



UNIVERSITI PUTRA MALAYSIA

**A COMBINED RECOMBINANT RETROVIRAL-BASED THERAPY
FOR COLON CANCER IN THE MURINE MODEL USING ANTI-
TUMORIGENIC AND ANTI-ANGIOGENIC AGENTS**

NIK MOHD AFIZAN NIK ABD. RAHMAN

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By

NIK MOHD AFIZAN NIK ABD. RAHMAN

**Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in
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January 2011

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Anti-tumorigenic gene therapy offers many benefits over than conventional therapy i.e., chemotherapy or radiotherapy including less toxic side effects, rapid delivery and more sensitive compared to target-based drugs. Anti-tumorigenic agents alone were not proven to be effective in promoting the delay or regression of tumor mass *in vivo* due to low therapeutic gene transfer efficiency and the formation of new blood vessels in tumors. In addition, the blood vessels are one of the major factors that reduce the effectiveness of anti-tumorigenic agents. Consequently, a new therapeutic approach that combined both anti-tumorigenic and anti-angiogenic agents was established and proven to provide superior and beneficial synergistic effect. In the present study, a complete open reading frame (ORF) encoding endostatin protein was RNA extracted from a Balb/c mice liver tissue and transcribed into cDNA. The gene was then cloned into TOPO vector prior to a retroviral vector, pMSCVneo. Upon cloning and sequencing of



the full length of the gene, it was revealed that the complete ORF contained 550 nucleotides, by which the size similar to that of established endostatin. While, anti-tumorigenic gene; VP3 was obtained from previous study (generous gift from Professor Mohd Azmi Mohd Lila) and verified by PCR approaches. The PCR amplification and restriction endonuclease analysis showed that the gene product was convenient and similar to the expected size. The gene obtained was also 98% homology to that the references strain, Cux-1 by DNA sequencing analysis. Both recombinant constructs were transfected into PT67 cells and the transiently produced virus used to treat Balb/c mice with established colon tumors. The method of treatments was given in either as single modality or combined treatment with standardized of tumor size in each mice group. Here, we also revealed a viral delivery vehicle named 'retroviral vector' to deliver the both anti-tumorigenic and anti-angiogenic agents into the tumor mass to achieve the effects of apoptosis. Furthermore, the retroviral-mediated gene therapies also showed no signs of adverse effect. Based on PCR approaches, the recombinant retrovirus particles were disseminated into the bloodstream and finally presented in various tissues but only expressed their therapeutic genes in tumors. As a result, the risk of undesirable characteristics of cancer gene therapy could be avoided in non-target tissues. The differential activities of VP3 and endostatin were investigated by the use of the immunohistochemistry analysis such as PCNA and vWF. The apoptosis activities upon expression of VP3 and endostatin were further studied by quantifying and identifying biochemical changes and number of affected cells due to apoptosis. Upon TUNEL and DNA fragmentation assays, typical apoptotic patterns were observed, to include formation of apoptotic bodies and visible DNA ladders, as early as 48 hours after



treatments. Since necrotic cells could be stained positively by TUNEL, a flow cytometry assay was developed for identifying and quantifying dead or dying cells in mode of cell death. The apoptosis percentage including early and late apoptotic quadrants showed that the combined tumor treatment was higher than VP3 and endostatin only. The effect of VP3 and endostatin protein expression in tumor and vital organ tissues were also confirmed upon examination by inverted microscopy analysis with H&E-stained sections. In addition, upon electron microscopic examination, typical morphological apoptotic features were observed including intact membranes and organelles, cell blebbing as well as condensed nuclear membranes in single- and combined-treated tumors not in other vital organ tissues. Interestingly, based on these constructive findings the combined treatment noted better responses in tumor bearing mice rather than single treatments. In conclusion, this study showed that the combined approach was more promising, effective and good governance on delay tumor growth and inducing apoptosis than introducing VP3 or endostatin alone. The combination treatment also offers potential benefits in control of tumorigenesis, and thus deserves further research as a preferred approach in cancer gene therapy.



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Oleh

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Januari 2011

Pengerusi: Zeenathul Nazariah Allaudin, PhD

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Terapi gen anti-tumorigenic menawarkan banyak manfaat lebih dari terapi konvensional iaitu kemoterapi, atau radioterapi termasuk kesan sampingan yang kurang toksik, penghantaran cepat dan lebih sensitif berbanding dengan ubat sasaran yang berpusat. Agen anti-tumorigenic saja tidak terbukti berkesan dalam mempromosikan kelewatan atau regresi massa tumor in vivo berhubung dengan kecekapan pemindahan gen terapeutik yang rendah dan pembentukan pembuluh darah baru pada tumor. Selain itu, pembuluh darah merupakan salah satu faktor utama yang mengurangkan keberkesanan agen anti-tumorigenic. Akibatnya, pendekatan terapi baru yang digabungkan baik agen anti-tumorigenic dan anti-angiogenik didirikan dan terbukti memberikan kesan sinergis unggul dan bermanfaat. Dalam kajian ini, suatu rangka baca terbuka lengkap (ORF) encoding protein endostatin adalah RNA diekstraksi dari RNA



