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We welcome journal submissions throughout the year but preferably by **March** and **September**. Articles submitted for publication are understood to be offered only to *JUMMEC* and which have not been sent to other journals for consideration.

Cover

An image of a female mosquito (*Aedes aegypti*). Image courtesy of the Department of Parasitology, University of Malaya.

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The **Journal of Health and Translational Medicine (JUMMEC)** publishes both basic and applied science as well as clinical research studies on any area of medicine that is of interest and relevance to the medical community. This is a peer-reviewed journal that publishes Reviews Articles, Original Articles, Short Communications, Clinico-pathological Conference Abstracts, Case Reports, Letters to the Editor and Book Reviews.

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Type manuscript double-spaced throughout. Number pages consecutively commencing on the title page.

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Limit the number of words to 150. It should state the purpose of the study, a brief description of the procedures employed, main findings and principal of conclusions. At the end of the abstract, please include an alphabetical list of 3-5 keywords and subjects for indexing. Choose the appropriate keywords as these will be used for subsequent retrieval.

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It should consist of an Introduction, Methods, Results, Discussion and Conclusion/Recommendation. Systeme Internationale (SI) Units should be used. Use only standard abbreviations. The full term for which an abbreviation stands should precede its first use in the text unless it is a standard unit of measurement.

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Number the references in the order of mention in text. References in the text should be indicated by a figure within parenthesis e.g. (1, 2,). Limit references to 30, if possible. Identify references in text, tables and legends.

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Foreword from the Editor

Dear JUMMEC readers,

In 2014, problems related to medicine and health has reached the main headline in various occasions both at national and international levels. Governments and scientists all over the world are relying on scientific research to better understand these diseases and hopefully provide the ultimate solution to cure or eradicate them. It is through medical journals like JUMMEC where findings of these researches can be reported and shared with peers who are working on related subjects worldwide. For the second issue of JUMMEC (Volume 17), it is my pleasure for me to introduce the contributions of a few authors in the fields of Health and Translational Research.

Dengue epidemic has reached an unprecedented scale in this country starting early 2014. It is very timely for Sekaran SD and her team to provide an overview on immunological basis of the pathogenesis of this condition. It is common knowledge that ultimate solutions for most viral infections including Ebola virus and HIV will most probably come from research laboratories, and we hope that review articles of this nature will stimulate more efforts towards eventual breakthrough in management of these diseases. Another common and chronic condition affecting many children is Asthma. In a case control study on this condition, Ghaempanah Z and co-authors highlighted the importance of assessing not only the clinical control of the disease, but also the psychosocial function of the whole family. Pillay Y and KL Goh reported an interesting case of self-limiting pneumoparotic following upper endoscopy.

MicroRNAs are non-coding RNAs that can influence cellular proliferation and associated with pathogenesis of some diseases. Wong PF and Jong HL provide a review on the role of MicroRNA in endothelial cell senescence and how it may be related to cardiovascular diseases. Autologous Chondrocyte Implantation has been an exciting subject that generated immense interest in the field of musculoskeletal research. Samsudin EZ and Kamarul T reported a summary of research projects in ACI (ACI) conducted by the Tissue Engineering Group of medical faculty of University Malaya since its inception in 2006. Finally, we have another review paper by Tang YQ and her team on the potential role of a tropical plant Phyllanthus sp that has been shown to have potential anti-viral and anti-cancer properties.

For the last four years, JUMMEC has been transformed from hard copy format to digital format following open access guidelines, and published material can be assessed and used by readers all over the world as long as proper attribution is given to the contributors. Continuing support from Medical Faculty and University Malaya for JUMMEC will allow publication of good quality articles on Health and Translational Medicine without article processing charge (APC). We should promote the journal among both academicians and researchers, especially for publication of reports or studies on subjects that are relevant to local or regional population.

Dr Saw Aik
Editor
JUMMEC – the Journal of Health and Translational Medicine

DENGUE: AN OVERVIEW

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ABSTRACT

Dengue is one of the highest occurring vector-borne diseases. It is caused by dengue viruses 1- 4. Currently, the disease is classified into dengue with or without warning signs and severe dengue based on WHO 2009 dengue classification. As of today, neither specific drugs nor commercial vaccine exist for dengue. The best treatment yet would be support, management and proper medical care. With no pathognomonic features that could differentiate it from other febrile illnesses, clinical diagnosis alone is insufficient. Yet, despite the current advances and existence of various laboratory diagnostic methods of dengue, a consensus singular method has not been established. There are several hypotheses or theories regarding the vaguely understood immunopathogenesis of dengue. Amongst these are the viral factors, host-immune factors and host-genetic factors. In addition to these, the occurrence of asymptomatic dengue has further complicated the disease. However, these individuals provide opportunities in the search for protective factors against dengue.

Keywords: *asymptomatic, dengue, diagnosis, immunopathogenesis*

Overview of Dengue

Dengue virus (DENV) infection is undoubtedly one of the most rapidly spread mosquito-borne viral diseases causing major health problems worldwide. The incidences of dengue has grown drastically around the world in recent decades with unprecedented geographic expansion as a result of increased global movement of humans, plants and hematophagous arthropods via shipping and air traffic (1). It is estimated that around 390 million people are infected each year which is more than triple the current estimate by WHO (2). This figure includes 96 million severe cases and 300 million mild or asymptomatic episodes. As a resulting implication, this suggests that the reservoir of the disease is far larger than expected. Before the 1970s, only nine countries had experienced severe dengue epidemics (3). However, at status quo, not only is the number of cases increasing as the disease spreads to new areas, explosive outbreaks are also occurring. The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, South-east Asia and the Western Pacific. The American, South-east Asia and the Western Pacific regions are the most seriously affected areas (3). This review provides our understanding of dengue in terms of the virus per se, its clinical manifestations, pathogenesis of the disease, tests that are used to diagnose it and management of the disease.

Dengue Virus

The DENV is a positive-sense single stranded RNA virus belonging to the genus *Flavivirus*, from the family of *Flaviviridae*, sharing the same category with other 70 different viruses (4). The genome of DENV is approximately 11 kb long and the mature virions are composed of three structural protein genes that encode the nucleocapsid or core protein (C), membrane-associated protein (M), envelope protein (E), and seven nonstructural (NS) protein genes. The gene order is 5'-C'prM(M)-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5-3'(5). There are four serotypes of DENV, DENV-1 through DENV-4 and all four serotypes are closely related but antigenically distinct. It is known that E protein is not only a functional protein molecule that binds to receptors on the host cell membrane but also a major antigen, which can induce neutralizing antibody and host specific protective immunity (6-8). As enveloped viruses, DENVs enter the cells through receptor mediated endocytosis (DC-SIGN, heparin-sulfate, clathrin-mediated) (9-12) where the plasma membrane invaginates to form an endocytotic vesicle around the enveloped virus (13-14). The deposition of the nucleocapsid into the cytoplasm occurs right after the virion envelope fuses with the plasma membrane and internal cell membranes rearrange to establish specific sites of replication (15- 17). The positive sense dengue virus RNA will be first translated

to make RNA polymerase, which is required for transcription of positive-strand RNA into negative-strand RNA. The negative-strand RNA will serve as template for the replication of positive-strand RNA. During the first 12 to 16 hours after infection, there is no encapsidation of RNA but the RNA serves as the template for replication and translation for the formation of dengue viruses. During the late eclipse period, the positive-strand RNA must be diverted to viral assembly (18). During late replication process, the affinity of RNA polymerase complexes towards positive-strand and negative-strand RNA changes, resulting in the predominance of positive-strand RNA later in infection (19). The assembly of nucleocapsids starts with the increasing concentration of C protein (20). The nucleocapsids first assemble from C protein and followed by envelopment through "budding" of nucleocapsids via membrane containing integral E and prM proteins (18). The virus is then released from the infected cells via secretory exocytosis when the virus-containing secretory vesicles fuse with the plasma membrane (21). At the end of the viral replication, cleavage of prM occur before or during the release of virus from infected cells, and this process is accompanied by reorganization of the virion envelope and also viral maturation (22).

The most common vectors that transmit DENV are *Aedes aegypti and Aedes albopictus*. However, DENV transmission are not limited to those with different species of *Aedes* mosquitoes such as *Aedes polynesiensis, Aedes scutellaris,* and*Aedes stegomyia* may act as vector for the disease as well (23). There are three types of transmission cycles for dengue virus, the primitive enzootic transmission cycle, epidemic transmission cycle and also urban endemic/epidemic transmission cycle (24). Most of the time, multiple virus serotypes are co-circulating in the same city (23, 25). Nevertheless, in recent years, human activities had enhanced the mosquito breeding activities, which has led to increased interaction between mosquitoes and humans, and eventually increased rate of viral dispersal between mosquitoes and humans (26-27). Slight movement will disrupt the feeding of the female mosquitoes, and only later it will return to the same or another person to continue feeding (24). Because of this feeding behavior, the female mosquitoes can feed on many persons in the same household in a short time. Therefore, it is common that several members of the same household might be infected, and usually the virus is being transmitted from the same mosquito to different people in the same household (25, 28-30). To this effect, many individuals are susceptible to being infected by DENV of which some may show clinical symptoms while others do not. Previous studies of asymptomatic infections have been reported to show varying ratios of symptomatic to asymptomatic dengue infection. This is clearly exemplified by different surveys such as in Thailand, during a prospective survey carried out in 1980–1981 in Bangkok amongst school children; it was estimated the ratio of asymptomatic/symptomatic cases to be 6.1:1. In addition, the DENV-4 infections that were detected during this survey were entirely asymptomatic (31). In contrast, another prospective study was conducted in Kamphaeng Phet, Northern Thailand, between 1998 and 2000 and the results were quite different since the ratio was only 1.1:1 (32). In Singapore, authorities assumed a ratio of asymptomatic/symptomatic infections between 2:1 and 10:1 (33). It had been shown in clinical-based study, asymptomatic dengue is common in primary DENV-2 or DENV-4 infections in children (34-35) while over 95% of primary infections of circulating Southeast Asian genotype DENV-2 in adults were asymptomatic during an outbreak in Santiago de Cuba in 1997 (36). Based on our preliminary findings from three different hospitals around Klang Valley as well, we observed that about 40% of the household members of the dengue patients are tested positive with dengue. These household members are not sick, but are actually having asymptomatic dengue infections. These differences might be explained by individual variations in susceptibility or variability in the virulence of DENV strains. The epidemiology of dengue may differ according to the region and country. The spread of the virus is imminent not only among the infected individuals but also among the healthy ones that are predisposed to the bites of the infected mosquito. Based on the data and reports mentioned above, it is undeniable that the asymptomatic DENV cases should not be taken lightly as they play a key role in helping researchers to decipher the immunological aspect of the human host in fighting against the DENV infection. To date, it is still unknown as to whether asymptomatic cases may be the reservoir of infection which will indeed spur the increase of DENV incidence rates. Besides that, this will also greatly increase the risk of occurrence of severe dengue in the future, as the previous DENV infection had gone undetected. However, these asymptomatic individuals provide avenues for researchers to look into the protective factors that protect them from developing symptoms during DENV infection.

Manifestation of Disease

DENV infection are mostly asymptomatic, however, a wide variety of clinical manifestations may occur, ranging from mild febrile illness to severe and fatal disease (37). The differential diagnosis is broad and varies as the disease evolves. Other diseases that should be considered as part of the differential diagnosis, depending on the clinical picture and local disease prevalence, include typhoid, malaria, leptospirosis, viral hepatitis, rickettsial diseases, and bacterial sepsis (38). Previously, patients were classified as having either dengue fever or dengue hemorrhagic fever, with the latter classified as grade 1, 2, 3, or 4. Over a number of years, there was increasing concern regarding the complexity and usefulness of this classification system (39). With the recent revision of the World Health Organization (WHO) dengue classification, patients are now classified as having either dengue or severe dengue (37, 39). Patients who recover without major complications are classified as having dengue, whereas those who have any of the following conditions are designated as having severe dengue: plasma leakage resulting in shock, accumulation of serosal fluid sufficient

to cause respiratory distress, or both; severe bleeding; and severe organ impairment.

The sudden onset of the symptoms starts after an incubation period of 3 to 7 days. Patient will undergo an initial febrile phase, a critical phase around the time of defervescence, and a spontaneous recovery (convalescence) phase. The initial phase is typically characterized by high temperature (≥38.5°C) which may be accompanied by headache, vomiting, myalgia, arthralgia and sometimes with a transient macular rash. This phase lasts for 3 to 7 days, after which most patients recover without complications. However, during the transition from the febrile to the critical phase, between days 4 and 7 of the illness, vascular leakage may develop in some patients. Signs of impending deterioration include persistent vomiting, increasingly severe abdominal pain, tender hepatomegaly, a high or increasing hematocrit level that is concurrent with a rapid decrease in the platelet count, serosal effusions, mucosal bleeding, and lethargy or restlessness (38). During this critical period, hemorrhagic manifestations are also most common. With proper management after approximately 48 to 72 hours, altered vascular permeability may be short-lived, spontaneously reverting patient to a normal level and concurrent rapid improvement in the patient's symptoms may be seen. The convalescence phase may be abrupt but prolonged with some having profound fatigue for several weeks after recovery.

