รายงานวิจัยฉบับสมบูรณ์

โครงการ การผลิตและศึกษาคุณลักษณะของโมโนโคลนอลแอนติบอดีต่อโปรตีน Mig-7 ซึ่งเป็นตัวชี้บ่งการแพร่กระจายของมะเร็งหลายชนิด

โดย ดร.สาธิตา ตปนียากร

1 กรกฎาคม 2557

สัญญาเลขที่ TRG5580019

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สำนักงานพัฒนาวิทยาศาสตร์และเทคโนโลยีแห่งชาติ

บทคัดย่อ

รหัสโครงการ: TRG5580019

ชื่อโครงการ: การผลิตและศึกษาคุณลักษณะของโมโนโคลนอลแอนติบอดีต่อโปรตีน Mig-7 ซึ่งเป็นตัวชี้บ่งการแพร่กระจายของมะเร็งหลายชนิด

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บทคัดย่อ:

โมเลกุลชีวภาพที่จำเพาะต่อเซลล์มะเร็ง (molecular target) สำหรับการรักษามะเร็งแบบที่เป็นก้อน (solid tumor) ที่ใช้ในปัจจุบัน คือ epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) และ human epidermal growth factor receptor 2 (HER2/neu) อย่างไรก็ตามโมเลกุลดังกล่าวมีข้อจำกัดในแง่ความจำเพาะเนื่องจากโมเลกุลเหล่านี้มีอยู่บนเนื้อเยื่อของคนปกติที่ไม่ใช่มะเร็งด้วย โปรตีน Migration-inducing gene 7 หรือ Mig-7 ซึ่งเป็นโมเลกุลใหม่ที่พบบนผิวเซลล์และมีความจำเพาะต่อเซลล์มะเร็งเท่านั้น มีความเกี่ยวข้องกับการแพร่กระจายของมะเร็งหลายชนิด ดังนั้นจึงมีความเป็นไปได้ที่จะใช้โมเลกุล Mig7 ในการรักษามะเร็งแบบมุ่งเป้า (targeted cancer therapy) ในการศึกษาครั้งนี้จึงมุ่งเน้นการผลิตโมโนโคนอลแอนติบอดีที่จำเพาะต่อ Mig7 โดยใช้เทคนิคไฮบริโดมา ซึ่งยังไม่มีรายงานก่อนหน้านี้ โมโนโคลนอลแอนติบอดีที่ผลิตขึ้นมี 2 ตัว ได้แก่ 12H11F9 (IgM, kappa) และ 10E1C12 (IgG2a, kappa) จากการทดสอบด้วยวิธี Western blot และ ELISA แอนติบอดีทั้งสองตัวมีความจำเพาะต่อโปรตีน Mig7 และเมื่อทำการทดสอบคุณลักษณะของแอนติบอดีทั้งสองตัวด้วยวิธี Immunofluorescence และ Immunocytochemistry กับเซลล์มะเร็งชนิดต่างๆ ที่มีการแสดงออกของโปรตีน Mig-7 พบว่า 12H11F9 สามารถจับบนผิวเซลล์มะเร็งแต่ไม่สามารถย้อมติดเซลล์เม็ดเลือดขาวและเซลล์กระพุ้งแก้ม ในขณะที่ 10E1C12 ไม่สามารถย้อมติดบนเซลล์ทุกชนิด แสดงให้เห็นว่า epitope ของแอนติบอดีทั้งสองตัวแตกต่างกัน และเฉพาะแอนติบอดี 12H11F9 เท่านั้นที่สามารถจับกับโปรตีน Mig-7 บนผิวเซลล์ นอกจากนี้ 12H11F9 ยังแสดงความเป็นพิษต่อเซลล์มะเร็งที่มีการแสดงออกของโปรตีน Mig-7 จากการศึกษาแสดงให้เห็นถึงความเป็นไปได้ที่จะนำโมโนโคลนอลแอนติบอดีที่จำเพาะต่อโปรตีน Mig-7 ไปใช้ประโยชน์ทางด้านการวินิจฉัยและรักษามะเร็ง

