIK SPINDYLO-EPIPHYSEAL DYSPLASIA TARDA (SEDT) DI BENGKULU

Oleh:
Dr. Aceng Ruyani

PROGRAM STUDI PENDIDIKAN BIOLOGI
FAKULTAS KEGURUAN DAN ILMU PENDIDIKAN
UNIVERSITAS BENGKULU
2011

SURAT KETERANGAN

Yang bertanda tangan di bawah ini, saya;

Nama	: Dr. **Agus Sundaryono,** M.Si
NIP	: 196008061987031005
Bidang Ilmu	: Kimia Organik
Unit Kerja	: Prodi Kimia, Universitas Bengkulu

Telah melakukan verifikasi akademik dan dokumen untuk rancangan karya teknologi atas nama Dr. Aceng Ruyani berjudul:

PETANDA OLIGONULEOTIDA (ACTTAATTGG GAGCCATATA) BERLABEL BIOTIN UNTUK DETEKSI DINI KELAINAN GENETIK SPINDYLO-EPIPHYSEAL DYSPLASIA TARDA (SEDT) DI BENGKULU

Demikian Surat Keterangan ini dibuat untuk dapat digunakan sebagaimana mestinya.

Bengkulu, 18 April 2011

Mengetahui
Dekan FKIP
Universitas Bengkulu

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Pembuat Keterangan

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Hasil Kerjasama Internasional:

Dr. Aceng Ruyani
Universitas Bengkulu

Professor R V Thakker
University of Oxford

Molecular Study of SEDT (Spondylo-Epiphyseal Dysplasia Tarda) Cases in Kedurang, South Bengkulu to Obtain Genetics Diversity and Candidate Marker for Early Detection
2009-2010

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UNIVERSITY OF OXFORD

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Dr.Ir. Fahrurrozi, M.Sc.
Pembantu Rektor Bidang Akademik
Universitas Bengkulu
Jalan Raya Kandang Limun,
Bengkulu
Indonesia.

7th April 2010

Dear Dr Fahrurrozi

Re: Work on the PAR-C Programme

I am writing to confirm that the work on the PAR-C Programme has been undertaken.

Dr Ruyani came to our laboratory this month and worked with UK group working on the project. DNA was extracted from the blood samples and RNA from the leukocyte cell lines that were established in the UK and used to undertake DNA sequencing and RT-PCR respectively to confirm a mutation in a family. DNA sequencing of the SEDT gene has been performed on a number of individuals and an abnormality has been identified and confirmed in several family members. Further work will be carried out in the UK with the aim of preparing diagrams suitable for publication. We have begun to prepare a manuscript.

It was agreed that Dr Ruyani, upon his return to Indonesia, will try to further establish the family history and obtain X-rays of an affected family member for the publication. This information is needed for inclusion in the manuscript we are preparing.

Yours sincerely,

Ms Louise Williams
NDM/OCDEM Personnel Administrator

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May Professor of Medicine
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March 14th 2009

I stated here that Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital Academic Endocrine Unit, Nuffield Department of Clinical Medicine, University of Oxford was the established institution and has a lot experiences to study molecular aspects of SEDT (SPONDYLO-EPIPHYSEAL DYSPLASIA TARDA) cases.

I would like to invite Dr. **Aceng Ruyani** (Civil service data: NIP. 131 615 506; Karpeg D 438503), an educative staff of Bengkulu University, Bengkulu, Indonesia to visit the institution and research together the entitled investigation; "Molecular Study of SEDT Cases in Kedurang, South Bengkulu to Obtain Genetics Diversity and Candidate Marker for Early Detection".

This Letter of Acceptance is required for completing Program of Academic Recharging (PAR) 2009 proposal which is applied by Dr. **Aceng Ruyani** to Directorate of Human Resource, Directorate General of Higher Education, Republic Indonesia Ministry of National Education, in Jakarta.

With best wishes,

Yours sincerely,

R. Thakker

Professor R V Thakker
May Professor of Medicine

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Technical Support Statement

Oxford, January 27, 2009

I stated here that Oxford Centre for Diabetes, Endocrinology and Metabolism, Churchill Hospital Academic Endocrine Unit, Nuffield Department of Clinical Medicine, University of Oxford was the established institution and has a lot experiences to study molecular aspects of SEDT (SPONDYLO-EPIPHYSEAL DYSPLASIA TARDA) cases.