Diagnosing dengue via laboratory assays

Clinical diagnosis alone for dengue is not sufficient because dengue has no pathognomonic clinical features from other febrile illnesses (40). This is where laboratory confirmation methods are vital to find out the etiological agent and to allow proper management and treatment of disease. Ideally, a laboratory assay for dengue detection should be rapid, simple, with high sensitivity and specificity, able to detect dengue at any phase of illness, preferably able to distinguish primary and secondary infections as well as the different serotypes. However, this ideal test has not been materialized yet due to (i) the complexity of dengue pathogenesis; (ii) hyperendemicity and multiple sequential infections and (iii) clinical conditions of patients including viremia and antibody response. Currently, at different phases of illness, different laboratory methods are being used to diagnose dengue. Virus isolation, genome and antigen detection is conducted during the early or febrile phase of illness; whereas at later phases, where antibodies have been formed serological-based assays are applied (Figure 1). The serological-based assays such IgM capture ELISA are favorable in hospitals of dengue endemic countries (41) due to its inexpensiveness, simplicity and (sometimes) rapid turnover as well as the narrow time frame of viremia. Nonetheless, serological assays can be a challenge in hyperendemic areas where pre-existing antibodies complicates diagnosis (42). These

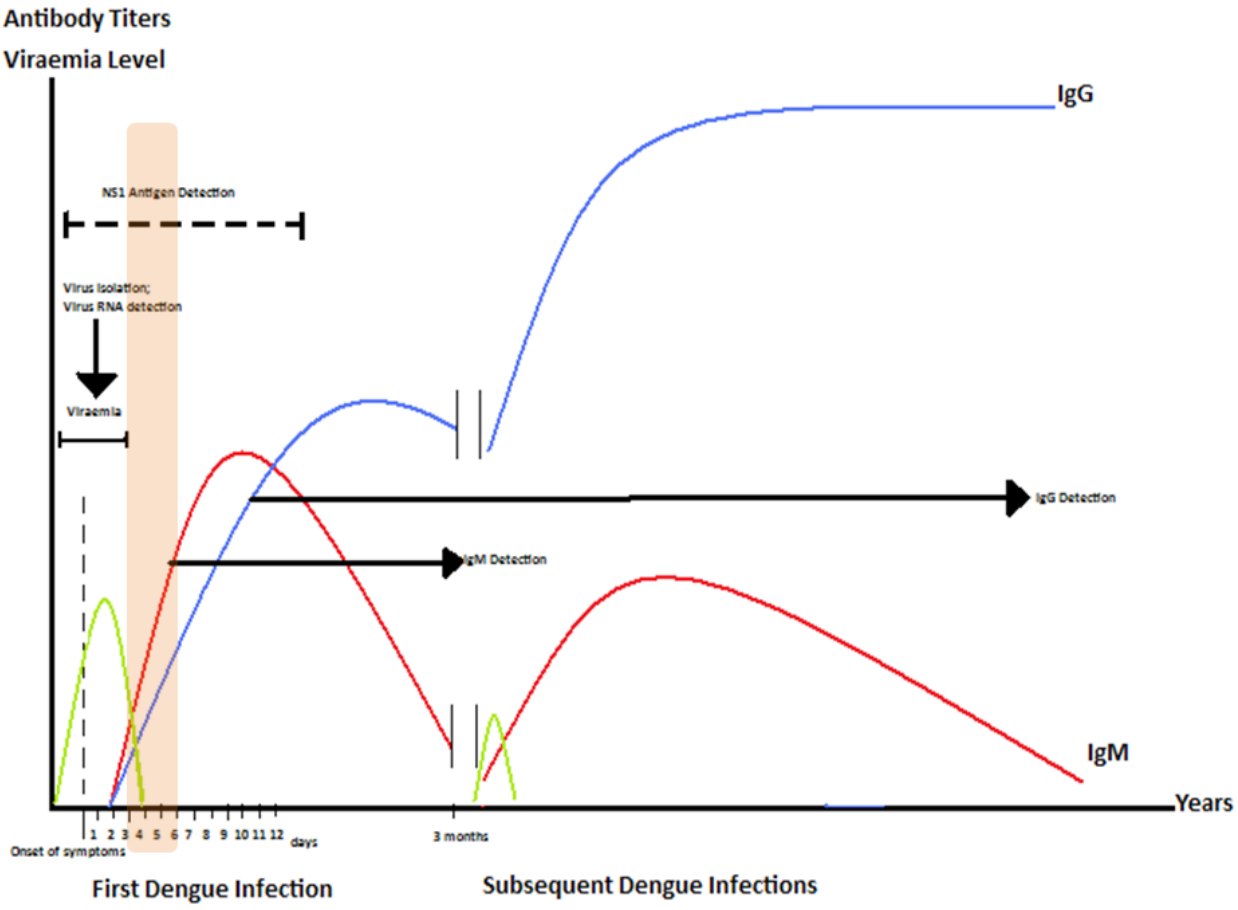

Figure 1: *Time course of primary/secondary dengue infections and the suitability of dengue diagnostics at different phases of illness.*

assays also require 2 samples (acute /defervescence and convalescence) to be able to confirm a patient via seroconversion as a single antibody positive sample may indicate an infection that has occurred in the past 3 months (43). Many serological assays have commercialized test kits in various formats including microplate, strips and cassettes. These kits have variable sensitivity and specificity, and may not have been evaluated against referenced serum panels (44). The sensitivity of the commercialized IgM ELISA kits ranged from 61.5 -- 99.0% with specificities ranging from 79.9 -- 97.8% when analyzed worldwide. The rapid diagnostics tests ideal for bedside diagnosis, had lower sensitivities ranging from 20.5 -- 97.7% and specificities of 76.6 -- 90.6%. To facilitate early diagnosis and confirmation of the etiologic agent, various polymerase chain reaction (PCR)-based techniques have been developed, optimized, innovated and simplified to detect dengue RNA including multiplex reverse-transcription (RT)-PCR (45-46), real time multiplex RT-PCR (47-51), nucleic acid sequence-based amplification (NASBA) (52), RT-Loop mediated isothermal amplification (LAMP) (53) and transcription-mediated amplification (TMA) (54). This method has minimized hazardous contact with live DENV and reduced the result turnaround time, enabling physicians to take early course of action in managing dengue patients. Despite many genomic detection methods for dengue detection, not all have been evaluated (42) and in one such external assurance study, only 10.9% of participating laboratories with different molecular-based method met all criteria for optimal performance (55). In this quality assurance study, 80.4% of participating laboratories needed to improve their DENV detection diagnosis procedures because (i) laboratories applying the same protocols had different reproducibility rates; (ii) false negative rates were high and (iii) false positive was detected in some laboratories (55). Furthermore, this technique is not favorable in poor endemic areas because the method is expensive with need of specialized equipment and skilled personnel. Ever since a decade ago, DENV NS1 detection has been pursued diligently with the invention of NS1 ELISA and rapid test kits. The NS1 antigen has been detected in serum and plasma of dengue infected patients from the onset of fever up to early convalescence (56-58). This antigen detection method is useful for low-resource settings as the kits are often lower priced and simple to use (59). Nevertheless, the commercialized ELISA and rapid NS1 kits had shown poor sensitivity ranging from 52 - 94% and 48- 91%, respectively when evaluated in different endemic countries. With the complicated immune status of dengue patients and assays that require more standardizations, combinations of different methods such as detection of both NS1 and IgM/IgG have proved to increase the overall performance (60). Hence, it seems that without the ideal diagnostic test for dengue, the best way currently to diagnose and confirm dengue is to run different assays or to obtain paired sera.

Dengue pathogenesis

Principally, disease manifestations often involve complex interactions between invading pathogen and the host immune response. Dengue infections undoubtedly fall in this category, where the clinical outcomes of dengue have been postulated to be intrinsically caused by both viral-induced and immune-mediated pathogenesis. Traditionally, it has been believed that secondary infections in dengue are more severe. This was first recorded in the Philippines in 1953/54, where a more severe form of dengue-associated disease was noted accompanied by bleeding, leakage, shock and death (61). Subsequently, various retrospective and prospective clinical studies showed evidence of dengue severity in secondary infections supported by appearance of DHF/DSS in infants with passively transferred maternal antibodies to dengue (36, 62-66). However, there are also cases where in DENV hyperendemic areas or in people with pre-existing antibodies, severe dengue had not occurred (67-68). Furthermore, severe dengue manifestations during primary infections have also been observed in children and adults (34, 69).

Immune cells such as macrophages and dendritic cells (DCs) are often recruited to sites of infection upon stimulation by chemokines. These are phagocytic cells which are supposed to kill DENV-infected cells. However, DENV preferentially targets these cells for infection and replication. As the cells circulate through the body, more DENV is released and viremia sets in. In a secondary infection, this phenomenon is further enhanced by non-neutralizing antibodies of the previous infection, a scenario termed as antibody-dependent enhancement. The non-neutralizing antibodies bind to DENV and via Fc receptors, viral entry into cells is increased (70-72), directly influencing the viremic state of the host (34). The innate immune system also responds to DENV infections by recognition of viral pathogen-associated molecular pattern (PAMPs) via pattern recognition receptors (PRRs) such as TLR-5, RIG-1 and MDA5 (73); consequently causing the secretion of type I interferons which have strong antiviral action (74). Nevertheless DENV have been shown to evade and modulate this pathway to inhibit/ delay the innate response. DENV can avoid interaction with cellular PRRs (75) or by expression of antagonist proteins to disrupt the type I interferon activation pathway including IRF-3 inhibition (76) as well as STAT1 and STAT2 down regulation(77-78). Subsequently, antiviral actions against DENV can be delayed, and furthermore this may ultimately impair the ability to evoke the adaptive immune response.

The DENV-infected tissue-resident DCs, working as an antigen presenting cells, will travel up to the lymph node to activate cells involved in the cell-mediated immunity. The bone marrow derived cell (B cells) will produce dengue specific antibodies to neutralize DENV. With a low-fidelity RNA-dependent RNA polymerase, DENV may be creating quasispecies to avoid recognition by the immune system (79). This was observed by sequence variation and epitope changes in DENV proteins, hence increasing viral fitness and decreasing neutralization abilities of dengue specific antibodies (80-81). Recently, memory B cells from previous DENV infections dominate the secondary infections and produces the non-neutralizing antibodies in antibody-

dependant enhancement (ADE) (82-83). The cell-mediated immune system involves activation of $CD4^+$ and $CD8^+$ T cells for viral clearance. T cells are activated when the T cell receptor and a co-stimulatory molecule binds to the major histocompatibility complex (MHC) molecule bearing the antigenic peptides on APCs. The activated cytotoxic T cells can recognize and kill DENV-infected cells. Antigenic variability and sequence variation by DENV may allow the virus to avoid/delay T cell detection. In secondary infections, the cross reactive memory T cells are postulated to be activated rapidly and in greater quantities (84). These cells are also hypothesized to have lower affinity and avidity towards the subsequent heterologous serotypes and are hence less efficient in viral clearance (85). Subsequently, these phenomena may trigger a cascade of events including skewed/overproduction of cytokines leading to increased vascular permeability. Cytokine irregularities have been observed via blood sera analyses where various cytokines have been implied as risk factors for increased dengue severity including IL-8, IL-10, IL-13, IL-18, IFN-γ, TNF-α, MCP-1, RANTES and MIP-1β (86-91). Many of these cytokines have been implied as modulators in vascular leakage and have influential impact on endothelium permeability. Obviously, these postulated hypotheses, one way or another can alter the physiological state of the host body which includes regulation of endothelium permeability, nevertheless less emphasis has been placed on the endothelium as a cause of DENV pathogenesis. DENV have been shown to infect endothelial cells both *in vivo* and *in vitro* (92-95). Morphological damage in the endothelium related to vascular leakage was seldom observed, despite the presence or increase in circulating endothelial cell markers, von Willebrand factor, pro-coagulants in severe dengue patients. In patients, the kinetic of DENV infection on the endothelial cells is obscure whereas *in vitro*, increase permeability in endothelial cells is believed to be contributed by the secretion of various factors triggered by immune response such as cytokines, chemokines, and also the complement activating factors, and not directly linked to the infection of DENV per se. However, the DENV infected endothelial cells (ECs) have shown to trigger various chemokine and cytokine responses and these may contribute to vascular permeability by modulating tight junctional changes (96-98). Moreover, oxidative stress in imminent in endothelial function and dysfunction where in dengue infections, oxidative stress markers such as glutathione peroxidase,glutathione,malo ndialdehyde, VCAM-1, high-sensitivity C-reactive protein and platelet-activating factor–acetylhydrolase have been found to be modulated as dengue severity increased (99)s. Nevertheless, endothelial permeability in dengue infection seems to be a transient effect when no extensive cell death and damage was observed upon DENV infection *in vitro* (96, 98, 100).

The modes whereby DENV evades our immune system are many; this combined with flaring up of the immune system are believed to cause pathological disease. But this may be the incomplete picture of dengue pathogenesis because various other factors have also been postulated as severity risk in dengue infections such as age (101), sex (102), nutritional status (103), immune status, co-morbidities (104), autoimmunity (105), genetic background (HLA and non-HLA molecules) and DENV fitness (or virulence). A great deal of effort has been devoted to understanding the immunopathogenesis of clinical dengue infections. Nevertheless, there remains a lack of specific ground rules to define the effect of viral virulence in dengue pathogenesis, as host susceptibility and also certain other factors might affect the pathogenicity of DENV infections. The factors conferring protection against clinical dengue infection have seldom been investigated. Therefore, in our recent study (unpublished data), we postulated that the molecular mechanisms underlying the asymptomatic DENV infection may confer protection towards the manifestation to clinical dengue infection. We found that the asymptomatic individuals elicited pre-existing neutralizing antibodies and certain immune response genes expressed in these individuals to be in contrast with severe dengue patients when gene expression studies were done. Our findings may highlight the potential association of certain host genes conferring protection against clinical dengue to better understand the immunopathogenesis of dengue infection.

Vaccine

There are currently no licensed dengue vaccines available; however, several vaccine candidates are under development. These include live attenuated virus vaccines, live chimeric virus vaccines, inactivated virus vaccines, and live recombinant, DNA and subunit vaccines (106). Live viral vaccines have advanced to clinical trials, but have shown problems, such as unequal immunogenicity of the four serotypes and viral interference among the four serotypes in tetravalent formulations (107). This includes subunit vaccines that mostly focused on the E protein or its derivatives. However, the difficulty of eliciting balanced levels of neutralizing antibodies to each of the four serotypes remains a major concern. The E protein is the major component on the surface of DENV virion and is a dominant target of Ab responses against DENV. Passive immunization with anti-E antibodies provides protection against DENV infection in mice (108). In addition, monoclonal antibodies against prM/M have been shown to provide protection against DENV challenge (109). However, this is contradictory to a study done by Dejnirattisai *et al.* 2010, which showed that prM antibodies do not neutralize infection but potently promote ADE instead (110). Besides that, in our unpublished data, the antibody titers towards prM and E antigens were seen to be higher than the neutralizing antibodies in dengue patients who showed heterotypic infection, indicating that there seems to be cross reacting antibodies that may hinder the neutralization of DENV infection. These observations may serve as a point to note that the development of dengue vaccine is yet a long way to go with much consideration on cross reactivity of antibodies and host immune response towards heterotypic infections.

Management

There is currently no effective antiviral agent to treat DENV infection, and treatment remains supportive, with particular emphasis on careful fluid management (37). In cases where patients who do not have complications and are able to tolerate oral fluids, they remain at home unless bleeding or warning signs suggestive of vascular leakage develop (38). Development of any warning signs indicates the need for hospitalization and close observation. Prompt fluid resuscitation to restore plasma volume is given if the condition progresses to dengue shock syndrome followed by ongoing fluid therapy to support the circulation at a level just sufficient to maintain critical organ perfusion. Blood transfusion can be life saving for patients with severe bleeding that compromises cardiovascular function, but it should be undertaken with care because of the risk of fluid overload. There are recent developments of establishments in therapeutics and design of randomized, controlled trials of drugs targeting the virus or the immune response (111). However, currently, there is no evidence in favor of the use of any specific therapeutic agent for dengue (38).

Conclusion

Over the past decades, the field of dengue research has been growing with the realization of the burden of disease coupled with the prospect of various antiviral therapeutics and vaccines. However, dengue has been problematic in an overall manner mainly because of its complexity. Being a disease with complicated features, it is of utmost importance that there should be more improved overall understanding of the disease to combat this global public health challenge. Besides that, no vaccine can be of immediate global curative, and efforts to improve treatment through application of existing best practices in triage and fluid management, along with efforts to develop new antiviral or other therapeutic drugs should be continued.

Acknowledgement

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THE IMPORTANCE OF ASSESING PSYCHOSOCIAL FUNCTIONS OF ASTHMATIC PATIENTS AND THEIR FAMILIES AS A COMPREHENSIVE ASTHMA CONTROL ASSESSMENT: A CASE CONTROL STUDY IN IRAN

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ABSTRACT

The present study was conducted to compare the physical and psychosocial functions of asthmatic patients presenting at different levels of asthma control to that of healthy children in Iran. The aim was to determine if a correlation could be made between the two thus suggesting that the level of asthma control can be used as an indirect indicator for the level of psychosocial impact. A case-control study involving 160 asthmatic children aged between 8 to 12 years-old, in comparison to an age-sex matched healthy control group was thus conducted.

The result demonstrates that asthmatic children are more likely to have emotional dysfunction associated with their disease. This in turn resulted in significantly negative effects on the parents' daily working activities. The PedsQL scale which categorizes asthmatic children into three levels of asthma (controlled, partly-controlled and uncontrolled asthma) demonstrated significant differences in their Physical Function scale scores ($p<0.05$). However, no significant correlation between asthma control to Emotional, Social and School Functioning levels were found.

