



An efficient approach to Enrich Glioma Stem Cells from Glioma Cell Lines in Culture

Corresponding Author:

Dr. Deepak Kamnasaran,
Assistant Professor & Scientist, Pediatrics, Laval University, G1V 4G2 - Canada

Submitting Author:

Dr. Deepak Kamnasaran,
Assistant Professor & Scientist, Pediatrics, Laval University, G1V 4G2 - Canada

Article ID: WMC00557

Article Type: Research articles

Submitted on: 13-Sep-2010, 11:20:23 PM GMT **Published on:** 14-Sep-2010, 12:56:31 AM GMT

Article URL: http://www.webmedcentral.com/article_view/557

Subject Categories: ONCOLOGY

Keywords: Cancer stem cells, Stem cell isolation, Cell culturing

How to cite the article: Ajeawung N, Kamnasaran D . An efficient approach to Enrich Glioma Stem Cells from Glioma Cell Lines in Culture . WebmedCentral ONCOLOGY 2010;1(9):WMC00557

Source(s) of Funding:

Funds for this research is provided by operating grants to DK from the CHUQ foundation, Fondation des étoiles, Canadian Foundation for Innovation (Leaders Opportunity Funds) and Fonds de la recherche en santé du Québec, and PhD studentships from Laval University to NA.

Competing Interests:

None

An efficient approach to Enrich Glioma Stem Cells from Glioma Cell Lines in Culture

Author(s): Ajeawung N, Kamnasaran D

Abstract

Within a glioma tumor mass and even in established glioma cell lines, resides a sub-population of cells known as glioma stem cells. These cells are postulated to contribute significantly to the treatment resistance among patients, and in fact are responsible for tumor re-growth. Furthermore, current therapies favorably enrich these cells, leading to a devastating clinical prognosis among the majority of patients. To date, several approaches were implemented to identify and isolate glioma stem cells based on exploiting the molecular and physical properties, but still continue to have major inherent pitfalls. We report herein an observation and a novel approach which is a rapid, simple and cost effective way to isolate and enrich glioma stem cells from human glioma cell lines in culture. An understanding of the biology of these cells and especially responses to current treatments will certainly assist in the discovery of improved therapies.

Introduction

Gliomas, like other tumors, comprise of undifferentiated cells referred to as cancer stem cells, that are characterized based on their abilities to express stem cell markers, are highly tumorigenic and are endowed with the ability to form spheres in culture which can self-renew and undergo multi-lineage cellular differentiation [1-4]. Several approaches were implemented to identify and isolate glioma stem cells based on exploiting the inherent molecular and cellular properties. One of the earliest reports on the isolation of glioma stem cells is from the selection of sphere forming cells in cultures established from dissociated glioma surgical specimens [1]. Shortly afterwards, glioma stem cells were isolated again from surgical specimens, in conjunction with xenograft tumors, using cell sorting for the expression of the CD133 stem cell marker [3,5]. The significance of these findings cannot be over emphasized since it is now well established that glioma stem cells are pivotal in conferring treatment resistance among glioma patients [6]. Therefore, further studies on these cells can inevitably lead to discovering more effective treatments. Isolation of glioma stem cells for translational research

represents a major challenge since this procedure is often expensive, cumbersome, and sometimes requires advanced technical expertise. In addition, since having access to fresh surgical specimens is difficult for most investigators, recent studies are aimed at finding more feasible ways of enriching glioma stem cells but with glioma cell lines grown in culture. An interesting approach involves isolating CD133+ cells from glioma cell lines [7] using magnetic cell sorting or flow cytometry. However, the CD133 marker is limited in its ability to identifying all cancer stem cells [4,8]. Other authors have reported using side population analysis to isolate glioma stem cells based on the ability to extrude the Hoechst 33342 dye [7,9], a procedure that warrants very careful optimization but with low success. Some studies have even reported the manual isolation of suspected glioma stem cells from glioma cell lines grown in culture using clone cylinders, a procedure requiring weeks to successfully accomplish [10]. In order to circumvent these major pitfalls in trying to isolate and enrich for glioma stem cells from glioma cell lines grown in culture, we report herein an observation and a novel approach which is rapid, simple and cost effective.