I would like to give technical support to Dr. **Aceng Ruyani** and Team, educative staff of Bengkulu University, Bengkulu, Indonesia to collaborate and research together the entitled investigation: *The Mutational analysis of SEDT (Spondylo-Epiphyseal Dysplasia Tarda) in Kedurang, South Bengkulu to find out genetic diversity among the Dwarfism for gaining their life quality*. Furthermore I stated also that the collaboration is really free from laboratory bench fees and supervisory salary.

This technical support statement is required for completing the HIBAH STRATEGIS proposal which is applied by Dr. **Aceng Ruyani** and Team to the research committee of Bengkulu University, Bengkulu, Indonesia.

With best wishes,

Yours sincerely,

R. Thakker

Professor R V Thakker
May Professor of Medicine

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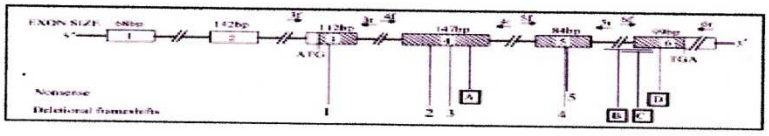
Judul Rancangan Teknologi:

PETANDA OLIGONULEOTIDA (ACTTAATTGG GAGCCATATA) BERLABEL BIOTIN UNTUK DETEKSI DINI KELAINAN GENETIK SPINDYLO-EPIPHYSEAL DYSPLASIA TARDA (SEDT) DI BENGKULU.

PENDAHULUAN

Di Bengkulu Selatan ditemukan penduduk berperawakan pendek terpaut kromosom X dengan ciri-ciri sebagai berikut (a) pertumbuhan abnormal *menyebabkan perawakan pendek ringan*, (b) *simptom perawakan pendek tidak* terdeteksi sampai umur 10 tahun, (c) pertumbuhan tulang spinal terhenti sehingga badan utama berhenti, sedangkan anggota badan tampak normal, (d) bahu tampak bungkuk, (e) leher kelihatan memendek, (f) dada melebar mengerucut ke depan, (g) wajah rata, dan (h) sering nyeri sendi pinggul serta sendi lain di tubuh.

Berdasarkan ciri-ciri tersebut penduduk berperawakan pendek itu termasuk *Spondylło Epiphyseal Displasia Tarda (SEDT; Purnamasari, 2006; Sumiyati, 2006;* Muslim *et al.*, 2007). Hasil studi anatomi menggunakan alat rontgen RSUD M.Yunus, Bengkulu menunjukkan bahwa penyandang SEDT dari Bengkulu Selatan, mengalami perubahan morfologi pertulangan akibat pemendekkan ruas vetebra dan penipisan diskus intervetebralis (Ruyani *et al.*,2007; Ruyani *et al.*, 2009). Populasi penyandang SEDT di Bengkulu Selatan sementara berhasil dicatat sebanyak 67 orang dan paling tinggi berasal dari kelompok usia 21-31 tahun (Karyadi *et al.*, 2008).



Gambar 1. Skema ilustrasi organisasi genomik dari gen SEDL yang tersusun atas 6 ekson, merentang sekitar 20 kb DNA, dan mengkode protein 140 asam amino. A, B, C, D menunjukkan letak titik mutasi yang bertanggungjawab terhadap kelainan tersebut (Chistie *et al.*, 2001).

Gene penyebab SEDT adalah gen SEDL yang terletak pada posisi Xp22.12-Xp22.31 (Gedeon *et al.*, 1999; Gecz *et al.*, 2000; Pearce *et al.*, 1995; Lloyd *et al.*, 1996) yang terekspresi luas pada berbagai jaringan, seperti; otot rangka, ginjal, katilago fetus, fibroblast, dan limfoblast. Gen SEDL tersusun atas 6 (enam) ekson (Gambar 1) yang merentang sekitar 20 kbp. Wilayah koding berukuran 420 bp dan terbagi menjadi ekson 3, 4, 5, dan 6 serta menghasilkan protein 140 asam amino yang kemudian disebut SEDLIN (Gideon *et al.*, 1999).