In conclusion, the present study suggests that asthma not only impairs the Physical and Emotional functions of asthmatic children, but also affects their parents' daily working activity. Tightening asthma control did not appear to improve the social functioning levels of these children although it does result in improved physical function. Therefore the level of asthma cannot be used as an indicator of heightened psychosocial impact. However, the authors of this paper would warrant that treating physicians should consider incorporating the psychosocial aspect of asthmatic children and their families in their treatment plan rather than aiming for only asthma control since this appears to be an important aspect of patient care.

Keywords: *asthma control level, children, family function, physical function, psychosocial function*

Introduction

Asthma is a chronic inflammatory disorder involving the airway that results in difficulty in breathing and in severe case even death. In several studies, it has been shown that asthma affects a large number of children resulting in many secondary effects, which have included poor growth and retarded personal development (1). This condition has also been shown to be a major contributor of school absenteeism and hospitalisation (2). In Iran, collective data suggests that the prevalence of asthma has been increasing since the early 1990s across all ages, sex and racial groups (3, 4). Earlier studies have somewhat indicated that asthma may have an effect on the psychosocial and physical functions of children with asthma as well, although the

exact relationship has never been demonstrated (5, 6). Children with asthma are exposed to a number of indirect health risk of which internalising behaviours seems to be of major concern more recently. These have included signs of depression, anxiety, dependency, somatisation, and social isolation (6). There is also evidenced that asthmatic children are more likely to have low score of self-esteem than that of healthy individuals (7). On the other hand, families with asthmatic children are also affected since they have other responsibilities that need to be cared for. This in turn causes emotional and physical distresses that may result in social isolation, and in many instances lead to loss in social opportunities (8). Despite numerous studies demonstrating that asthmatic children and their families are subjected to psychosocial assault, treatment for this condition has been generally limited to reducing physical symptoms only. So much so that there is even a classification that defines asthma control, but not its psychosocial impact. One of the more famous is the Global Initiative for Asthma guideline classified which defines asthma control levels as controlled, partly controlled or uncontrolled. In the absence of physical symptoms asthmatic patients are considered as having well-controlled asthma (9). With limited resources especially in country like Iran, it may not be prudent to have every child and their family to undergo psychosocial therapy and management, and therefore a simpler approach to identifying these problems would be required. This is with the aim to be able to ensure that those in need would be given preference. We hypothesized that the increase the severity of the symptoms, or in other words the control of the disease, is strongly related to the level of psychosocial impact. Thus the present study was conducted to assess psychosocial functions of asthmatic children based on asthma control level and thereby suggesting that if a correlation exists, the severity of asthma can be used as an indirect indicator of psychosocial impact.

Method

Participants

The study is a case-control study, questionnaire-based survey among 80 asthmatic children and 80 healthy children as control ranged in age from 8 to 12 year-old. The sample size calculations were made based on power of 95%, confidence 95% and the results of a Dutch Study (10). Asthmatic children enrolled in this study was referred to the Immunology Asthma and Allergy Research Institute in Tehran between December 2011 to June 2012.. These children were diagnosed at least 2 years prior to the study (mean 2.5 years). Using GINA, asthma level of patients was classified as “well controlled” ,“partly controlled” and “uncontrolled” by an asthma specialist.(9)

For the control group, healthy children who had never diagnosed with asthma or other respiratory problem were selected randomly from local schools. The age, gender, and socioeconomic status of this group were matched to those in the asthmatic group.

Children were excluded from the study if they had psychiatric disorders, chronic medical illness (e.g., diabetes, congenital anomalies, obesity, cystic fibrosis), as well as neurological disorders, physical or intellectual disability, based on parent reports.

Instrument

PedsQL™4.0 Generic Core Scale:

The PedsQL™4.0 Generic Core Scale is an instrument with 23 items containing four subscales: Physical Functioning (8 items), Emotional Functioning (5 items), Social Functioning (5 items) and School Functioning (5 items). Likert response scale with five categories was used which are 0 for never a problem to 4 for almost always a problem and items were transformed to a 0-100 score (11). Three summary scores can be calculated: a Physical Health score (8 items), a Psychosocial Health score (15 items), and a Total Core score (23 items) (12). This questionnaire was previously translated and validated in Iran (13, 14).

PedsQL™Family Information Form

The PedsQL™ Family Information Form was developed by James W.Varni et al and completed by parents and contains general socio-demographic information including the child’s date of birth, gender, disease history, disease severity and the parent’s marital status, education, occupation and the impact of child’s health on parents daily works (11). The final questions of this form (in the past 30 days, has your child’s health interfered with your daily routine/ability to concentrate at work) defined as the affects of child health status on housekeeper and employed parent’s daily works.

Ethics

This study was approved by Research and Ethics Committee at Immunology Asthma and Allergy Research Institute (IAARI), Tehran University of Medical Sciences. Informed consent was obtained for all participates (Patient and controls).

Statistics

The data was analysed using the SPSS statistical software. Basic descriptive statistics (mean, standard deviation) were examined for all individual items and scales. The means comparisons of scales for PedsQL in asthmatic and healthy groups were performed using the independent samples of *t*-test. One-way analysis variance (ANOVA) and Tukey

Post Hoc Tests were used to assess differences on PedsQL dimensions among the various asthmatic groups. *P* value (p<0.05) relate to differences /associations between all existing scales.

Result

There were 160 asthmatics and healthy children enrolled in this study with girls (51.4%) outnumbering boys marginally. Their mean age was 9.09 yr (range= 8-12). Sixty-three percent of the patients had well-controlled asthma, 20.7% had partly controlled asthma and 13.4% had uncontrolled asthma. In this study 86.7% of mothers answered to questionnaires. The results of Family Information Form questionnaires imply that the asthma disease has significantly impact on their parentss work and daily activities (p<0.001) (table 1).

Table 1: Comparison of Demographic Characteristics between asthmatic and healthy children and their mothers

	No. of children (%)	
	Asthmatic children (n=80)	**Healthy children (n=80)**
Characteristics of children		
Age (years) Mean(SD)	9.07(3.12)	9.12(3.51)
Gender		
Boys (%)	41(51.4)	41(51.4)
Girls (%)	39 (48.6)	39(48.6)
Characteristics of parents	9(11.2)	8(10)
Father (%)	71(88.7)	72(90)
Mother (%)		
Parent's education		
<Middle school	18(22.5)	10(12.5)
High school	45(56.2)	48(60)
>University	17(21.25)	22(27.5)
Employment status		
Full or part time	12(15)	14(17.5)
Not employed	68(85)	66(82.5)
Impact of child health status on parents daily work Mean(SD)	62.80(32.45)	98.19(18.07)[a]
Asthma status		
Well controlled(%)	52(63.4)	-
Partly controlled(%)	17(20.7)	-
Uncontrolled(%)	11(13.4)	-

[a] Analysis of *t*-test , *P* value=0.001

Basic descriptive information and mean score of PedsQL scales is presented in table 2. The total score in healthy children is 79.69 whilst in asthmatic children it is 56.78. Lowest scores in asthmatic children and highest scores in healthy children were reported in our Emotional Functioning scale. Our study showed that, Emotional Functioning scores in the asthma group were significantly lower than those in the healthy group (p<0/001). However, we found that the comparisons in the Physical, Social, and School Functioning between the asthmatic and healthy children demonstrated no statistically significance (table 2). Our results also suggest that Physical Functioning was significantly better in well-controlled asthma group (p<0.001); however no association between asthma control level to Emotional, Social and School Functioning was found (table 3).

Table 2: Mean and standard deviation for PedsQL scales in asthmatic and healthy group

scales	**Asthmatic children Mean (SD)**	**Healthy children Mean (SD)**	***P* value**
Physical Functioning	77.36 (27.35)	83.33 (22.91)	.169
Emotional Functioning	20.41 (31.59)	89.99 (21.48)	.001
Social Functioning	65.39 (17.13)	81.32 (15.80)	.092
School Functioning	66.77 (22.36)	78.02 (19.81)	.137
Psychosocial Health	64.90 (11.02)	82.57 (13.53)	.164
Total score	56.78 (10.97)	79.69 (11.33)	.655

Table 3: The comparisons of PedsQL mean scores in three asthma subgroups

Scales	**Well control[a] Mean (SD)**	**Partly control[b] Mean (SD)**	**Uncontrolled[c] Mean (SD)**	**P value*.**
Physical Functioning	84.61 (19.82)	57.51 (33.84)	73.73 (33.43)	.001
Emotional Functioning	69.87(19.46)	65.68 (24.09)	53.78 (29.19)	.081
Social Functioning	65.74(17.43)	65.80(16.84)	63.06(17.55)	.885
School Functioning	43.38(18.74)	49.63(14.90)	42.04(11.21)	.391
Psychosocial Health	66.24(10.80)	64.32(11.87)	59.49(9.86)	.163
Total score	58.20(10.02)	52.10(13.35)	57.32(10.26)	.220

*The mean difference is significant at the .05 level. a,b (p=0.001), a,c (p=0.404), b,c (p=0.230)

Discussion

The present study had three key findings. First, children with asthma are more prone to have impaired Emotional Functioning states when compared to healthy children.

Similar findings were observed in previous studies (15, 16, 17). Blackman also reported that children with asthma had significantly higher incidence of associated behavioural problems (18). There is significant evidence that suggests childhood asthma may be associated with not only functional effects, but also negative psychosocial changes which in turn, have wide-ranging effects on all aspects of the patient's life (19). Asthmatic children have been reported to show a variety of characteristics: depression, anxiety, lack of self-esteem, dependency, immaturity and latent aggression (20, 21). Gordon et al found that stress, as a factor related to childhood asthma; mediate its effect through the asthmatic children in their psychophysical functions (22).

Second key finding in our study was that the child's health status dramatically impacts the parents' daily works and activities. Our result shows that the asthma as a disease in children can affect parents' functions as well. They cannot develop their personal needs effectively and deal with the limitations of their working hours, since most of their time is spent providing care for their sick children. These findings are compatible with the study Hein Raat (10) showing that the families' psychological elements and performance are related to the increase in responsibility. Wolf et al showed that the parents' psychological statement predicts the asthmatic attacks among their sick children (23). In more severe cases, Sinta perry had shown that in several cases mothers of children with asthma are prone to depression, which in turn rapidly promotes the need for more intensive care for their asthmatic children (24).

Third main finding in our study is that there is no significant difference between psychosocial scales among three asthmatic groups (well controlled, partly controlled, uncontrolled). This suggests that the asthma as a chronic disease regardless of the level of control not only impairs the child's physical functions but also affects psychosocial functions of the child. This is despite having their asthma under control. A systematic review of 14 studies by Everhart et al showed that asthma severity is significantly related to child Quality of Life in nine of these studies (25). In contrast, five of fourteen studies as Annett at al did not find significant relationship between asthma severity and child's QOL (26). It is indeed interesting to note that the parent's behaviours of asthmatic children appears different to that of healthy ones. Asthma brings about considerable changes in family behaviour approach insofar as the asthma control levels had no influence over them. Asthma causes irreversible affects in terms of the psychosocial manners on child, which despite seeing improvement in symptoms; they do function as similar to that of healthy children. It is therefore not wrong to suggest that asthma has long-term effects on children which not only affect their physical state of but that of their state of mind. What is worrisome is that specialist are more likely to provide medical treatments to overcome physical limitation while psychosocial dysfunctions caused by the disease remains to be at large.

In conclusion, asthma disease causes some limitations on the functions of asthmatic children and their family as compared to healthy individuals. Clinically, the current study suggests that not only asthma and allergy specialists treat physical disease symptoms, but they should consider the psychosocial functions of asthmatic children and their parents. Although our study does not indicate that the level of asthma control can be used as an indirect indicator of psychosocial impact, it does demonstrate that all asthmatic children are significantly affected psychosocially. In many cases, psychologists in health care team could improve the psychosocial functions of asthmatic child as well as their medical treatment. We suggest that psychosocial assessment should be considered as part of the assessment to provide better level of asthma control since this appears to be an integral part of treating chronic asthma in children, regardless whether they are well controlled or not.

Acknowledgement

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TRANSIENT UNILATERAL PNEUMOPAROTID FOLLOWING UPPER ENDOSCOPY

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ABSTRACT

Salivary gland swelling is a rare complication of upper endoscopy with less than twelve cases reported in the literature. The swelling is usually transient in nature, with complete resolution in a few hours .While all the major paired salivary glands have been implicated, the exact aetiology remains obscure. In this case report, a sixty one year old female presents with unilateral swelling of the right parotid gland immediately following an upper endoscopy. There was complete resolution of the pneumoparotid with no neurological sequelae.

Keywords: *Endoscopic complications, pneumoparotid, salivary gland, transient salivary gland swelling, sialadenopathy*

Introduction

A 61 year old Malay woman was referred for an upper endoscopy to our gastrointestinal unit. She complained of epigastric pain with relation to food. Symptomatic medical treatment did not alleviate her symptoms. Her medical history included Diabetes and Hypertension. She also suffered from Systemic Lupus Erythematosus (SLE) over the past thirty years but this was well controlled. Her premedication for the upper endoscopy consisted of Midazolam 2.5 mg intravenously and Xylocaine spray to the mouth and oropharynx. The gastroscope was then performed and findings included multiple duodenal ulcers in the first part of the duodenum. There was no stigmata of bleeding. The rest of the endoscopy was normal. Post-operatively in the observation ward the patient complained of unilateral swelling to the right side of the face, immediately after the procedure.

Clinical examination revealed a swelling over the right parotid gland. The swelling was soft and mobile with no bruit or palpable thrill. There was no dysphagia or dysphonia. There was no bleeding from the mouth or any surgical crepitus around the neck. No obvious facial nerve injury was noted on either side. The patient remained clinically stable and her oxygen saturation was 95% on room air. Her upright chest roentgenogram was normal. The patient was kept under observation for three hours and remained stable throughout. The swelling subsided spontaneously with complete resolution. The patient was then sent home and put on eradication therapy for her duodenal ulcers. Follow-up a week later revealed no further swelling and the patient has remained asymptomatic.

Discussion

The term pneumoparotid was initially described by Hyrtl in 1865, in musicians who were learning to play wind instruments (1, 2). Other terms for this condition include pneumatocele glandulae parotis, wind parotitis, surgical mumps, anesthesia mumps, and pneumosialadenitis (3). The anatomy of Stensen's duct usually prevents a retrograde influx of air. The normal intraoral pressure is 20 mmHg which increases to 150mmHg with forceful exhalation of air within a closed mouth. The redundant mucosal folds that surround the slit-shaped orifice, and smaller diameter of the duct orifice prevent large increases in intraoral pressure (1, 2). Transient parotid swelling has been documented as a rare complication of upper endoscopy (4).There is thought to be an association with retching, though there have been cases without any retching recorded. The two types of submandibular swelling is attributed to air distention of blind branchial cleft remnants or salivary gland swelling e.g. the parotid gland (4). The hypothesized aetiology for the salivary gland swelling is the ductal compression between the mylohyoid and hyoglossus muscles (5). This is due to pressure from above during increased salivary flow as a result of oral stimulation during the endoscopy.

This transient sialadenopathy has also been reported during the induction of anaesthesia with endotracheal intubation. Parasympathetic stimulation of the salivary glands is thought to cause vasodilatation and hyperemia. All the reported cases in the literature indicate that the salivary gland swelling is transient, painless and requires no treatment after removal of the endoscope (6). It is incumbent upon endoscopists to be aware of this rare complication so as to reassure the patient of its transient nature and lack of any real morbidity.