Methods

Enrichment for glioma stem cells

Human glioma cell lines were cultured using conventional tissue culture media: DMEM (Invitrogen, Canada) supplemented with 10% Fetal bovine serum (Fisher, Canada). These glioma cell lines were grown to confluence and dissociated using 0.1% trypsin (Invitrogen, Canada) or accutase (Stem cell technologies, Canada). About 1.0×10^6 of each glioma cell line was suspended in sphere enrichment medium, specifically, human NeuroCult NS-A basal medium supplemented with human 10% NeuroCult NS-A proliferation supplement (Stem cell Technologies, USA), 20ng/ml EGF and FGF-2, (Millipore, USA). These cells were then plated in 100 mm tissue culture plates (Sarstedt, Canada). After 24 hours of growth in the sphere enrichment medium, both populations of adherent and non-adherent spheres were visible in culture. Non-adherent spheres were harvested, and to further enrich for tumor sphere

formation, a non-trypsin dissociation in the sphere enrichment medium was performed. This was followed by resuspension of the pellet in a fresh aliquot of the same medium. These suspended cells were then transferred to 100 mm plates, which were subsequently mixed gently on a belly dancer laboratory shaker for a 5 minute period to facilitate dissociation of the tumor spheres. Following overnight incubation at 37°C with 5% CO₂ and 100% humidity, distinct non-adherent human glioma stem cells were apparent in culture. These spheres were collected, then gently centrifuged at low speed (1000 rpm), passaged and maintained for growth in the sphere enrichment medium.

RT-PCR analyses

Total RNA from cells were isolated using the GenElute Mammalian Total RNA miniprep kit (Sigma-Aldrich, Canada) and 20 µl reverse transcription reactions were performed according to the manufacturer using the Superscript II reverse transcriptase kit (Invitrogen, Canada). 2 µl of cDNA synthesized from each cell specimen were used in standard PCR reactions with the following human primer pairs: Bmi1 (F-GATGCCACAACCATAATAGAA; R-TCATTCACCTCCTCCTTAGAT); Melk (F-CAGAAACAACAGGCAAAC; R-GAGGGTGTAGTGTAAACGATT); Nestin (F-GTGGGAAGATACGGTGGAGA; R-ACCTCCTCTGTGGCATTTCAG); Msi1 (F-GATGGTCACTCGGACGAAGAA; R-CAAACCCTCTGTGCCTGTTG); and β-actin (F-ATGTGGCTTGGTCACTTCGT; R-CAGAAGACTCAGCCCTGA). The PCR cycle condition was: an initial denaturation at 94°C- 4 minutes, followed by 35 cycles of 94°C-30 seconds denaturation, 59°C-30 seconds annealing, 72°C-30 seconds extension; and a final extension of 72°C- 7 minutes.

Immunofluorescent Staining

Dissociated glioma stem cells were seeded in 4-well laminin coated chamber slides (BD Biosciences, USA), then incubated at 37°C- 24hrs. Cells were fixed in 4% paraformaldehyde for 20 minutes, permeabilized for 15 minutes with 0.5% triton-X in PBS, then incubated in 0.5% Roche western blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany) for 30mins. The primary antibodies used were a rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) (Dako, USA), mouse monoclonal anti-Beta 3-tubulin (Santa Cruz, USA) and a mouse monoclonal anti-CNPase (Abcam, USA). Hybridizations in the primary antibody were for 60 minutes in 0.5% Roche western blocking reagent (Roche Diagnostics GmbH, Mannheim, Germany),

followed by 2X washing in 0.1% PBS-Tween. A rabbit polyclonal protein G-FITC secondary antibody (Abcam, USA) was used. After a final 2X washing with 0.5% PBS-tween, the slides were treated with Vectashield hard set mounting stain containing DAPI (Vector Laboratories, USA). Semi-quantitative analyses were undertaken using a Nikon eclipse 80i microscopy system with bright field and fluorescence features, a Digital Sight DS-Ri1 high resolution camera and the NIS-elements BR software.