Kajian genomik terhadap kasus SEDT di Inggris menunjukkan adanya mutasi pada ekson 4 (TGG –TGA, Trp70Stop) dan ekson 6 (CGA–TGA, Arg122Stop) dari gen SEDL tersebut, sehingga terjadi percepatan osifikasi pada berbagai ruas tulang (Christie *et al.*, 2001). Laporan lain telah mengidentifikasi 4 missense mutasi yang menyebabkan SEDT yaitu: tiga mutasi (S73L, F83S, V130D) yang memetakan bagian dalam protein. Mutasi ini mengganggu struktur, dan mutasi keempat yaitu (D47Y), suatu mutasi yang terjadi pada bagian permukaan yang dapat menggagalkan interaksi fungsional dengan suatu protein mitra (Jang *et al*, 2002). Kasus SEDT telah

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dilaporkan juga oleh sejumlah pakar dari beberapa negara (Mumm *et al.*, 2000; Christie *at al.*, 2001; Shi *et al.*, 2002; Gambar 1).

Oleh karena SEDT tergolong cacat karena faktor genetik yang hanya muncul kerika laki-laki mencapai akil balig, maka kajian genomik diperlukan untuk mengetahui titik mutasi yang telah terjadi. Agar dampak mutasi dapat antisipasi, diperlukan petanda molekular yang mampu mendeteksi secara dini bagi mereka yang secara kekerabatan memiliki resiko tinggi. Atas dasar tersebut dilakasanakan kerjasama penelitian: "Molecular Study of SEDT Cases in Kedurang, South Bengkulu to Obtain Genetics Diversity and Candidate Marker for Early Detection"

CARA KERJA

Patients

Probands of three extensive Indonesian SEDT families all from the Kedurang region, were ascertained (Figure 2). A family history of SEDT could be established in each case. Affected males had been diagnosed, usually in the second decade of life when they were investigated for short stature. They all had disproportionate short stature with short trunks and adult heights of 120-140 cm as well as typical symptoms including platyspondyly with hump-shaped central and posterior portions, narrow disc spaces and moderate epiphyseal dysplasias.

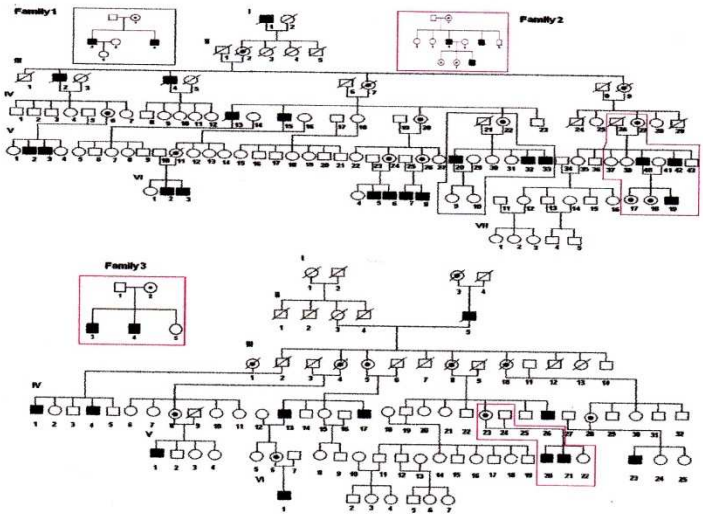

Figure 2. Family trees showing the families examined in this study. Individuals are represented as males (squares), females (circles), unaffected (open symbol), affected (filled in symbol) and unaffected carrier (dot in middle of symbol). Originally there were believed to be three families, but genealogical analysis subsequently showed families 1 and 2 to be related. The three original probands are indicated by an arrow.

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Mutational Analysis

DNA samples were obtained (from venous blood samples, by standard methods (Pearce *etal.* 1995) from the SEDT probands and other relatives, with informed consent and after approval had been obtained from the relevant ethical committees.

DNA sequence abnormalities were initially sought in each of the SEDT probands. Four pairs of primers (Gedeon *et al.* 1999) were used for the PCR amplification of the four coding exons of the *SEDL* gene and of their corresponding intron/exon boundaries with conditions described previously (Gedeon *e al.* 1999). PCR products were gel purified and *Taq* polymerase cycle sequencing performed as described previously (Lloyd *et al.* 1996, Lloyd *e tal.* 1997). All coding exons and the intron/exon boundaries were sequenced in both forward and reverse directions.

In addition, DNA sequence abnormalities were confirmed by Amplification refractory mutation system (ARMS) PCR (Hannan *et al.* 2008, Turner *et al.* 2010). These were demonstrated not to be common polymorphisms (by sequencing) in DNA obtained from 50 male and 30 female normal individuals (110 alleles). RT-PCR analysis performed as previously described (Nesbit *etal,* 2004) to determine the splicing effect of the *SEDL* mutation. Total RNA from blood lymphocytes was extracted using Trizol reagent (Invitrogen) and was reverse transcribed using the SuperScript first-strand synthesis system (Invitrogen) and random hexamers. Subsequently, RT-PCR was performed using exon1F 5'CTCATTGCGTTTCCGTTG-3' and exon4R 5'-AGTGACAAATGCCGACACAA-3' primers. RT-PCR products were sequenced in both directions.