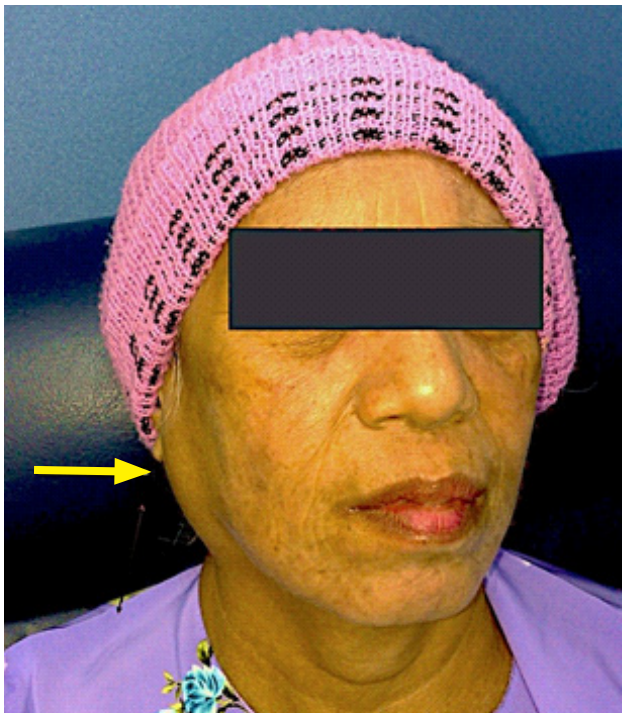

Figure 1: *Right parotid swelling (yellow arrow)*

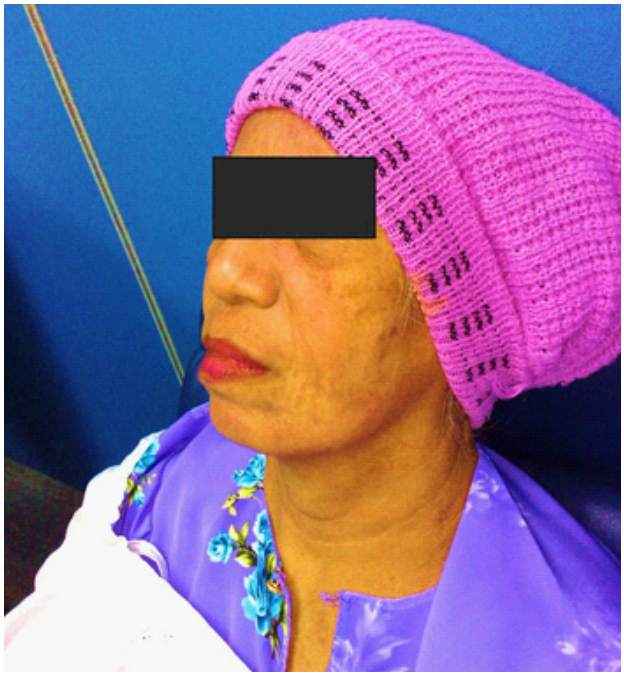

Figure 2: *Normal left parotid gland*

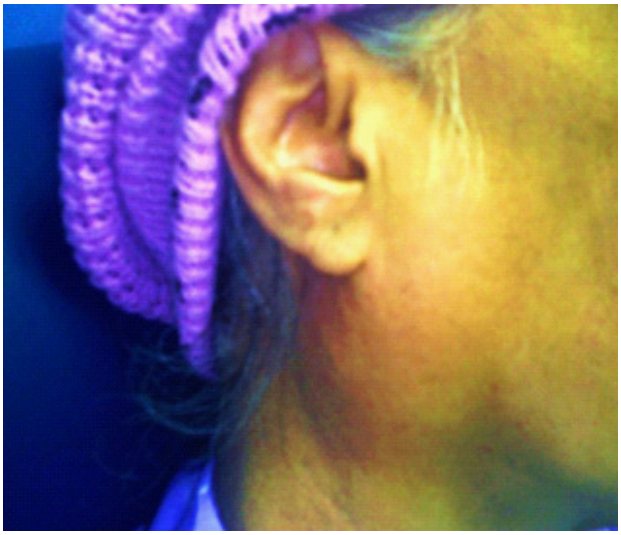

Figure 3: *Right pneumoparotid lateral view*

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THE RELEVANCE OF MICRORNAS IN VASCULAR AGING

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ABSTRACT

MicroRNAs (miRNAs) are short, single-stranded non-coding RNAs that control gene expression by annealing to complementary sequences in mRNAs. They are estimated to regulate at least one third of human transcripts and hence, manipulation of these miRNAs can profoundly affect the proteome and ultimately cellular phenotypes. A substantial amount of work has shed light on the crucial roles of miRNAs in diseases. miRNA expression profiles between normal and diseased tissues have identified miRNA signature patterns that correlate to disease development and progression. This review discusses some of the important miRNAs that are involved in endothelial cell senescence and dysfunction that contribute to the development and progression of cardiovascular diseases.

Keywords*: Cellular senescene, endothelial cells, miR-299-3p*

Introduction

Cellular senescence is defined as the limited ability of cells to proliferate after a finite number of population doublings. Senescent cells do not divide but remain viable and metabolically active. These cells have enlarged and flattened morphology, increased granularity and vacuolization as well as adhesion to extracellular matrix while losing cell-to-cell contacts, altered patterns of gene and protein expression, and shortened telomere length. They characteristically express senescence-associated-β-galactosidase (SA-β-gal) activity at pH 6.0 (1). Organismal aging is associated with cellular senescence as increased occurrence of senescent cells can be found in aged human tissues including the vasculature (2), skin (3), liver (4), kidney (5), astrocytes (6) and prostate (7), contributing to age-related diseases such as atherosclerosis, Alzheimer's disease and benign prostatic hyperplasia. Senescence can be triggered by the shortening of telomere during each cell cycle division which eventually leads to telomere "uncapping" which restricts cell division, hence, the term cellular senescence (8, 9). An imbalance in the biological redox reactions between the high level of oxidative stressors, such as hydrogen peroxide (10) and the low level of anti-oxidants can damage cellular DNA, RNA and proteins and induces cellular senescence (11), Other triggers of senescence include aberrant activation of oncogenes such as *Ras* and *Myc*, or silencing of tumor suppressor genes such as *PTEN* and *pRb*, can result in oncogenic stress and cause oncogene-induced senescence (OIS) *via* three intrinsic pathways: p16/pRb-, Arf/p53/p21- and DNA damage response (DDR)-pathway. Oncogene-induced senescence often occur when the stress surpasses a threshold level, the cells are deprived of nutrients or growth factors, there are improper cell-cell contacts, and the cells are exposed to sub-lethal dose of DNA damaging agents such as anticancer drugs and gamma irradiation (8, 12). Cellular senescence has been described as a double edge sword since it does not only suppresses uncontrolled proliferation for cells at risk of neoplastic transformation (2), but also contributes to aged-related loss of tissue function or pathologies such as atherosclerosis in aged individuals (13).

MicroRNAs (miRNAs) are a well-recognized group of short non-coding RNAs that regulate gene expression by targeting 3' untranslated region (UTR) of mRNA transcripts and inhibit mRNA translation. In-depth details on the biogenesis and the mechanism of actions of miRNAs are reviewed at length elsewhere (14). Post-transcriptional regulation mediated by miRNA is pervasive in human because they are estimated to regulate at least one third of human transcripts as they can bind to multiple targets and suppress mRNAs translation (15). Although most miRNAs repress gene expression *via* translational inhibition or degradation of mRNA, some miRNAs can activate gene expression *via* upregulation of the translation of mRNA (16). Emerging evidence suggest that miRNAs play crucial roles in diverse biological processes including somatic

and stem cell differentiation, cell proliferation, organ development, cell death, signaling in various diseases (17). Hence, it is not surprising that deregulation of miRNAs are associated with numerous diseases. These include cardiovascular diseases such as myocardial infarction and atherosclerosis (18), various types of cancer such as breast, lung, nasopharyngeal and thyroid carcinoma (19-22), neurodegenerative diseases such as Alzheimer and Parkinson diseases (23, 24) and type 2 diabetes mellitus (25).

An important risk factor for cardiovascular diseases is aging (26). Accumulation of senescent cells in the vasculature leads to impaired vascular homeostasis. It has been reported that loss of vasomotor function of the major vasculature among elderly can contribute to the risk of development of cardiovascular diseases (27). It has been demonstrated that senescence of endothelial cells can lead to endothelial dysfunction due to an imbalance between vasodilating and vasoconstricting mediators released by (or acting on) the endothelium which subsequently results in reduced vasodilatation, a pro-thrombotic and pro-inflammatory state (28, 29). Endothelial dysfunction is further associated with atherosclerosis (30), hypertension (31), coronary artery disease (32), chronic heart failure (33) and peripheral artery disease (34). Previous studies have shown that deleterious changes in senescent endothelial cells lead to impairment of endothelium-dependent vasodilation and thrombogenesis that contribute to the pathogenesis of atherosclerosis (35). In addition, studies have shown that telomere shortening and activation of Notch pathway modulate senescence of endothelial cells in atherosclerosis (36).

In vitro studies using isolated human blood vessels and *in vivo* studies in animal and human demonstrated that there is a decrease in the release of endothelium-derived relaxing factors such as nitric oxide (NO), prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) but an increase of endothelium-derived vasoconstrictors such as prostanoids and endothelin-1 (ET-1) in senescent endothelium (29). NO, the major relaxing factor is synthesized by endothelial nitric oxide synthase (eNOS) and the decline in NO level produced by senescent endothelium is reported to be due to a decline in the phosphorylation of eNOS by Akt in senescent endothelium (37). Impaired release of endothelium-derived relaxing factors leads to progressive endothelial dysfunction (29).

The importance of senescence in age-associated diseases highlights the need to study and further understand the molecular signaling of endothelial cell senescence, particularly on the role of miRNAs and their target genes in regulating senescence given that miRNAs are found deregulated in these diseases. This review discusses the involvement of selected well known miRNAs in endothelium senescence and vascular aging and their roles in cardiovacular diseases.

miR-34a

miR-34a is consistently upregulated in both cell culture and various organs of aged mouse (38-40). Overexpression of miR-34a has been shown to inhibit proliferation and to induce senescence of endothelial cells (39). The prime target of miR34a is Sirtuin 1 (SIRT1) (41) which is a NAD^+-dependent protein/histone deacetylase that regulates memory and plasticity, cell cycle progression, angiogenic functions, cellular senescence, apoptosis and various cellular metabolisms through its interaction with a number of molecules, including Ku70, p53, PML and FOXO (42-44). SIRT1 is essential for endothelial functions and it promotes longevity by preventing cellular senescence. Downregulation of SIRT1 by miR-34a is detrimental for endothelial and progenitor cells functions (40, 45). SIRT1 expression level decreased with age inversely to miR-34a in mouse brain tissues (39). More recently, miR-34a was found induced in aging mouse and human heart, and its silencing reduced age-associated cardiomyocyte cell death and improved myocardial function (46). This study further showed that silencing of miR-34a released the suppression of its other target, protein phosphatase 1 nuclear-targeting subunit (PNUTS), which reduces telomere shortening, DNA damage responses and cardiomyocyte apoptosis, and improves functional recovery after acute myocardial infarction (46). In coronary artery disease (CAD) patients, levels of miR-34a were also higher compared to the non-CAD group, whereas levels of SIRT1 protein were lower in the CAD group than in the non-CAD group. In patients treated with atorvastatin, a drug that lowers blood cholesterol, marked decrease in miR-34a levels and increase in SIRT1 levels were noted (45). Results from this study show that miR-34a is a valuable biomarker and should be explored for therapeutic monitoring of cardiovascular diseases and other age-related diseases.

miR-217

The expression of miR-217 is upregulated in senescent endothelial cells and in human atherosclerotic plaques. Its expression level inversely correlates to those of SIRT1 and eNOS. Hence, overexpression of miR-217 downregulates SIRT1, increases eNOS acetylation and reduces eNOS protein expression leading to the reduction of the major endothelium relaxing factor, NO (38, 47). miR-217 also reduces eNOS through the downregulation of FOXO1 (forkhead box O1) (38) and FOXO3 (48), which negatively regulates angiogenesis and vascular growth.

miR-29 family members

The miR-29 family members are upregulated in senescent endothelial cells (38), aortas of aged mice (49) and atrial tissues of dogs (50). The upregulation of the expression of miR-29 family members during senescence involves the

activation of retinoblastoma tumor suppressor protein (Rb) pathway, which is a central pathway essential for the induction of senescence (51). Its overexpression is reported to be triggered by DNA damage and is mediated by p53 (52). Furthermore, miR-29 family members repressed Ppm1d phosphatase which in turn enhanced the activity of p53 (52). In normal aged ICR mouse and klotho-deficient senescence mouse models, the upregulation of miR-29 resulted in reduced type IV collagen, which can weaken the basal membrane of aged tissues (53). Using an aneurysm mouse model, it was shown that interference of miR-29 expression enhanced the expression of matrix protein and improved the integrity of the vascular wall, suggesting that miR-29 may be a valuable target for the maintenance of the vascular wall integrity (49).

miR-146 family members

Two members from this family, namely miR-146a and -146b, are known for their roles in regulating inflammation. Their expressions are inducible by pro-inflammatory cytokines in cultured endothelial cells (54) and are found elevated in human atherosclerotic plaques (55). It was further shown that miR-146a and -146b suppressed the pro-inflammatory NF-κB pathway and downstream EGR and AP-1 transcription factors that drive inflammatory gene expression. This then suppressed endothelial activation and response to pro-inflammatory cytokines which in turn protected the vascular cells from cytokines-induced damages (54). Moreover, they also directly target and inhibit the expression of HuR, an RNA binding protein that promotes endothelial activation by increasing eNOS expression (54). By inhibiting HuR, endothelial activation can be suppressed. Other studies also revealed that miR-146 family members control inflammation by inhibiting interleukin-1 receptor-associated kinase 1 (IRAK1), an enzyme required for the upregulation of NF-κB in fibroblast cells (56) and mouse heart tissues (57). Inhibition of IRAK1, hence, can inhibit the pro-inflammatory NF-κB pathway. miR-146a also targets NADPH oxidase 4 (NOX4) which is a predominant NOX in endothelial cells. NOX4 generates ROS and contributes to endothelial senescence and repression of NOX4 by miR-146, hence reduced oxidative stress and replicative senescence in endothelial cells (58). These studies collectively suggest that enhancement of miR-146 family members may be a potential strategy to protect vasculature against inflammation and oxidative stress-induced senescence and damages.

miR-17-92 family

miR-17-92 cluster is a well-defined group of miRNAs involved in endothelial functions and angiogenesis. This is a polycistronic miR cluster that encodes the miR-17, miR-18a, miR-19a/b, miR-20a, and miR-92a. The miR-17-92 cluster is commonly linked to tumor angiogenesis, where overexpression of the miR-17-92 cluster promotes tumor angiogenesis (59). Specifically, miR-17, 18 and 19 exert their pro-angiogenic activities through the downregulation of the extracellular matrix proteins connective tissue growth factor (CTGF) and thrombospondin-1 (TSP-1), an anti-angiogenic molecule (60). This is also observed in aged mice, where the expressions of miR-18/19 concomitantly increased with the decreased in their targets, CTGF and TSP-1, which contributed to a failure-prone heart (61). On the other hand, miR-92a has anti-angiogenic activity where overexpression of miR-92a suppressed angiogenic sprout formation *in vitro* and interfered with intersegmental vessel growth in zebrafish (62). However, the role of each member in angiogenesis may be distinct in selective cells or environments. Combined overexpressions of miR-17, miR-18a, and miR-20a in endothelial cells can rescue the impaired endothelial functions caused by the inhibition of Dicer, an RNA endonuclease that regulates miR maturation (63). In contrast, overexpression of individual miR, namely, miR-17, miR-18a, miR-19a, and miR-20a alone, reduced sprouting of endothelial cells (64). It is unclear why their effects differed as a group and as individuals but the authors suggested that they have cell-intrinsic activities (64) and that they may also function differently in physiological and tumor angiogenesis (64). Hence, these data suggest that manipulation of these miRNAs for therapeutic purposes requires careful consideration since they are important modulators in both cancer and aging pathologies.

miR-299-3p

miRNAs discussed in this review represent some of the highly studied and consistently reported ones. As science advanced with more powerful and highly sensitive tools such as next generation sequencing and microRNA array system, it is not impossible to uncover novel miRNAs and to map their functions. We used an integrated miRNA and gene profiling approach to identify miRNAs and their targets that are associated with endothelial senescence (65). We found that miR-299-3p is up-regulated in senescent HUVECs compared to the young cells and one of its target genes could be *IGF1*, which is largely known to be associated with aging. Knockdown of miR-299-3p also resulted in significant reduction in the percentage of cells positively stained for senescence associated-b-galactosidase, increased in cell viability measured using MTT assay but marginal increases in cell proliferation and cell migration capacity measured by real-time growth kinetics analysis. Moreover, knockdown of hsa-miR-299-3p also increased proliferation of H_2O_2-induced senescent endothelial cells. Our findings showed that knockdown of miR-299-3p can delay or protect against cellular senescence of endothelial cells by improving the metabolic activity of the senesced cells and this miRNA also acts in concert with miR-338-3p and miR-134 (65).