hati mencit Balb/c dan ditranskripsi menjadi cDNA. Gen ini kemudian diklon ke vektor TOPO sebelum vektor retroviral, pMSCVneo. Setelah pengklonan dan sekuensing panjang penuh gen, terungkap bahawa ORF lengkap mengandungi 550 nukleotida, di mana saiz serupa dengan endostatin ditetapkan. Sementara, gen anti-tumorigenic; VP3 diperolehi daripada kajian sebelumnya (hadiah yang murah hati dari Profesor Mohd Azmi Mohd Lila) dan disahkan oleh pendekatan PCR. Amplifikasi PCR dan analisis sekatan endonuklease menunjukkan bahawa produk gen nyaman dan serupa dengan saiz yang diharapkan. Gen diperolehi juga 98 homologi% untuk bahawa strain rujukan, Cux-1 dengan analisis DNA sekuensing. Kedua-dua rekombinan pembinaan tersebut transfected ke PT67 sel dan virus transiently yang dihasilkan digunakan untuk mengubati tikus Balb/c dengan tumor usus besar yang ditetapkan. Kaedah perlakuan diberikan dalam baik sebagai modalitas tunggal atau kombinasi perlakuan dengan standard saiz tumor pada setiap kumpulan tikus. Di sini, kita juga mendedahkan sebuah kenderaan penghantaran virus bernama 'vektor retroviral untuk memberikan yang baik agen anti-tumorigenic dan anti-angiogenik ke dalam massa tumor untuk mencapai kesan-kesan apoptosis. Selanjutnya, gen terapi retroviral-dimediasi juga tidak menunjukkan tanda-tanda kesan yang merugikan. Berdasarkan pendekatan PCR, zarah retrovirus rekombinan disebarkan ke dalam aliran darah dan akhirnya dihidangkan dalam pelbagai rangkaian tetapi hanya menyatakan gen terapeutik mereka pada tumor. Akibatnya, risiko ciri-ciri yang tidak diinginkan dari terapi kanser gen boleh dielakkan dalam rangkaian non-target. Kegiatan pembezaan dari beberapa VP3 dan endostatin diteliti dengan menggunakan analisis imunohistokimia seperti PCNA dan vWF. Kegiatan apoptosis ini atas ekspresi VP3 dan endostatin yang melanjutkan



pengajian dengan mengukur dan mengenalpasti perubahan biokimia dan jumlah sel yang terkena kesan akibat apoptosis. Setelah Túnel dan ujian fragmentasi DNA, apoptosis pola khas diamati, untuk memasukkan pembentukan badan apoptosis dan tangga DNA terlihat, pada awal 48 jam selepas rawatan. Kerana sel nekrotik boleh diwarnai secara positif oleh TUNEL, alat ujian flow cytometry dibangunkan untuk pengenalan dan kuantifikasi mati atau sekarat sel dalam mod kematian sel. Peratus apoptosis termasuk kuadran apoptosis awal dan akhir menunjukkan bahawa kombinasi perlakuan tumor lebih tinggi dari VP3 dan endostatin saja. Pengaruh endostatin VP3 dan ekspresi protein pada rangkaian tumor dan organ vital juga disahkan pada pemeriksaan dengan analisa mikroskop inverted dengan H & E-bernoda. Selain itu, pada pemeriksaan mikroskopik elektron, ciri khas morfologi apoptosis yang diamati termasuk selaput utuh dan organel, blebbing sel serta selaput nuklear berlawanan pada tumor tunggal dan gabungan yang dirawat tidak dalam rangkaian organ vital lain. Menariknya, berdasarkan penemuan konstruktif kombinasi perlakuan mencatat respon lebih baik pada tikus bantalan tumor daripada perlakuan tunggal. Sebagai kesimpulan, kajian ini menunjukkan bahawa pendekatan gabungan lebih menjanjikan, berkesan dan pemerintahan yang baik terhadap pertumbuhan tumor dan menunda apoptosis inducing dari memperkenalkan VP3 atau endostatin saja. Kombinasi perlakuan juga menawarkan potensi manfaat mengendalikan tumorigenesis, dan dengan demikian layak penelitian lebih lanjut sebagai pendekatan yang lebih disukai dalam terapi kanser gen.



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I certify that an Examination Committee met on 18th January 2011 to conduct the final examination of Nik Mohd Afizan Bin Nik Abd. Rahman on his Doctor of Philosophy thesis entitled “A Combined Recombinant Retroviral-based Therapy for Colon Cancer in The Murine Model using Anti-Tumorigenic and Anti-Angiogenic Agents” in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The committee recommends that the candidate be awarded the relevant degree. Members of the Examination Committee are as follows:

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DECLARATION

I declare that this thesis is my original work except for quotation and citations which have duly acknowledged. I also declare that it has not been previously, and is not concurrently submitted for any other degree at Universiti Putra Malaysia or other institutions.

NIK MOHD AFIZAN NIK ABD. RAHMAN

Date: 18 January 2011



Dedicated to my family:
Haimah, Nik Abd. Rahman and Norriah Hani
For their endless supports



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