HASIL DAN PEMBAHASAN

Hasil dan pembahasan dari kerjasama penelitian secara pada paper berjudul: "Characterisation of mutation of the *SEDL (TRAPPC2)* gene in patients with X-linked spondyloepiphyseal dysplasia tarda from a region of Indonesia", sebagaimana terlampir.

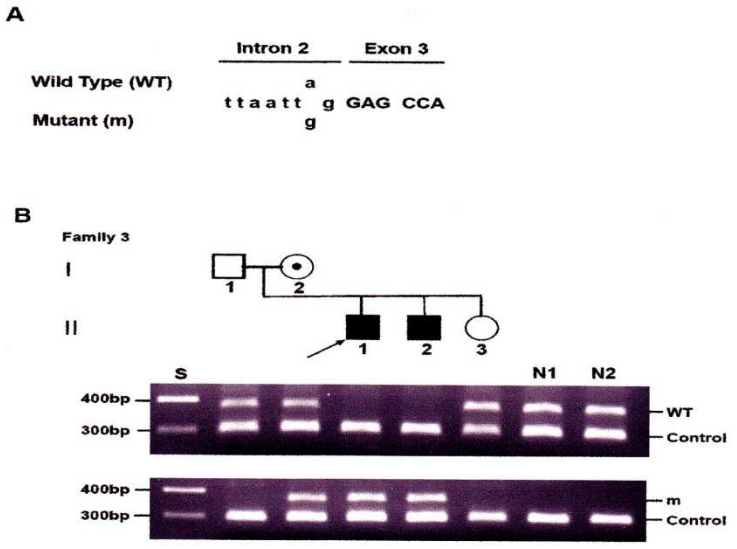

Figure 3. Detection of mutation in intron 2 of the *SEDL* gene in family 3. DNA sequence analysis of an affected male individual (II.2) revealed an a to g transition of the invariant ag dinucleotide of the acceptor splice site consensus sequence of intron 2 (A). The a to g transition did not alter a restriction enzyme site, thus ARMS-PCR was used to confirm the presence of the wild-type (WT) and mutant (m) sequences in lymphoblastoid DNA (B). The affected male individuals (II.1 and II.2) were homozygous for the m sequence, whereas their mother (I.2), a carrier, was heterozygous having both the WT and m sequences. A control primer employed in both reactions confirmed that the ARMS-PCR was amplifying correctly (lower band on both gels). The positions of the size markers (S; 100-bp ladder) at 300 and 400 bp are shown. Cosegregation of this *SEDL* mutation with SEDT in the family was demonstrated and its absence from 110 alleles of 80 unrelated normal individuals, 50 males and 30 females (N1 and N2 shown) indicated that it is not a common DNA sequence polymorphism. Individuals are represented as males (squares), females (circles), unaffected (open symbol), affected (filled in symbol) and unaffected carrier (dot in middle of symbol). The proband (II.2) is indicated by an arrow.