Conclusion

Evidence from various studies discussed above strongly support the notion that miRNAs are relevant and play

important roles in endothelial cells senescence and vascular aging. While some of their target genes are known and some information on the associated mechanisms are defined, there remains much to be investigated. Prior to the development into miRNA-based therapeutics, many questions remain and these include the mechanism of increased or decreased expression of these miRNAs during senescence and aging, how and what regulate these miRNAs and whether their deregulation is direct effector of disease development or part of the feedback mechanisms. With the use of advanced technology such as next generation sequencing, more novel miRNAs are being identified and their functions validated. The answers to these questions will have significant implications to their use as therapeutics and biomarkers.

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CELL-BASED THERAPY FOR THE TREATMENT OF FOCAL ARTICULAR CARTILAGE LESIONS: A REVIEW OF SIX YEARS OF STUDIES IN A MALAYSIAN UNIVERSITY MEDICAL CENTRE

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ABSTRACT

Autologous chondrocyte implantation (ACI) is a significant technique that has gained widespread use for the treatment of focal articular cartilage damage. Since its inception in 2004, the Tissue Engineering Group (TEG) of the Faculty of Medicine, University Malaya has been dedicated to carrying out extensive research on this cell-based therapy. The objective of this report, comprising one clinical case report, six animal studies and one laboratory study, is to summarise and discuss TEG's key findings. On the whole, we observed that the ACI technique was effective in regenerating hyaline-like cartilage in treated defects. Autologous chondrocytes and mesenchymal stem cells (MSC) were found to produce comparable tissue repair irrespective of the state of MSC differentiation, and the use of alginate-based scaffolding and oral pharmacotherapy (Glucosamine and Chondroitin Sulphate) was shown to enhance ACI-led tissue repair. ACI is suggested to be an efficient therapeutic option for the treatment of articular cartilage defects of the knee.

Keywords: *articular cartilage, cell therapy, autologous chondrocyte implantation, animal studies, review*

Introduction

The treatment of articular (hyaline) cartilage injuries is one of the most difficult challenges for orthopaedic surgeons. As aptly stated by William Hunter in 1743, 'an ulcerated cartilage is a troublesome problem and once destroyed, it never repairs'. This is because articular cartilage is avascular, with very limited healing capacity. Injury to articular cartilage begins an inexorable cascade of pathobiological and pathomechanical events that lead towards loss of tissue and formation of defect, in contrast with the process of inflammation and repair found in vascularized tissue. If left untreated, articular cartilage lesions invariably progress in size and lead to degenerative changes typical of osteoarthritis (1-2).

Articular cartilage lesions can result from sports injuries, trauma, osteoarthritis or osteochondritis. In Malaysia, symptomatic knee osteoarthritis is responsible for the largest burden of joint pain and is the single most important rheumatological cause of disability and handicap. A Community Oriented Program for Control of Rheumatic Diseases (COPCORD) survey conducted in 2007, involving a total of 2594 community members from Banting, revealed that 9.3% of adult Malaysians complain of knee pain, with clinical evidence of osteoarthritis in more than half those examined for knee pain (3). The incidence was noted to be higher in the aging population, with a sharp increase in pain rate to 23% in those over 55 years of age, and 39% in those over 65%. For those who suffer from degenerative joint disease, the ability to regenerate cartilage would reduce the need for joint replacement, decrease pain, increase mobility and lead to a better quality of life.

As such, the restoration of articular cartilage defects is of paramount importance due to an urgent and expanding clinical need, especially with the expected 269% increase in the elderly (above 65 years) population in Malaysia between the years 2008 and 2040 (4). In recent years, the cell-based therapy termed autologous chondrocyte implantation (ACI) has emerged as a promising technique to repair full-thickness chondral defects. The early work done by Hui *et al* of the National University of Singapore (NUS) in 1999 involving 52 Singaporean patients reported a symptomatic improvement in 90.2% of patients, with no adverse reaction to surgery (5). However, there are minimal data on the efficiency of the ACI technique in Asian populations available at present. Since its inception in 2004, the Tissue Engineering Group (TEG) of the Faculty

of Medicine, University Malaya has been dedicated to carrying out extensive research on the ACI technique, with the aim of advancing knowledge and repair outcomes in the treatment of articular cartilage defects. In particular, we have directed our investigations towards focal articular cartilage lesion of the knee, defined as a circumscribed, full-thickness cartilage defect down to, but not through, the subchondral bone on a load-bearing surface of the femoral condyle or patellar facet.

The objective of this report is to present TEG's six years of experience in engineering cartilage cell therapy from the year 2007 to 2012. We have embarked on a collective appraisal of our centre-led trials (clinical, animal, laboratory studies) and scientific literature to discuss and compare our findings with previously published data. In particular, we aim to: 1) provide an overview of cartilage surgical repair techniques; 2) examine the clinical outcome and regenerative profile of conventional ACI; 3) discuss the chondrogenic potential of transplanted cells; 4) discuss the role of cell carriers and scaffolds; and 5) discuss the role of local and systemic pharmacological agents.

An overview of cartilage surgical repair techniques

A number of orthopaedic surgical procedures have been developed in an attempt to alter the otherwise dismal natural history of chondral defects. Arthroscopic debridement was the earliest technique utilised to treat articular cartilage defects of the knee. Although this procedure was shown to provide clinical improvements (6-9), the benefits were short-lived and the technique did not address the underlying pathology (10-12). Attempts were then made to improve the long-term results of cartilage repair by directing treatment towards exploiting the natural intrinsic repair response, observed upon penetration of the subchondral bone in full-thickness defects. Methods that accomplish this type of repair are termed marrow stimulation treatment (MST) techniques, and include subchondral drilling, abrasion arthroplasty and microfracture. In MST, subchondral bone is perforated to induce mesenchymal stem cell infiltration into a chondral defect, which leads to the formation of a clot that may differentiate into repair tissue. While MST procedures have demonstrated good to excellent results in 60-80% of patients (13-14), they typically promote the development of fibrocartilage that is biomechanically inferior to articular cartilage (10-12,15-16) and deteriorates over a short period of time postoperatively (16-18).

In 1965, Audrey Smith was able to culture chondrocytes in vitro after freezing, culminating in the advent of cryopreservation. This led to modern cartilage transplantation techniques, which include perichondral and osteochondral grafts. Perichondral grafts harvested from donor sites and implanted into chondral defects have been shown to lead to marked functional improvement (19, 20). Osteochondral grafts, which harvest osteochondral plugs from low weight-bearing areas within the knee joint for implantation into chondral defects, have shown promising results with a success rate of up to 80% (21-25). Although both these techniques are able to retain the viability of hyaline tissue unlike previous surgical interventions (20, 26), perichondral grafts are susceptible to ossification and graft failure (10, 20) while osteochondral grafts are limited by donor site morbidity concerns (10) and the lack of lateral integration of mosaic plugs and recipients, which may lead to degeneration of the graft over time (17, 27).

In recent years, the numerous experimental attempts to reestablish structurally and functionally competent repair tissue which is of an enduring nature has led to the advent of autologous cell extraction for implantation into chondral defects. A Swedish group led by Lars Peterson and Mats Brittberg was the first to describe the technique of autologous chondrocyte implantation (ACI) in 1984 (28), which was the first application of a cell-based approach in orthopaedic surgery. As previous studies had shown the ability of autologous cells to regenerate cartilage, Peterson *et al* refined a three-step procedure where chondrocytes isolated from cartilage biopsy were harvested arthroscopically from a non weight-bearing area, isolated enzymatically and expanded in a monolayer culture, and then implanted into the cartilage defect using a periosteal patch over the defect as a method of cell containment. Encouraged by their early success in rabbit models, the Swedish group refined the technique to culture autologous chondrocytes from a small sample of patients' own normal articular cartilage, and began a clinical trial to treat full-thickness defects in patients' knees.

In a variation of ACI, Wakitani *et al* exploited the self-repair potential of mesenchymal stem cells (MSC) in treating chondral defects (29). MSC are pluripotent progenitor cells that can be ex vivo expanded and induced, either in vitro or in vivo, to terminally differentiate into chondrocytes. They have emerged as a potential cell source for cartilage cell therapy due to their multipotential capacity, easy isolation and culture, and high ex vivo expansive potential in governing the rapid and specific repair of skeletal tissues (30, 31). Aside from cells, recent advancements in cartilage cell therapy have focused on exploring the potential of chondro-conductive scaffolds and biofactors in further improving articular cartilage repair, giving rise to the terms 'second-generation' and 'third-generation' ACI.

The usage of ACI in the repair of cartilage has generated much interest within the orthopaedic community, and there have been numerous studies reporting on ACI. A systematic review published in 2011 by our centre suggested that there were at least 568 scientific papers reporting controlled and randomised clinical studies related to ACI, involving 1,644 patients in total (32). In this paper, comparison with other treatments suggested that ACI is associated with superior structural regeneration of cartilage tissues and better clinical outcomes when compared with microfracture, similar or superior outcomes

when compared with mosaicplasty, and comparable outcomes when compared with MSC transplantation and matrix-assisted autologous chondrocyte implantation (MACI), both of which are techniques adapted from ACI. Many authors comparing the ACI technique with another treatment modality reported more favourable results for ACI (33-38). However, there were also authors who reported comparable (39-41) or less favourable results (42) for ACI. Early systematic reviews examining the evidence for ACI concluded that there was insufficient evidence from the existing literature to determine whether conventional ACI was superior in the treatment of focal articular cartilage defects (43-49). However, the ACI technique has evolved considerably over the past few years. As mentioned earlier, the induction of biomaterial-based scaffolding, alternative cell sources and biofactors are anticipated to improve the outcome of ACI-led cartilage repair, which will be discussed in the following text.

An overview of the studies performed in TEG

The TEG studies investigating the ACI technique for the treatment of focal articular cartilage defects are illustrated in Table 1. There is a total of eight studies included in this paper, comprising a clinical case report, six animal studies and one laboratory study. There were no adverse effects observed in any of the studies.

The clinical outcomes and regenerative profile of the conventional ACI technique

Our first attempt to establish the clinical outcomes of conventional ACI (i.e. utilising autologous chondrocyte and periosteal patch) in our centre was a preliminary case report of two patients (50). In this study, we observed clinical improvements in both patients following ACI-led repair of articular cartilage defects as evidenced by the International Knee Documentation Committee score, Oxford Knee Score and American Knee Society Score at nine months postoperatively (Table 1). Complete filling of treated chondral defects with repair tissue was also demonstrated via magnetic resonance imaging at six months. No complications were observed in both patients during the study period. Our early clinical findings suggested that the ACI technique could be applied in clinical practice for the treatment of chondral defects.

As the compositional changes in repair tissues are known to have major influences on their physiological function, we sought to determine the quality of cartilage regenerated following conventional ACI repair. In order to perform a quantitative and qualitative assessment of regenerated tissue, we opted to examine the glycosaminoglycan (GAG) content and Brittberg score. GAG plays a significant role in regulating the chondrocyte phenotype and is a determinant of the biochemical and mechanical quality of regenerated cartilage (51), whilst the Brittberg score has been found to be statistically reliable and repeatable for the macroscopic assessment of cartilage repair (52). In our later studies, we also used the O'Driscoll score, a validated measure for histological scoring (53), as an outcome measure.

Using nine rabbit models, identical full-thickness defects were created in the articular cartilage of both knees, and a month later the right knee was repaired via ACI while the left knee was left untreated (control group) (54). At three months post-surgery, enhanced macroscopic regeneration, significantly ($p=0.008$) higher cellular expression of GAG and significantly ($p=0.007$) higher mean Brittberg repair score were observed in the ACI-treated knees compared to the control group (Table 1). The study suggested that articular lesions treated by ACI repaired better than those which were not treated.

The work done by other centres has also reported a significant amount of cartilage reconstitution in ACI-treated defects in both preclinical (28,55-56) and clinical studies (57-61). The clinical trial conducted by the pioneering Swedish group led by Peterson and Brittberg, spanning a mean follow-up of 39 months, showed good or excellent clinical outcomes in 87% of cases (n=23) and 'hyaline-like' results in 11 out of 15 treated lesions (57). A more recent publication from this group has shown durable results up to 11 years, with 96% clinical durability (60), and a correlation of good and excellent clinical results with the generation of hyaline-like repair tissue (62). However, not all reports have been favourable. Horas *et al* (42) compared ACI with osteochondral cylinder transplantation, and reported slower recovery and fibrocartiliginous defect filling for ACI-treated patients. Moreover, despite its promising tissue repair potential, several complications arising from the use of conventional ACI have been reported. These include periosteal hypertrophy, disturbed fusion of regenerative cartilage and healthy surrounding cartilage, and dedifferentiation of cellular phenotypes cultured in a monolayer culture (63-67). Although ACI has been shown to produce satisfactory short and medium-term results, further technique improvements are needed to address the typical complications associated with ACI.