Flow Cytometry Analysis of CD133 expression

Glioma cell lines were dissociated in 0.1% trypsin-EDTA (Invitrogen, Canada), while glioma stem cells were dissociated with Accutase (Stem cell Technologies, Canada), prior to suspension in Neurocult NS-A basal medium. The cell suspension was passed through a 0.70 µm nylon cell strainer (BD Falcon, Bedford, USA), then 400 µl of 1.0 X 10⁶ cells was added to 100 µl of FcR-blocking reagent (Miltenyi Biotec, Germany) and incubated for 30 minutes at room temperature on a belly dancer laboratory shaker. This was followed by the addition of 50 µl of the CD133/2 (293C3)-phycoerythrin (PE) antibody (Miltenyi Biotec, Germany) and then a one hour incubation with gentle mixing on a belly dancer laboratory shaker. 2X washes were done using the FACS buffer consisting of 0.1% sodium azide and 2% Neurocult NS-A basal medium in PBS. The cells were finally resuspended in the FACS buffer and analyzed using a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, California) at our institution.

Soft agarose assays

Agarose (1.8 %, Roche Diagnostics, USA) and low melting agar (0.7%, Bishop, Canada) were completely melted in a microwave and kept at 42°C to prevent solidification. 1.8% agarose was added to Neurocult NS-A complete medium (Stem cell technologies, Canada) to form a 1:1.8 bottom agar master mix, from which 2mls were removed and rapidly added to each well of a six well plate. Solidified bottom agar mix was overlaid with 2 mls of top agar mix which was prepared by adding equal volumes of neurocult NS-A complete medium containing 5.0 x 10⁴ cells/ml and 0.7% low melting agar. The plates were allowed to solidify in the laminar flow hood after which, 500 µl of Neurocult NS-A complete medium was added. Following incubation at 37°C for 21 days, transformed colonies were stained with 0.005% crystal violet and examined using an inverted microscope and digital camera unit (Nikon, Canada).

In-vivo tumor assays

Between 3 x 10³ to 5 x 10⁴ cells were stereotactically delivered into the frontal lobe of Nod-Scid mice. All mice were sacrificed at 3 weeks post-injection, as the

study endpoint. The brains were harvested and fixed in 10% formamide, then embedded in paraffin. 10mM sections were subjected to standard immunohistochemical analyses.

Statistical Analysis

All experiments were performed in triplicate and quantitative data was represented as mean +/-SD. Student t-test was used for analysis of quantitative data and $p \leq 0.05$ was reported as being significant.

Results

Since glioma stem cells can still grow in DMEM/10% Fetal Bovine Serum but in extremely low yields [10], we investigated whether these cells can be enriched by switching to a medium that promotes favorable growth. To accomplish this, we grew 106 glioma cell lines overnight in human NeuroCult NS-A basal medium with human 10% NeuroCult NS-A proliferation supplement, 20 ng/ml EGF and FGF-2 to induce a heterogeneous mixture of transient tumorsphere formations (Figure 1). These transient tumorspheres were harvested and re-grown in the tumorsphere medium for another 24 hours. Within 48 hours, we observed full tumorsphere formation such as with the U87 glioma stem cells (Figure 1), however the size and morphology of sphere formation was cell line dependent. In this manner less aggressive glioma cell lines such as U251 developed smaller and more loosely associated tumorspheres, in comparison to other aggressive cell lines (U87, U373, DB54MG). Most importantly, these tumorspheres were clonal, and in fact can be stably passaged for long terms without any significant changes in the self-renewal and proliferation capabilities as judged from proliferation and cell cycle analyses. In order to validate whether these tumorspheres in culture were enriched glioma stem cells, we quantified the percentage of CD133+ cells. As similarly identified with other glioma cell lines we tested, we noted that with U87 at least a 10% increase and enrichment of CD133+ cells in the glioma tumorsphere pool occurred (Figure 2). Other stem cell markers (Bmi1, Msi1, Melk, Nestin) were also identified to be expressed in these enriched tumorspheres, with elevated expression in comparison to the parental glioma cell line. Overall, this procedure seemed to efficiently enrich for both CD133+ and CD133- cells which are also present in glioma tumorspheres [4].

Furthermore, to validate whether these tumorspheres can differentiate in-vitro, we added 10% Fetal Bovine Serum to the growth medium and grew the cells for

another 48 hours at 37°C, 5%CO₂. Within this period, we observed a 63%, 50% and 62% increase in the expression of the GFAP, Tuj1 and CNPase respectively, in comparison to the expression of these neuroglial markers in the parental U87 cell line (Figure 3). Similar findings were noted with other glioma cell lines we tested. In addition, these molecular and cellular features appeared to be conserved even in late passaged tumorspheres. Finally, one of the eminent features of glioma stem cells in comparison to neural stem cells arises from their ability to transform [1-4]. With the U87 glioma stem cells, we identified that these cells can efficiently grow as anchorage independent clones in soft agarose and into intracranial tumors having pathologically similarities to the parental glioma cell line (Figure 4), findings also documented in glioma stem cells from other glioma cell lines we tested. However, the rate of transformation of the enriched glioma stem cells was significantly higher compared to the parental glioma cell lines, and with dependency on the inherent aggressive properties of the cells. Moreover, these transformation hallmarks were still conserved in late passaged tumorspheres.