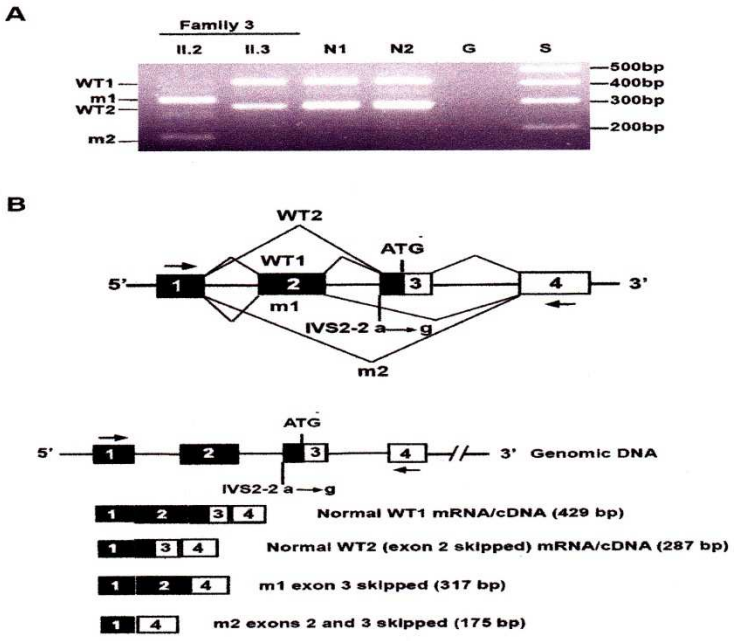

Figure 4. Exon skipping due to an intron 2 acceptor splice site consensus sequence mutation. The transcription of exons 1-4 of the *SEDL* gene was detected by RT-PCR using RNA obtained from EBV-transformed lymphoblastoids of an affected male (II.2), an unaffected female (II.3) from family 3 and two normal individuals (N1 and N2) (A). RT-PCR products were not obtained from the genomic control (G), thereby confirming the specificity of the primers (forward primer from exon 1 and reverse primer from exon 4). In the normal individuals (N1 and N2) and individual (II.3), two bands were observed – a correctly spliced *SEDL* mRNA of 429 bp (WT1) consisting of exons 1 to 4, along with another *SEDL* mRNA of 287 bp (WT2), which is 142 bp less than WT1 and corresponds to the size of exon 2 (B). DNA sequence analysis of this product confirmed exon 2 skipping, with the splicing of exon 1 to exon 3. However, in the proband (II.2) with SEDT, two abnormal cDNAs were observed. The mutant bands (m1 and m2) were both smaller than the WT bands (WT1 and WT2) by 112 bp respectively, and in both cases correspond to the size of exon 3. DNA sequence analysis of this cDNA confirmed exon 3 skipping in both the m1 and m2 cDNAs (B), Thus mutant cDNA m1 consisted of exons 1, 2

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and 4, whereas mutant cDNA m2 consisted of only exons 1 and 4. The positions of the size markers (S; 100-bp ladder) at 200, 300, 400 and 500 bp are shown. Non coding exons are filled in, and the translation start site (ATG) is indicated. The predominant SEDL transcript from several normal tissues has been reported to lack exon 2 (Gedeon *etal.* 1999; Gecz *etal.* 2000; Tiller *etal.* 2001).

Hasil analisis mutasi menunjukkan bahwa penyandang SEDT di Bengkulu mengalami point mutasi yang tidak sama dari hasil penelitian sebelumnya. Adanya bukti historis antara masyarakat Bengkulu dengan kolinial Inggris tidak berarti penyandang SEDT di Inggris memiliki kekerabatan dengan penyandang yang sama di Bengkulu. Perubahan A pada kondisi normal menjadi G pada mutan memiliki dampak menunculkan fenomena SEDT, sebagai temuan baru berpotensi untuk dipatenkan (Gambar 5).

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Pat3-2pcr-3F ATTGACTACT AAAAATATGT ACTATTTTTC ACTTAATTAG GAGCCATATA
             ---------- ---------- ---------- ---------- ----------

Pat4-2pcr-3F ATTGACTACT AAAAATATGT ACTATTTTTC ACTTAATTGG GAGCCATATA
             ---------- ---------- ---------- --------*- ----------
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Gambar 5 Perubahan A pada kondisi normal menjadi G pada mutan memiliki dampak menunculkan fenomena SEDT, sebagai temuan baru berpotensi untuk dipatenkan

Dari temuan baru itu dihasilkan rancangan petanda oligonukleotida ACTTAATTGG GAGCCATATA yang secara komersial didapat diproduksi oleh pabrikan bereputsi tinggi seperti Genisphere Inc (www.Genisphere.com). Petanda oligonukleotida yang dihasilkan dapat dilabel (dengan Biotin misalnya) sehingga dapat digunakan dalam analisis Southern blotting. Metode ini mengkombinasikan elektroforesis gel agarosa untuk memisahkan DNA berdasarkan ukurannya dan kemudian ditransfer ke membran filter untuk selanjutnya dilakukan hibridisasi dengan probe. Untuk mengidentifikasi ataupun melacak suatu fragmen DNA spesifik, diperlukan suatu pelacak (probe). DNA dipisahkan terlebih dahulu dengan elektroforesis. Probe yang dilabel akan hibridisasi pada pita-pita DNA untuk mengetahui apakah DNA tersebut mengandung gen yang diinginkan. Blot Southern mendeteksi DNA rantai tunggal dengan menggunakan DNA sebagai pelacak. Selain Blot Southern, metode lain yang mirip dan dikembangkan dari Blot Southern adalah Blot Western, Blot Northern, dan Blot Southwestern yang memiliki prinsip yang sama, namun molekul yang akan dideteksi dan pelacak yang digunakan berbeda.[1] Kegunaan dari Blot Southern adalah untuk menganalisis keberadaan mutan yang ada pada suatu organisme dan dapat diketahui ukuran dari gen yang menjadi mutan pada organisme tersebut (Wikipedia).