The chondrogenic potential of transplanted cells

Chondrocytes, responsible for the secretion of extracellular matrix, were initially the logical and preferred cell source for cell-based cartilage engineering. Although found to produce good outcomes, their use in various models was shown to be limited by issues of donor site morbidity and cellular dedifferentiation when cultured in vitro (65-67). In recent years, MSC has emerged as a promising alternative cell source for cartilage repair. MSC, responsible for bone and cartilage formation in the embryo, and repair and turnover in the adult, are multipotent stem cells with the potential of differentiating into adipocytes, chondrocytes or osteocytes. The multipotency of MSC enables them to be stable phenotypes in vitro as well as progenitors for

Table 1. TEG papers investigating the ACI technique for the treatment of focal articular cartilage defects, in chronological order

Authors	Year	Type	n	Objective	Assessment tools	Follow up	Findings	Adverse events	Conclusions
Abbas et al	2008	Clinical case report	2	To examine the clinical efficacy of ACI	IKDC score OKS AKSS MRI	9 months	Improved clinical post-operative scores Patient 1: IKDC (34.5→79.3), OKS (22→12), AKSS (90→100) Patient 2: IKDC (29.9→87.4), OKS (22→12), AKSS (90→100) MRI: complete filling of treated knee defects with repair tissue	nil	Clinical and structural improvements seen after ACI at 9 months
Kamarul et al	2008	Animal study	9	To compare the efficacy of ACI vs. non-operative measure	GAG expression Brittberg score	3 months	Significantly higher GAG expression in ACI-treated knees (1.12 vs. 0.81µg GAGs/mg protein, p=0.008) Significantly higher Brittberg score in ACI-treated knees (6.00 vs. 1.89, p=0.007)	nil	ACI superior to non-operative measure for repairing focal cartilage defects
Kamarul et al	2010	Animal study	18	To compare the efficacy of chondrogenic differentiated MSC (C-MSC) vs. undifferentiated MSC vs. autologous chondrocytes	Brittberg score, O'Driscoll score, GAG expression	3 and 6 months	Higher scores (all outcome measures) for defects treated with undifferentiated MSC. Treated knees produced significantly (p=0.0024) higher scores compared to untreated (control) group at 6 months. Hyaline cartilage regeneration at treated sites.	nil	Undifferentiated MSC shown to have better tissue repair outcomes compared to C-MSC and autologous chondrocyte
Tay et al	2011	Animal study	30	To compare the efficacy of allogenic undifferentiated MSC (allo-MSC) vs. autologous chondrocyte (auto-C)	Brittberg score, O'Driscoll score, GAG expression	6 months	Similar regenerative profile in both groups except for significantly higher Brittberg scores in the allo-MSC group (8.8 vs. 6.6, p=0.04) All outcome measures significantly higher (p<0.05) in both treatment groups compared to control group (untreated)	nil	Allo-MSC and auto-C have similar efficacy in repairing focal cartilage defects Both treatments superior to non-operative measure
Dashtdar et al	2011	Animal study	12	To compare the efficacy of allogeneic derived chondrogenic pre-differentiated MSC (C-MSC) vs. undifferentiated MSC	Brittberg score, O'Driscoll score, GAG expression	3 and 6 months	No significant difference in all outcome measures between C-MSC and undifferentiated MSC Mean Brittberg score is significantly higher (p<0.05) in treated knees compared to untreated knees (control) at 6 months, with hyaline-like cartilage regeneration	nil	C-MSC and MSC produced comparable treatment outcomes
Kamarul et al	2011	Animal study	18	To examine the effect of GS and CS on healing of ACI-treated articular cartilage defects	Brittberg score, O'Driscoll score, GAG expression	3 and 6 months	Significantly higher (p<0.05) Brittberg score and O'Driscoll score for GS and GS+CS groups compared to groups without pharmacotherapy at 3 and 6 months, and GAG values at 6 months. Histology revealed typical hyaline cartilage structure.	nil	Combination of ACI and pharmaco-therapy may improve healing of focal cartilage defects
Ab-Rahim et al	2013	Lab Study	9	To compare the extracellular matrix expression produced by chondrocyte-alginate constructs ± TGF-β1 vs. monolayer culture	Immuno-histochemical evaluation, histology exam, TEM, SEM	-	Significant increase in GAG/mg protein levels in chondrocyte cultures grown in alginate than in monolayer cultures (1.071µg/mg protein vs. 0.435µg/mg protein, p<0.05) and an abundance of ECM protein distribution was observed	nil	Use of alginate hydrogel beads in chondrocyte cultures (± TGF-β1 supplement) produced superior ECM expression than monolayer cultures
Dashdtar et al	2013	Animal study	24	To compare repair outcomes of MSC seeded in novel PVA-chitosan composite hydrogel vs. established alginate-transplanted models	Brittberg score, O'Driscoll score, GAG expression	6 months	Significant (p<0.05) results for groups treated with PVA-chitosan-MSC or alginate-MSC compared to PVA-chitosan-scaffold and untreated controls. No significant difference between PVA-chitosan-MSC and alginate-MSC group.	nil	Comparable outcomes with PVA-chitosan-MSC and alginate-MSC for cartilage repair.

TEG Tissue Engineering Group; *ACI* autologous chondrocyte implantation; *IKDC* International Knee Documentation Committee; *OKS* Oxford Knee Score; *AKSS* American Knee Society Score; *MRI* magnetic resonance imaging; *GAG* glycosaminoglycan; *MSC* mesenchymal stem cell; *GS* Glucosamine sulphate; *CS* Chondroitin sulphate; *TEM* transmission electron microscopy; *SEM* scanning electron microscopy; *ECM* extracellular matrix; *PVA* polyvinyl alcohol

other cell types despite being removed from their original environment (68-69). MSC can be isolated from various tissues, but bone marrow-derived MSC have been found to have better chondrogenic differentiation compared with other sources and are easily attainable (70-71).

To determine the cell source with the highest chondrogenic potential, we conducted a study to compare the treatment outcomes of alginate-embedded allogenic undifferentiated MSC against autologous chondrocytes in the repair of focal articular cartilage defects in 30 rabbit models (72). We found that, apart from significantly (p=0.04) higher Brittberg scores in the allogenic MSC treatment group, both treatments showed similar cartilage regenerative profiles (Table 1).

Similar to the findings of our study, many authors have reported that autologous chondrocytes and MSC produce comparable repair outcomes (29,73-74). Hui *et al* (73) from NUS compared the efficacy of chondrocytes, MSC, periosteal grafts and mosaicplasty for treating osteochondritis dissecans in rabbits, and reported that cultured chondrocytes and MSC had comparable enhancing effects on the repair of chondral defects, whereas mosaicplasty did well initially and periosteal grafts did less favourably. Yan *et al* (74) from Peking University evaluated the repaired tissue formed in 36 full-thickness rabbit cartilage defects implanted with chondrocytes, MSC, fibroblasts and human umbilical cord blood stem cells (hUSC-SC), and reported hyaline-like cartilage repair and significantly higher histologic scores in groups treated with chondrocytes and MSC compared with groups treated with fibroblasts and hUSC-SC. Similarly, Nejadnik *et al* compared the clinical outcomes of 72 patients treated with conventional ACI with patients treated with autologous MSC and found no discernable differences between the MSC and ACI group in terms of clinical outcomes (75).

Another issue to consider with regards to the choice of transplanted cells is the suggestion that lineage committed cells have superior chondrogenic expression markers compared with undifferentiated MSC (76-77). As in-vivo assessments of chondrogenic pre-differentiated MSC to repair cartilage defects had not yet been initiated, we decided to perform a preliminary study in which full-thickness focal cartilage defects were created in 18 rabbit models and treated with either chondrogenic differentiated MSC (CMSC), autologous chondrocytes or undifferentiated MSC (78). Quantification analyses demonstrated higher scores in terms of mean Brittberg score, GAG quantification and histological score for defects treated with undifferentiated MSC compared with ones treated with CMSC and autologous chondrocytes, although these findings were not significant (Table 1). On the other hand, in a subsequent study comparing cartilage repair potential between allogenic-derived CMSC and undifferentiated MSC for full-thickness cartilage defects of 12 rabbits, we observed no significant differences between the Brittberg, O'Driscoll scores, GAG and total protein content when comparing defect sites treated with MSC and CMSC (79) (Table 1). These findings suggest that transplantation of MSC for the repair of articular cartilage defects produces a comparable repair to autologous chondrocytes, irrespective of their state of differentiation. This may have clinical implications forthe wider use of undifferentiated MSC in cell-based therapy, as this option is associated with more simplified and economical laboratory processing. However, as there are no available papers at present with which to compare our findings, these observations are preliminary.

The role of cell carriers and scaffolds

First-generation (conventional) ACI utilising periosteal grafts, as developed by Peterson *et al*, was observed to produce good repair outcomes, but this was complicated by periosteal hypertrophy, local morbidity for periosteal harvest and graft failures (63, 64, 80, 81). Second-generation ACI explored the use of collagen membranes as a replacement for periosteal grafts, and was found to eliminate the drawbacks associated with the use of periosteal flaps with no statistical reduction in clinical outcomes (61, 82). However, both first- and second-generation ACI techniques have been reported to be limited by the risk of cell leakage with inadequate sealing (83) and dedifferentiation of cellular phenotypes cultured in a monolayer culture (65-67).

Recent advances in tissue engineering have led to the development of third-generation ACI, which relies upon utilising scaffolds and cell carriers to mimic a matrix and provide the necessary information or signaling for cell attachment, proliferation and differentiation (84-85). Chondrocytes are embedded into three-dimensional (3D) constructed scaffolds for cell growth, resulting in an 'all-in-one' graft that does not need a periosteal cover or fixing stitches and can be trimmed to exactly fit the cartilage defect, effectively avoiding spillover or asymmetric distribution of chondrocytes following implantation. Additionally, it has been suggested that the use of certain scaffolds in chondrocytic cultures can eliminate the phenotypic drift associated with monolayer culture (65, 86). This is of particular importance considering that an alteration in the ability of chondrocytes to maintain extracellular matrix expression (ECM) may ultimately lead to inferior repair outcomes in patients.

We decided to study the use of alginate, one of the most studied and applied polymers in the tissue engineering and drug delivery field (85) which is known to improve chondrocyte differentiation in cultures (87-90). To this end, our centre conducted a controlled laboratory experiment where chondrocytes were isolated from rabbit knee articular cartilage and expanded in vitro using a monolayer culture system or alginate chondrocyte construct suspension (91) (Table 1). Results demonstrated a significant increase ($p<0.05$) in GAG/mg protein levels in chondrocyte cultures grown in alginate construct compared with monolayer cultures. In addition, an abundance of ECM protein distribution surrounding the chondrocytes cultured in alginate hydrogel was observed.

To support our findings, many other authors have reported encouraging alginate outcomes in in vitro and in vivo studies and concur that cultures of chondrocytes in alginate beads enable chondrocytes to maintain their spherical shape and typical chondrotypic appearance (87-88), resulting in significantly raised cartilage formation and particularly increased expression of type II collagen (89-90). Correspondingly, the application of alginate in ACI-treated patients has produced significant clinical improvements at two years, with predominantly hyaline-like repair tissue observed in the majority of patients (92). Other matrix and scaffold materials such as collagen (84, 93-95), hyaluronic acid (96-98), agarose (99), fibrin (100) and polyhydroxyacids (101) have shown equally favourable clinical results in cartilage regeneration. Four comparative studies comparing the use of periosteal-ACI with collagen (102-103), hyaluronic acid (104) and polyhydroxyacids (105) produced comparable short-term clinical results, suggesting that at present there is no obvious ranking among the currently available scaffolds for clinical use.

In recent years, hybrid scaffolds that combine synthetic and natural polymer have recently been developed in an effort to further enhance the clinical outcome of ACI-led cartilage repair. Naturally derived materials such as alginate often have desirable biological properties but limited mechanical strength or fat degradation profiles (for eventual integration into surrounding tissue). In contrast, synthetic polymers provide an appropriate 3D environment and have the desired mechanical strength but lack the bioactive properties of natural scaffolds. Composite hydrogels are thought to be able to retain the desirable characteristics of both materials, and have been observed to produce encouraging results in in vitro and in vivo experiments (106-109).

Poly-vinyl alcohol (PVA)-chitosan application has been previously described for wound healing (108, 109) but its role as a scaffold for MSC in cartilage tissue engineering has not been demonstrated. Our group investigated the efficacy of a novel PVA-chitosan composite hydrogel compared with previously established alginate-transplanted models (107). In this study, a medial femoral condyle defect was created in both knees of 24 rabbit models and transplanted, after three weeks, with PVA-chitosan-MSC, PVA-chitosan scaffold alone, alginate-MSC or alginate alone. We observed that the morphological and histological analysis showed significantly better ($p<0.05$) tissue repair when treated with PVA-chitosan-MSC or alginate MSC compared to the scaffold alone and the untreated control (Table 1). No significant difference was observed between the PVA-chitosan-MSC and alginate-MSC groups. These findings suggest that the use of scaffolds and cell carriers (e.g. alginate, PVA-chitosan) in the ACI technique may provide more favourable ECM expression compared to conventional ACI utilising monolayer cultures, by maintaining cell morphology within a conductive culture environment.

Although the induction of scaffolds and cell carriers into the ACI technique has been reported by many authors to be favourable, evidence suggests that while third-generation ACI, utilising scaffolds and cell carriers, seems to promote chondrocyte differentiation and formation of cartilage matrix, it has not resulted in superior clinical results when compared with conventional ACI. The five comparative controlled clinical trials available at present demonstrated similar results when comparing second- and third-generation ACI against first-generation ACI (82, 102-105). However, the authors did note that the use of scaffolds reduced the incidence of periosteal hypertrophy (82, 102).

The role of local and systemic pharmacological agents

Another approach in augmenting tissue differentiation and cell activity in cartilage tissues is via the introduction of biochemical modulators. Glucosamine sulphate (GS) and chondroitin sulphate (CS) are the two most frequently prescribed nutritional supplements for the treatment of osteoarthritis (110), and have been shown to have substantial clinical efficacy in several osteoarthritis studies (111-114). In vitro, GS and CS have been reported to stimulate the synthesis of proteoglycans by cultivated human chondrocytes (115-118). Although investigated extensively in osteoarthritis, the use of these pharmacological agents for the repair of focal chondral lesions has not been explored.

To elicit the role of GS and CS in enhancing ACI-led focal cartilage repair, our centre conducted a preliminary laboratory experiment to establish the effects of oral administration of GS and CS on the healing rate of ACI-repaired chondral lesions in 18 rabbits (119). We observed significantly higher ($p<0.05$) Brittberg scores and modified O'Driscoll scores in groups treated with a combination of ACI and pharmacotherapy (Group ACI+GS and Group ACI+GS+CS) compared with the ACI without pharmacotherapy group at three months, and significantly higher ($p<0.05$) GAG content at six months (Table 1). Based on the results of our study and those reported by others (120, 121), it is suggested that there may be a functional role for GS and CS in augmenting the restoration of ACI-repaired chondral lesions. However, as there are no available papers at present with which to compare our findings, these observations are preliminary.