Discussion

Within the last decade [1-3], the isolation of glioma stem cells represents an important hallmark in the identification of a cell type within a glioma tumor mass which has changed our biological and therapeutic understanding of gliomas. Indeed what we know about the isolation and characterization of the properties of glioma stem cells, can also be usefully implemented in purifying and studying cancer stem cells from other solid tumors. To this date, numerous efforts have been undertaken to isolate and purify glioma stem cells from various sources, including from fresh surgical specimens, xenografts and even from glioma cell lines [1-10]. However, the overall yield and ease of obtaining glioma stem cells still remains to be dependent on the type of method and source of tissue/cell line chosen. Furthermore, due to the fact that most investigators are not privileged by having access to fresh surgical specimens, efforts are now being directed to purifying glioma stem cells instead from conventional glioma cell lines which have been extensively utilized in understanding the biology and therapeutic responses of patients with gliomas.

Our procedure to isolate and enrich glioma stem cells from glioma cell lines grown in culture offers many advantages in comparison to other existing methods [1-10]. More specifically, our procedure has the

advantages of being very cost-effective, fast and can enrich glioma stem cells which can be stably maintained in culture without losing their pathological properties. Most importantly, due to the simplicity of this procedure, personnel with advanced technical skills are not required to isolate glioma stem cells from glioma cell lines in culture. The yield of glioma stem cells however is dependent on the pathological aggressiveness of the glioma cell line. In this manner, higher yields of glioma stem cells can be more easily harvested from very pathologically aggressive glioma cell lines. In addition, what constitutes a cancer stem cell or a glioma stem cell is still being debated, but our understanding of these cells is currently based on their inherent molecular, cellular and physical properties, features which have been exploited to isolate these cells from tissues or cell lines in culture [1-10]. In fact, different types of informative molecular biomarkers or even physical properties are being continuously updated as more ongoing studies are undertaken on glioma stem cells. Another important facet of our procedure originates from the fact that it is not biased towards isolating CD133+ or CD133- glioma stem cells. This procedure may therefore offer an alternative approach to isolating a population of cells which can be useful in identifying other molecular and physical properties of cancer stem cells that can inevitably lead to the discovery of improved therapies for the treatment of this deadly disease.

Acknowledgement(s)

We thank Drs. Bigner for providing us glioma cell lines, and Shi for undertaking in-vivo assays. Funds for this research is provided by operating grants to DK from the CHUQ foundation, Fondation des étoiles, Canadian Foundation for Innovation (Leaders Opportunity Funds) and Fonds de la recherche en santé du Québec, and PhD studentships from Laval University to NA.

Authors Contribution(s)

Norbert Ajeawung¹

Deepak Kamnasaran*^{1,2}

¹Pediatrics Research Unit, Centre de recherche du CHUL, Québec, Québec

²Department of Pediatrics, Laval University, Québec, Québec

*Correspondance:

Dr. Deepak Kamnasaran

Assistant Professor & Scientist

Pediatrics Research Unit

Centre de Recherche du CHUL (CHUQ)