Tahap awal dari metode Blot Southern adalah pendigestian DNA dengan enzim restriksi endonuklease sehingga terbentuk fragmen-fragmen DNA yang lebih kecil. Kemudian DNA dipisahkan sesuai ukuran dengan elektroforesis agarosa. Setelah DNA terpisah, dilakukan pemindahan DNA ke membran nitroselulosa, tahap ini disebut dengan tahap blotting. Membran nitroselulosa diletakkan pada bagian atas dari gel agarosa. Pada teknik blotting dengan menggunakan vakum, membran diletakkan pada bagian bawah gel. Tekanan diberikan secara merata pada gel untuk memastikan terjadi kontak antara gel dengan membran. Proses transfer berlangsung dengan memanfaatkan daya kapilaritas. setalah DNA ditransfer ke gel, membran nitroselulosa dipanaskan dengan suhu tinggi (60°C-100°C) kemudian membran diberi radiasi UV agar terbentuk ikatan kovalen dan permanen antara pita-pita DNA dengan membran. Lalu, membran dicampur dengan probe (pelacak) yang telah dilabel radioaktif, tetapi dapat juga digunakan label nonradioaktif yang dapat berpendar.

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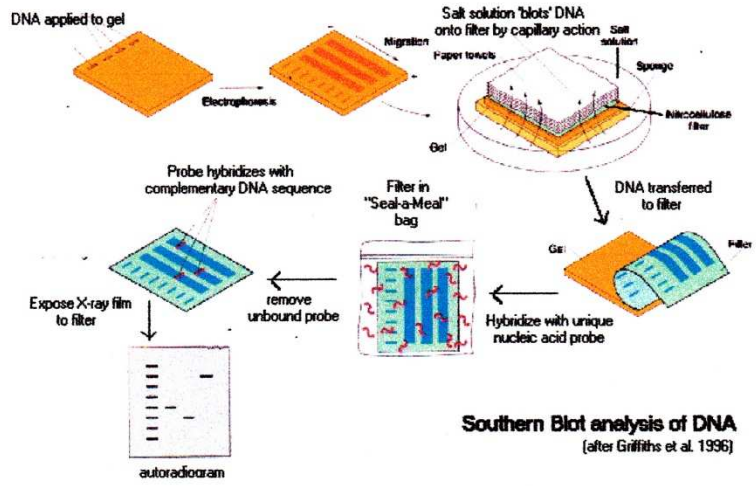
Probe yang digunakan adalah DNA utas tunggal yang memiliki sekuen yang akan dideteksi. Probe diinkubasi dengan membran agar dapat berhibridisasi dengan DNA yang ada pada membran. Setelah proses hibridisasi, probe yang tidak terikat dicuci dari membran sehingga yang tinggal hanya probe yang hibrid dengan DNA di membran. Pola hibridisasi kemudian dideteksi dengan visualisasi pada film X-ray melalui autoradiografi (Wikipedia; Gambar 6).



Gambar 6 Tahapan Southern blotting yang dapat diimplementasikan dalam mendeteksi dini SEDT menggunakan petanda oligonukleotida ACTTAATTGG GAGCCATATA.

Setiap individu yang secara kekerabatan memiliki riwayat penyandang SEDT sebaiknya secara dini (sebelum akil balig) sebaiknya ditentukan status genotipnya menggunakan petanda oligonukleotida ACTTAATTGG GAGCCATATA. Status genotip itu selanjutnya akan menjadi bahan pertimbangan dalam memberi tindakan agar dampak klinis dari penyandang SEDT dapat dikurangi. Upaya ini merupakan langkah awal dalam mewujudkan harapan hidup sejahtera dengan menyandang *SEDT*.

PENUTUP

Kesimpulan

Petanda oligonukleotida ACTTAATTGG GAGCCATATA berlabel Biotin adalah temuan baru berpotensi paten untuk medeteksi dini penyandang SEDT di Bengkulu.

Saran

Penelitian aspek genomik dari kasus SEDT di Bengkulu Selatan perlu ditindaklanjuti dengan riset melalui pendekatan proteomik agar dapat mengungkap perubahan biokimia dan klinis yang terjadi untuk suatu tindakan yang terbaik.

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