Conclusion

Earlier systematic reviews examining the evidence for conventional ACI by other authors concluded that there was insufficient evidence in the existing literature to determine whether or not conventional ACI was superior in the treatment of focal articular cartilage defects (43-49). However, the ACI technique has evolved considerably over the past few years. Not only have biomaterials been introduced into the field, MSC has emerged as a promising potential alternative cell source to autologous chondrocytes. Our six years of experience in cell-based therapy, which consists of one clinical study, six animal

studies and one laboratory study, suggests that the ACI technique is a feasible technique for the treatment of focal articular cartilage defects. Our study has shown that the ECM expression produced by a chondro-conductive scaffold construct (e.g. chondrocyte-alginate construct) is superior to the ECM expression produced by chondrocytes grown in monolayer cultures. Moreover, by utilising a 3D chondro-conductive scaffold, the issues of periosteal hypertrophy, cell leakage and dedifferentation of cellular phenotypes cultured in a monolayer culture (63-67, 80-81, 83) associated with conventional ACI can be avoided. Our study has also demonstrated that autologous chondrocytes and MSC have comparable regenerative profiles, irrespective of the state of MSC differentiation. Owing to the ease of harvesting and their multi-potential ability, MSC represent a feasible alternative cell source that can be exploited in repairing articular cartilage defects. Finally, our study has also preliminarily shown the potential of oral pharmacotherapy (GS and CS) in enhancing the healing of ACI-led repair of focal cartilage defects. No adverse reactions were observed in any of our clinical and animal studies. On the whole, these findings collectively suggest that the ACI technique incorporating 1) autologous chondrocytes or MSC; 2) chondro-conductive scaffolds and cell carriers; and 3) oral pharmacotherapy, is a safe and efficient therapeutic option for the treatment of articular cartilage defects of the knee. We are excited to be embarking, in the near future, on research into further methods of refining the ACI technique, including mechanotransduction and gene manipulation. Based on our six years' experience in cell-based therapy, we are optimistic that we will be able to achieve an effective and reproducible solution to the repair of the notoriously difficult to treat chondral lesions.

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PHYLLANTHUS SP A LOCAL PLANT WITH MULTIPLE MEDICINAL PROPERTIES

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ABSTRACT

The plants of the genus *Phyllanthus* (*Euphorbiaceae*) are distributed in most tropical and subtropical regions of world. This plant has been long used as a traditional medicine to treat problems such as stomach, intestinal infections, kidney and urinary bladder disturbances, diabetes, and hepatitis B. There has been considerable interest in these plants in recent years. This review discusses the antiviral and anticancer aspects of *Phyllanthus species*. Scientific studies have demonstrated that extracts and purified isolated compounds (flavonoids, lignans, phenols, and terpenes) obtained from these plants possess antiviral effects against herpes simplex (HSV) and dengue virus infections (DENV). These observations are associated with the disruption of essential proteins needed during viral cycle, thus halting the viral replication. In addition, the *Phyllanthus* species have also been shown to exert inhibitory effects against selected cancers types. In these studies anti-proliferative, anti-metastatic, anti-angiogenic effects and induced apoptosis of human cancers cell lines were observed. These may be explained by the disruption of multiple survival pathways and differential protein expression. CONLCUSION:As a conclusion, tThe *Phyllanthus* plant possesses multiple medicinal properties, including antiviral and anticancer activities which may potentially be used as a medicinal source for many disease locally.

Keywords: *anticancer, antiviral, broad spectrum antimicrobial, dengue, Phyllanthus*

1. *Phyllanthus*

Besides being sources of food supply, plants and herbs also play important roles in the treatment of illnesses indigenously (1-3). One of the best examples is the discovery and development of Artemisinin (antimalaria drug) from *Artemisia annua L* (4). Since our ancestral times, they have discovered and primarily used a vast range of natural compounds to improve healing or treat diseases and injuries. It is only during the past decades where the importance of natural products has emerged second to the designing of synthetic compounds. However, scientists have found a new role for natural products as a basis for drug development and hence a renewed attention to natural products has emerged (5).

Phyllanthus is a small annual plant that is widely distributed throughout the tropical and subtropical regions of world (6). It is a very large genus containing approximately 550-750 species including *P. accuminatus, P. amarus, P. pulcher, P. niruroides, P. anisolobus, P. orbiculatus, P. emblica, P. oxyphyllus, P. flexuosus, P. raticulatus, P. fraternes, P. simplex, P. mullernus, P. urinaria, P. mytrifolis, P. virgatus, P. niruri and P. watsonii* (7-11). In Ayurvedic medicine, *Phyllanthus* has a long tradition of use to treat jaundice, gonorrhea, frequent menstruation, dysentery and diabetes, as well as skin ulcers, sores, swelling, and itchiness (8). In Traditional Chinese Medicinal (TCM), *Phyllanthus* has been used to treat gallstones and kidney stones. In addition, this plant has been thought to stimulate the immune system (9).

The bioactive components present in various plants and foods have a prominent role for diseases prevention. *Phyllanthus* extracts have been reported to have several beneficial pharmacological effects, which include antiviral activity against hepatitis B and related hepatitis viruses. Others have also reported that the plant provides antibacterial, antihepatotoxic, and antidiabetic activities (10-11). This could be associated with the present of different bioactive compounds such as geraniin, gallic acid, rutin and quercetin (10-15).

It has been demonstrated that these plants are potentially good sources for the production of antiviral agents as compared to synthetic analogues. More interestingly is

that by using plants as the source of antiviral agents, supply would not be a problem since the majority of these plants are usually found abundantly in developing and third world countries, where coincidentally, the incidences of infectious diseases are more widespread. Many of these diseases are still waiting for a miraculous vaccine or drug. Among these include herpes simplex (HSV) and dengue (DENV) virus infections. Besides that, plant has also been proposed by various research groups to demonstrate anticancer activities on different cancers (8, 9). In this particular review, the antiviral and anticancer aspects of *Phyllanthus* species carried out in our laboratory are highlighted.

2. Antiviral activities of *Phyllanthus*

2.1 Anti-Herpesviridae (HSV) Properties

2.1.1 Introduction on HSV

Herpesviridae family consists of at least eight species of single large double stranded DNA enveloped viruses that are pathogenic to humans (16-20). Among these species, HSV-1 and HSV-2 are more intriguing due to the enormous clinical symptoms that they may manifest, ranging from gingivostomatitis to keratoconjunctivitis, genital disease, encephalitis. In addition, these viruses are likely to infect newborn and immunocompromised patients, which is more alarming (20). The main concern however, are their ability to remain latent and produce lifelong infections, get reactivated whenever triggered by a particular stimuli; which can include sunlight, stress or due to the weakened immunity, resulting in secondary infections (21).

2.1.2 Treatments of Herpes Simplex Virus

The primary treatment for HSV infections involves mainly nucleoside analogs such as acyclovir, valaciclovir, famciclovir and cidofovir. The only drawback of these limited anti-HSV drugs is the development of viral resistance against these treatments and drug toxicity (19-21). Drug-resistant viral strains were increasingly isolated from patients, in particular immunocompromised individuals (20). Although more HSV inhibitors (both nucleoside and non-nucleoside analogues) were established, very few of them were approved for therapeutic use due to their relatively high toxicity (20). Thus, it is imperative to search for new antiherpetic agents that are less toxic and have lower probability of resistance development for the prevention or treatment of herpes virus infections.

2.1.3 Role of Phyllanthus species in HSV infection

2.1.3.1 Phyllanthus orbicularis

In 2003, Fernandez Romero and his research group prepared two butanol- and acetic acid-soluble fractions from the leaves and stems of *Phyllanthus orbicularis* and tested these fractions against acyclovir-sensitive and -resistant HSV-1 strains in both human foreskin fibroblast (HFF) and green ape kidney (Vero) cell lines. Both the fractions exhibited antiviral selectivity indexes (SI) ranging from 10.3 to 22.8, while their extracellular virucidal activities reached SI values ranging from 371 to 1040 (22). In another study, a fraction that contains flavan-3-ol gallates, procyanidin B1 and B2, procyanidin dimer gallates, procyanidin trimers and procyanidin trimer gallates exerted strong anti-HSV-2 activity. As compared to this fraction though, its crude methanol extract showed higher antiviral selectivity index, suggesting the loss of some active components contributing to the antiviral activity during separation (23). The third fractionation studies revealed inhibition of acetic ether, methanol and aqueous extracts from *Phyllanthus emblica* on HSV-1 at IC_{50} values 25.28, 66.17 and 100.94µg/ml, respectively. Whereas on HSV-2, the IC_{50} value of acetic ether, methanol and aqueous extracts from *Phyllanthus emblica* are 31.7, 180.3 and 112.1µg/ml, respectively (24).

2.1.3.2 Phyllanthus urinaria

Subsequent studies involved isolation of pure compounds from acetone extract (leaves and branches) of *Phyllanthus urinaria* and their results showed that hippomanin A blocked only HSV-2 infection but corilagin inhibited neither HSV-1 nor HSV-2 replication although both these compounds displayed similar structures (25). Another polyphenolic compound, 1,2,4,6-tetra-O-galloyl-β-d-glucose (1246TGG) isolated from *Phyllanthus emblica* was found to inhibit both HSV-1 and HSV-2 infections at varying magnitudes of activity *in vitro*. This compound has been shown to inactivate HSV-1 particles directly, thus this leads to the inhibition of viral attachment and penetration during early infection. Other anti-HSV mechanisms of 1246TGG include an antiviral effect mainly within 3 hours post-infection as well as intracellular growth suppression of HSV-1 up to 12 hours post-infection. This is mainly due to the inhibition of HSV-1 E and L gene expressions and viral DNA replication without interrupting the RNA synthesis of IE gene, thus this leads to the reduction of viral protein synthesis. Taken together, 1246TGG might exert anti-HSV activity both by inactivating extracellular viral particles and by inhibiting viral biosynthesis in host cells (26). A similar structure purified from the acetone extract of *Phyllanthus urinaria*, 1,3,4,6-tetra-O-galloyl-β-d-glucose (1346TOGDG) effectively reduced HSV-1 infection with IC_{50} values of 19.2±4.0µM. Geraniin, a more well established pure compound isolated from the same extract actively suppressed HSV-2 infection with IC_{50} values of 18.4±2.0µM (27).

Activities of three *Phyllanthus urinaria* (whole plant) extracts, acetone, ethanol and methanol, inhibited HSV-2 infections at IC_{50} values of 4.3±0.5, 5.0±0.4 and 4.0±0.9 mcg/ml, respectively. All three extracts showed no cytotoxic effect against Vero cells at concentrations of 10.0 mcg/ml and below. However, the time-of-addition study showed that these three extracts were only effective during simultaneous treatment, suggesting that they affect the initial stage of HSV-2 infection (20). Similar phenomenon was observed in Tan et al's (28) study whereby two mode

of treatments (simultaneous- and post-treatments) of four whole *Phyllanthus* plant (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) aqueous extracts effectively inhibited both HSV-1 and HSV-2 via inhibition of viral entry, binding and replication. Pure isolation of excoecarianin from the acetone extract of whole *Phyllanthus urinaria* plant protected Vero cells from HSV-2 infection with IC_{50} values 1.4±0.1μM without affecting the viability or the morphology of Vero cells. This inhibitory effect was most prominent when excoecarianin was added concurrently with the virus as compared to pre-treatment and post-viral entry treatment that did not yield any antiviral effects. This revealed that excoecarianin primarily inactivate HSV-2 virus particles to prevent viral infection. It was also able to act synergistically with acyclovir to exhibit antiviral effect against HSV-2 infection in Vero cells (21).

2.1.3.3 Phyllanthus emblica

The effects of pentagalloylglucose (PGG), a hydrolyzable polyphenol isolated from the branches and leaves of *Phyllanthus emblica* were tested on HSV-1-infected MRC-5 cells with or without PGG-treatment, as well as between non-infected MRC-5 cells with or without PGG-treatment by two-dimensional gel electrophoresis coupled to mass spectrometry analysis. Significantly, PGG downregulated cofilin1, a key regulator of actin cytoskeleton dynamics which is important for HSV-1-induced actin-skeleton rearrangements for infectivity, leading to a reduced HSV-1 DNA, mRNA, protein synthesis and virus yields (29). Other antiviral mechanisms of the active *Phyllanthus orbicularis* (aerial parts) extracts and fractions involved inhibition of several HSV-2 early replication events and DNA synthesis (30).

2.1.3.4 Summary

Of importance it is observed that both whole extract and pure compounds derived from *Phyllanthus* plants exerted very minimal toxicity without changing cellular morphologies or affecting cells viability (8-9, 17, 20-22, 25-28). Various pure compounds were successfully isolated from the *Phyllanthus* extracts, each of these compounds demonstrating specific inhibitory activities towards the different herpes simplex viruses, unlike the whole extract that possessed a broader spectrum of antiviral activity against both HSV-1 and HSV-2. Nonetheless, most of these research groups reported that whole *Phyllanthus* plant extracts inhibits the herpes simplex viruses most effectively at the time of infection (simultaneous treatment). They postulated the blocking of viral entry or replication during early HSV infection as the antiviral mechanisms. In actual case scenario however, majority of HSV infections were only diagnosed in patients present with clinical symptoms, signifying a post-infection stage rather than simultaneous infection. In such circumstance, *Phyllanthus* might not be a valuable anti-HSV agent and hence, further assessment of *Phyllanthus* in other pharmacological effects is essential before it could attract public interest as a plant possesses multiple medicinal properties.

2.2 Anti-Dengue Properties

2.2.1 Introduction on Dengue

Dengue is a globally important mosquito-borne arboviral infection that has spread to many tropical and subtropical areas, endangering an estimated 2.5 billion people and hence causing an increasing public health concern in endemic countries. There is approximately 50 to 100 million infections reported each year, of which 500,000 cases were severe and potentially life-threatening (31, 32). The causative agent for dengue disease is an icosahedral, enveloped virus that contains a single-stranded positive sense genome (31). Due to the serological and genetic relatedness, dengue virus (DENV) can be grouped into four serotypes: DENV-1 to DENV-4. It appears that all of these variants can co-circulate in endemic areas. Infection by a particular serotype confers lifelong protection against similar serotype, however protection against secondary infection by another heterologous serotype is only temporary (32, 33). Human population of all ages are susceptible to dengue virus infection, leading to diverse illness symptoms, often involving an abrupt onset of fever, myalgia, headache, severe retro-orbital pain, and petechiae. In a more severe scenario, the individual might present with capillary leakage, hemorrhage, and circulatory shock (31, 32). Severe disease is mainly observed in secondary, heterologous DENV infections. The mechanism by which this occurs can be explained using the antibody-dependent enhancement (ADE) of infection theory, which clearly describes the primary mechanism of dengue immunopathogenesis (33). Recognition of these clinical symptoms is crucial for successful patient management, mainly involving careful fluid infusion (31).

As specific dengue treatments are not yet available, dengue vaccine development is considered a major advance in the disease control. The only drawback for this vaccine development is the potentially detrimental role of immune enhancement (33). Therefore, the need for new antiviral agents is of paramount in light of the need to prevent the manifestation of severe dengue. Further, this will also ensure that outbreaks can be prevented and, in future to complement possible vaccination programs. The therapeutic properties against dengue infection is currently still in its early stages. However, understanding of the molecular mechanisms behind the dengue virus life cycle and elucidation of the specific functions for each dengue viral proteins has allowed the design of novel antiviral targets. Some of these potential therapeutic intervention targets include viral entry, viral RNA polymerase/methyltransferase, nucleotide synthesis, viral serine protease, α-glucosidases and kinases (34). Nonetheless, plants that are rich in bioactive components remain the basis for these novel antiviral compounds.

2.2.2 Antiviral activity of Phyllanthus

Klawikkan and his research group first investigated the effects of various medicinal plant extracts from Thailand against DENV2 in Vero cell. Amongst these plant extracts, dichloromethane and ethanol extracts of *Phyllanthus urinaria* did not exhibit any DENV inhibitory activity when tested at 12.5µg/ml (35). Two years later, cocktail (aqueous and methanolic) extracts were prepared from whole four *Phyllanthus* plants (*P. amarus, P. niruri, P. urinaria, and P. watsonii*). The maximum non-toxic dose on Vero cells for both aqueous and methanolic extracts were determined to be 250µg/ml and 15.63µg/ml respectively. At these concentrations, *Phyllanthus* showed strongest inhibitory activity against DENV2 with more than 90% of virus reduction in simultaneous treatment through regulation of various proteins that play important roles in cell-virus attachment, viral entry, viral polyprotein production, viral RNA replication as well as viral assembly and maturation (17, 37). Inhibition of the viral entry into the host cell via binding of viral E protein to heparin sulfate during early infection is normally the first point of intervention (34).