2705, boulevard Laurier, Local RC-9800

Québec, Québec, Canada

G1V 4G2

E-mail: deepak.kamnasaran@crchul.ulaval.ca

References

1. Ignatova TN, Kukekov VG, Laywell ED, Suslov ON, Vrionis FD, Steindler DA. Human cortical glial tumors contain neural stem-like cells expressing astroglial and neuronal markers in vitro. *Glia* 2002; 39:193-206.
2. Yuan X, Curtin J, Xiong Y, Liu G, Waschmann-Hogiu S, Farkas DL, Black KL, Yu JS. Isolation of cancer stem cells from adult glioblastoma multiforme. *Oncogene* 2004; 23:9392-400.
3. Singh SK, Hawkins C, Clarke ID, Squire JA, Bayani J, Hide T, Henkelman R, Cusimano MD, Dirks PB. Identification of human brain tumour initiating cells. *Nature* 2004; 432:396-401.
4. Pollard SM, Yoshikawa K, Clarke ID, Danovi D, Stricker S, Russell R, Bayani J, Head R, Lee M, Bernstein M, Squire JA, Smith A, et al. Glioma stem cell lines expanded in adherent culture have tumor-specific phenotypes and are suitable for chemical and genetic screens. *Cell Stem Cell* 2009; 4:568-80.
5. Kondo T, Setoguchi T, Taga T. Persistence of a small subpopulation of cancer stem-like cells in the C6 glioma cell line. *Proc Natl Acad Sci* 2004; 101:781-786.
6. Kamnasaran D. Stem cells and models of astrocytomas. *Clin Invest Med* 2009; 32:E166-79.
7. Wu A, Oh S, Wiesner SM, Ericson K, Chen L, Hall WA, Champoux PE, Low WC, Ohlfest JR. Persistence of CD133+ cells in human and mouse glioma cell lines: detailed characterization of GL261 glioma cells with cancer stem cell-like properties. *Stem Cells Dev* 2008; 17:173-84.
8. Clément V, Marino D, Cudalbu C, Hamou MF, Mlynarik V, de Tribolet N, Dietrich PY, Gruetter R, Hegi ME, Radovanovic I. Marker-independent identification of glioma-initiating cells. *Nat Methods* 2010; 7:224-8.
9. Qiang L, Yang Y, Ma YJ, Chen FH, Zhang LB, Liu W, Qi Q, Lu N, Tao L, Wang XT, You QD, Guo QL. Isolation and characterization of cancer stem like cells in human glioblastoma cell lines. *Cancer Lett* 2009; 279:13-21.
10. Zhou ZH, Ping YF, Yu SC, Yi L, Yao XH, Chen JH, Cui YH, Bian XW. A novel approach to the

identification and enrichment of cancer stem cells from a cultured human glioma cell line. *Cancer Lett* 2009; 281:92-9.

Illustrations

Illustration 1

Figure 1: Example of enrichment for glioma stem cells from the U87 parental cell line.

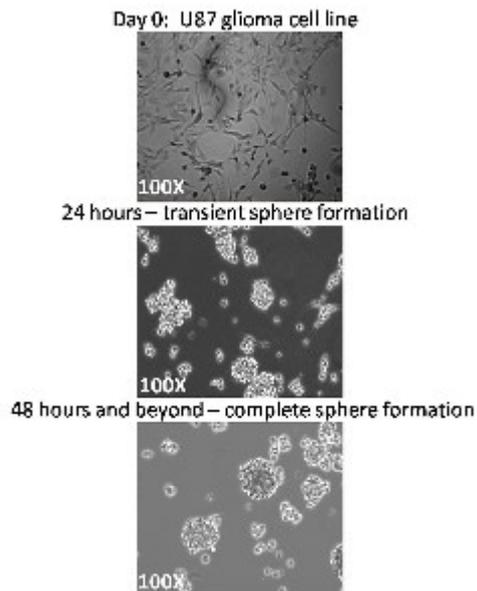


Illustration 2

Figure 2: FAC analyses to quantify CD133 + cells.

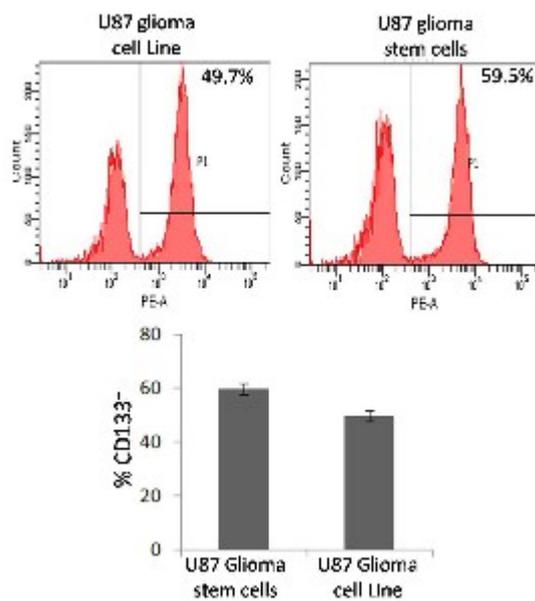


Illustration 3

Figure 3: U87 enriched glioma stem cells can differentiate.