2.2.3 Larvicidal activity of *Phyllanthus*

Management of dengue disease vectors provides a better means of controlling disease outbreak. Transmission of all four dengue virus serotypes to humans occurs via infected *Aedes spp.* mosquito bite, mainly *Aedes aegypti*, an anthropophilic mosquito. Outbreak transmission could also be caused by other members including *A. albopictus*, *A. polynesiensis*, *A. mediovittatus* and members of the *A. scutellaris*. Hence, knowledge of mosquito vectors and the interactions between virus and vector is essential to establish methods to interrupt transmission, for instance, by targeting elimination or suppression of target mosquito populations in endemic areas (34). It is envisaged from studies conducted using *Phyllanthus* that it has a putative role in reducing viral load in mosquitoes. In 2012, Sarita evaluated the larvicidal activity of 15 Indian plant species and the preliminary screening showed that only 10 plants possessed larvicidal potential as they could result in 100% mortality at 1000ppm. Among these plants, they showed that the hexane extract from *Phyllanthus emblica* fruit was the least effective with an LC_{50} value of 298.93ppm. However, Jeyasankar and his group performed a similar experiment by examining the mosquitocidal potential of *Phyllanthus emblica* against *A. aegypti* and his results obtained were more promising. The hexane extract from the leaves of *Phyllanthus emblica* was tested against the fourth instar larvae of *A. aegypti*, where 86.0% of the larval mortality was observed at 250ppm concentration with the LC_{50} of 111.34ppm (LCL=93.07 - UCL=133.20). Meanwhile, the ethyl acetate extract of *Phyllanthus emblica* exhibited the maximum larvicidal activity (99.6% larval mortality) with LC_{50} value of 78.89ppm (37).

In summary, *Phyllanthus* has the potential to be a candidate in the development of anti-dengue agents. It may work either by causing toxicity to the mosquito larvae as a part of vector control management, or by inhibiting the infection of dengue virus in the host cells. Different parts of the same *Phyllanthus* species may also give rise to the different magnitude of anti-dengue effects, probably attributed to the varying composition of bioactive components present. Nevertheless, the *in vitro* antiviral activities of *Phyllanthus* studied so far focused only on a particular dengue virus serotype (DENV-2) and more detailed investigations on other DENV serotypes is crucial since these serotypes may present different infectivity and virulence. Besides that, *in vivo* (toxicity and efficacy) investigations and pre-clinical studies need to be conducted in order for the plant to be accepted as a prospective anti-dengue agent.

3. Anticancer activities of *Phyllanthus*

3.1 Cancer

Cancer refers to a group of diseases that arise from a single (mutated) cell when it starts to grow uncontrollably to form a tumor (group of undifferentiated cells). Different stages of the tumor exhibit different responses to treatment thus making treatment very difficult. Most of the chemotherapeutic drugs including doxorubicin and cisplatin, mainly affect the fast-dividing cells of the body causing undesirable side effects such as pain, nausea, vomiting, alopecia, and anaemia (38).

Evasiveness of apoptosis is a hallmark of cancer and is critical for cancer development and survival of tumor cell (3). This has led the cancer cells to possess uncontrolled proliferation and form a large mass of abnormal (mutated) cancer cells: tumor. During transformation of malignant tumor, tumor will triggers angiogenesis: a process whereby new blood vessels are formed from pre-existing blood vessels, to provide the route for tumor cells to exit from the primary tumor and enter the circulation and spread to other parts of the human body. Therefore, angiogenesis and metastases are intrinsically connected. This ability of malignant tumors to metastasize is responsible for the poor prognosis and the apparent high mortality rate in cancer patients (3). Therefore, metastasis and angiogenesis remain a major clinical challenge in cancer treatment.

Development of chemoresistance to the anticancer drugs by cancer cells is another major obstacle in cancer treatments and has resulted in high mortality rate among cancer patients. Currently, there is no effective treatment for cancer, so intense research is required to obtain new anticancer agents for this cancer. Thus, scientists have begun to focus on natural product as an alternative to cancer treatment following successful development of natural-based anticancer agents such as zapotin, apigenin and resveratrol (38, 39).

3.2 Anti-proliferative effect

The anti-proliferative effects of four whole *Phyllanthus* plants (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) have been identified in different cancer cell lines including breast, lung, melanoma, liver, lung, leukemia and prostate

(8, 9, 40-44). The selective anti-proliferative effect of *Phyllanthus* was observed when whole extract of four *Phyllanthus* species (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) inhibited growth of four different cancer cell lines; A549, MeWo, MCF-7 and PC-3, without cytotoxic effects on their respective normal cells [8, 9, 40]. Most of the polyphenol compounds present in *Phyllanthus* plant have been reported to possess anti-proliferative effects on cancer cells including gallic acid (44).

The anti-proliferative effect of anticancer agents has also been associated with cancer cell cycle arrest (G0/G1, S or G2/M) that finally leads them to apoptotic cell death (45-46). *Phyllanthus* plants have been reported to induce cell cycle arrest in cancer cells, which leads to inhibition of cancer cell proliferation and eventually induces apoptosis.

The whole plant extract of four *Phyllanthus* species (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) have been reported to induce G1- and S-phase arrest in PC-3 and MeWo cells, respectively (40). For instance, the G1-phase arrest in *Phyllanthus* treated PC-3 cells was believed to be due to the disruption on several cellular pathways including MAPKs, Wnt, NFκB and Myc/Max (Figure 1) (8). Down-regulation of these pathways could lead to the activation of p27 proteins, which in turn inhibit the activation of cyclin E/CDK2 and/or cyclin D/CDK4 complexes, thus halting cell cycle progression at G1 phase (47). In addition, degradation of c-myc and β-catenin proteins by GSK3β could reduce the production of cyclin D, which is an important initiator of cell cycle. Several studies have shown the involvement of Wnt and Myc/Max pathways in the regulation of cyclin D to induce cell growth arrest (Figure 1) (8, 47-49).

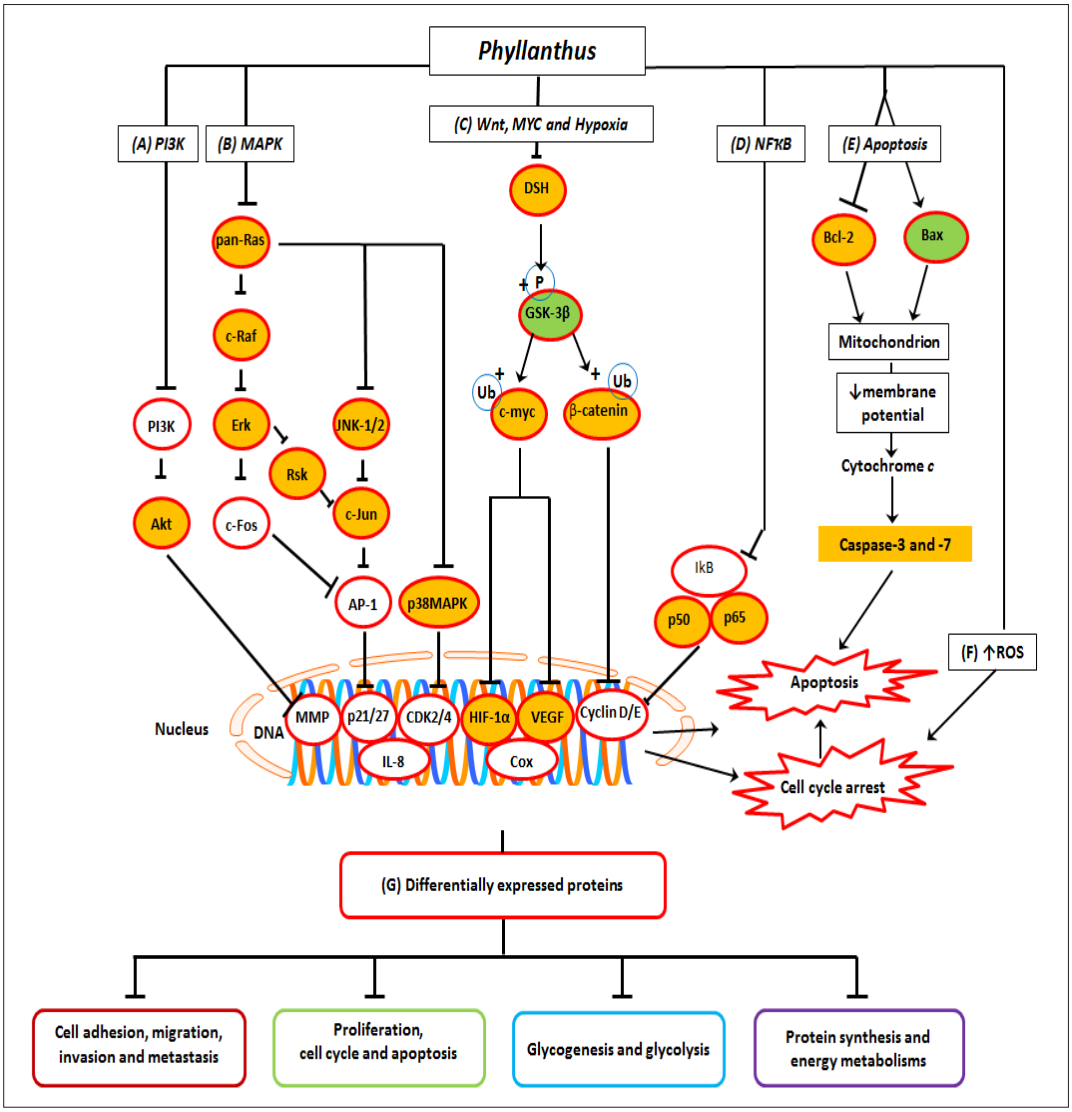

Figure 1: Schematic diagram illustrating that Phyllanthus regulates multiple survival signalling pathways and protein activities in cancer cells. The inhibition of (A) PI3K/Akt (Akt protein), (B) MAPKs (pan-Ras, c-Raf, RSK, Elk1, c-Jun, JNK1/2, and p38 MAPK proteins), (C) Wnt (DSH, Gsk3, and -catenin proteins), Myc/Max (c-myc protein), hypoxia (HIF-1α and VEGF proteins) and (D) NFkB (p50 and p52 proteins) pathways by Phyllanthus plant extracts in cancer cells. (E) Apoptosis induction via up-regulation of Bax protein and down-regulation of Bcl-2 to induce caspase-3/7 activation in treated cancer cells. (F) Induction of reactive oxidative stress by Phyllanthus leads to cell cycle arrest and apoptosis. (G) A number of proteins involved in proliferation, cell cycle, apoptosis, metastasis, glycogenesis and glycolysis, protein synthesis as well as energy metabolism were found altered in cancer cells upon Phyllanthus treatment.

3.3 Apoptosis inducer

Whole extract of *Phyllanthus* plants (*P.amarus, P.emblica, P.niruri, P.urinaria* and *P.watsonii*) have been reported to induce apoptosis in various types of cancer cells including melanoma, breast, lung, colorectal, cervical, liver and prostate (8, 9, 40, 44, 50-55). Apoptosis induction by these *Phyllanthus* is always associated with the activation of effector caspases such as caspase-3 and -7. These activated effector caspases will in turn activate other degradative enzymes such as DNases to cleave the DNA into fragments in an apoptotic cell. Besides involvement of caspases activation, *Phyllanthus* plant has been reported to utilize other mechanisms to induce apoptosis in cancer cells. For instance, the methanolic extract of hairy root of *P.amarus* increased the levels of intracellular reactive oxygen species (ROS) and decreased mitochondrial membrane potential (MMP) to induce apoptosis in MCF-7 (50). In addition, whole plant extract of *P.amarus, P.niruri, P.urinaria* and *P.watsonii* have also been reported to increase the expression of pro-apoptotic protein, Bax, and reduce expression of anti-apoptotic protein, Bcl-2, which in turn allow apoptosis to occur in cancer cells (Figure 1) (8, 51-52). In addition, whole plant extract of three *Phyllanthus* species (*P. urinaria, P. amarus* and *P. debilis*) also induced TNF-α production and inhibited expression of other anti-apoptotic genes including IL-8 and COX-2 in human hepatocarcinoma cells (43).

3.4 Anti-angiogenic and anti-metastatic effects

Endothelial cells are primary cells that form the lining of blood vessels. Although *Phyllanthus* showed no cytotoxic effect on endothelial cells, it decreased the migration and invasive ability of endothelial cells, thus inhibiting the formation of new blood vessels (41). The anti-angiogenic effect of water extract of whole *Phyllanthus* plant was observed when it inhibited microcapillary tube-like formation of endothelial cells cultured on extracellular matrix (ECM), which mimics the *in vivo* lining of blood vessels and in addition, it decreased the vessel density in both *in vivo* and *ex vivo* studies (53).

Phyllanthus plants have also shown the potential to reduce the metastazing ability of several cancer cell lines (8, 9, 54-55). For example, gallic acid found in *Phyllanthus* plant has disrupted cancer cell-cell interaction in a mechanical scratch-wound cellular monolayer healing assay (8, 9, 54). In addition, whole *Phyllanthus urinaria* extract also inhibited the invasion ability of cancer cells in a dose-dependent manner through the ECM gel as matrix barrier, which mimiced the *in vivo* basement membrane of blood vessel (41, 55, 56). This anti-metastatic effect of whole *Phyllanthus* plant extracts (*P.amarus, P.emblica, P.niruri, P.urinaria* and *P.watsonii*) was further observed when *Phyllanthus* showed inhibitory effects on different matrix metalloproteinases (MMPs) in various types of cancer cells (8, ,, 53, 56-57). Four whole plant extracts of *Phyllanthus* (*P.amarus, P.niruri, P.urinaria* and *P.watsonii*) were noted to inhibit the glycolytic pathway and energy production in PC-3 cells by down-regulating HIF-1α protein which leads to reduction of pro-angiogenic VEGF and thus inhibit tumor angiogenesis and thereby decrease cancer progression (Figure 1) (8).

3.5 Anticancer overview

In summary, cumulative results from experimental and predictive studies suggest that whole plant extract of *Phyllanthus* can interfere with (1) multiple survival signalling pathways (PI3K/Akt, MAPKs, Wnt, Myc/Max, hypoxia and NFkB), and (2) protein regulations involved in tumors' cellular function and biological processes (tumour cell adhesion, apoptosis, glycogenesis and glycolysis, metastasis, angiogenesis, and protein synthesis and energy metabolism) in cancer cells. However, most of these results were derived from *in vitro* studies which are insufficient and less convincing since all these experiments were performed in an artificial environment. Thus, pre-clinical study using experimental cancer animal model is needed to determine the pharmacological, toxicological as well as anti-tumor effects of *Phyllanthus*, to provide more information on the safety usage and effectiveness of this plant against cancer.

4. Conclusion

In a conclusion, the *Phyllanthus* plant possesses multiple medicinal properties against viruses and cancer cells. Further investigations into the antiviral and anticancer properties of *Phyllanthus* are required to provide more information on the safe use and effectiveness of this plant. This may create opportunities for the plant to, not only be designed and developed as antiviral and anticancer agents, but also as a dietary supplement for the prevention of disease.

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