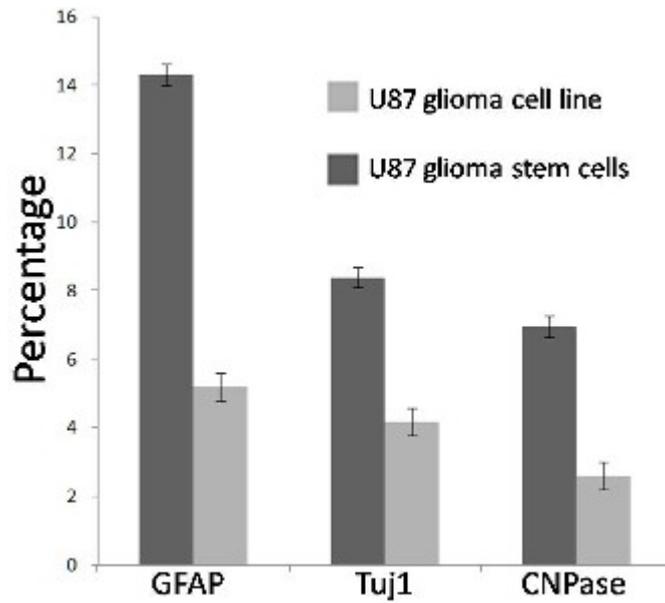
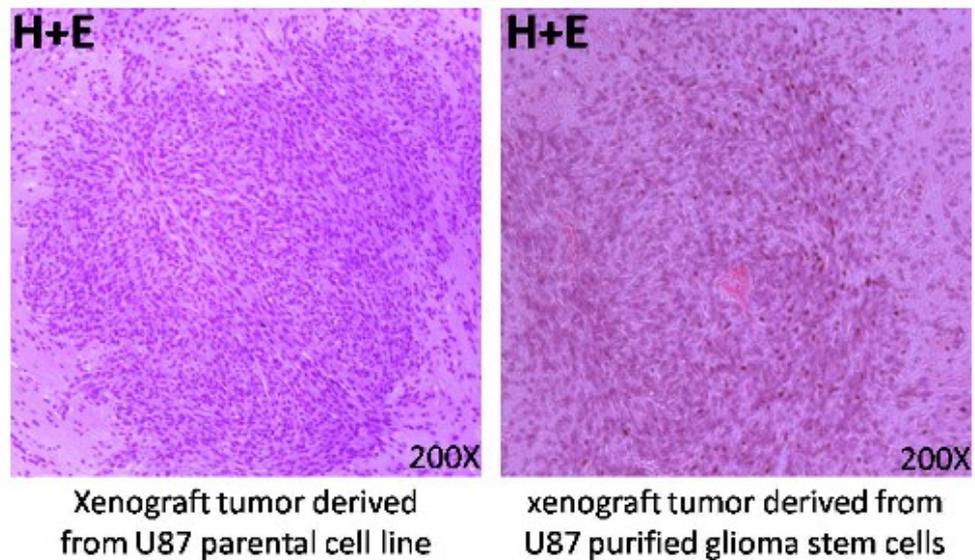


Illustration 4

Figure 4: Tumor properties of the enriched U87 glioma stem cells versus the U87 parental cell line.



Disclaimer

This article has been downloaded from WebmedCentral. With our unique author driven post publication peer review, contents posted on this web portal do not undergo any prepublication peer or editorial review. It is completely the responsibility of the authors to ensure not only scientific and ethical standards of the manuscript but also its grammatical accuracy. Authors must ensure that they obtain all the necessary permissions before submitting any information that requires obtaining a consent or approval from a third party. Authors should also ensure not to submit any information which they do not have the copyright of or of which they have transferred the copyrights to a third party.

Contents on WebmedCentral are purely for biomedical researchers and scientists. They are not meant to cater to the needs of an individual patient. The web portal or any content(s) therein is neither designed to support, nor replace, the relationship that exists between a patient/site visitor and his/her physician. Your use of the WebmedCentral site and its contents is entirely at your own risk. We do not take any responsibility for any harm that you may suffer or inflict on a third person by following the contents of this website.