คำหลัก : ไบโอมาร์คเกอร์; Mig-7; โมเลกุลชีวภาพที่จำเพาะต่อเซลล์มะเร็ง; โมโนโคลนอลแอนติบอดี; การรักษามะเร็งแบบมุ่งเป้า

Abstract

Project Code : TRG5580019

Project Title : Production and characterization of a novel monoclonal antibody against Mig-7, a promising biomarker for cancer metastasis

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Project Period : 1 year 6 months (plus 6 month extension)

Abstract:

Current molecular targets for the treatment of solid tumors are epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and human epidermal growth factor receptor 2 (HER2/neu). However, these targeted molecules are also present on normal tissues. Migration-inducing gene 7 (Mig7), a membrane protein found in various types of carcinoma cells, is a cancer-specific biomarker and a new potential marker for circulating and migrating cancer cells. Therefore, Mig7 is a promising molecular target for targeted cancer therapies. Here, a novel monoclonal antibody specific to Mig7 was prepared by hybridoma cell technique. Isotype of this monoclonal antibody was identified as IgM heavy chain and kappa light chain. The specificity and sensitivity of the antibodies were determined by Western blot and enzyme-linked immunosorbent assay (ELISA). Immunofluorescence and immunocytochemistry assays demonstrated that the monoclonal antibody could specifically recognize Mig7 expressing on RL95, TK6, HT29, A431, A549, HepG2, Hela, MCF7 carcinoma cell lines but not on human white blood cells and buccal mucosa cells. Moreover, the monoclonal antibody revealed cytotoxicity against Mig7-expressing cells *in vitro*. This monoclonal antibody may have important applications for cancer diagnosis and targeted therapy.

Keywords: biomarker; Mig-7; molecular target; monoclonal antibody; targeted cancer therapy

1. Introduction to the research problem and its significance

Cancer is one of the leading causes of death in the world, particularly in developing countries. Effective cancer treatment is urgently needed in order to increase the rate of survival. Chemotherapy is one of major cancer treatment strategies. This treatment involves the use of drugs to destroy cancer cells in the body. The drugs used in chemotherapy work by stopping or slowing the growth of cancer cells, which have a high proliferation rate. In addition to destroying cancer cell, they can also kill normal cells that divide rapidly, such as blood-producing cells in bone marrow, hair follicles, and cells in mouth. Recently, targeted cancer therapies *via* specific targeted molecules have been introduced. The treatment is more selective for cancer cells than normal cells, thus harming fewer normal cells, reducing side effects, and improving quality of life. Targeting tumor cell proteins that facilitate their invasion could provide additional efficacy and reduce recurrence of diseases. Nowadays, several targeting molecules have been developed in order to deliver drugs to specific cancer cells by binding to targeting drugs and cancer-associated markers. Development of such antibodies with high specificity and affinity against cancer-specific biomarkers will enhance the level of success in targeted therapies.

Current molecular targets for the treatment of solid tumors are epidermal growth factor receptor (EGFR), vascular endothelial growth factor (VEGF) and human epidermal growth factor receptor 2 (HER2/neu). However, these targeted molecules are also present on normal tissues. Migration-inducing gene 7 (Mig-7), a membrane protein found in various types of carcinoma cells, is a cancer-specific biomarker and a new potential marker for circulating and migrating cancer cells. 87% of tumors from breast endometrial, colon, lung, ovary, stomach, kidney, thyroid, cervix, small intestine and prostate (n>200 patients), and blood from untreated metastatic cancer patients possess cells expressing Mig-7 mRNA. However, the Mig-7 mRNA was not detectable in tissues or blood from 25 normal subjects. Therefore, Mig-7 is a promising molecular target for targeted cancer therapies.

2. Literature review

Targeted cancer therapies are drugs or other substances that block the growth and spread of cancer by interfering with specific molecules involved in tumor growth and progression. By focusing on molecular and cellular changes that are specific to cancer, targeted cancer therapies may be more effective than other types of treatment, including chemotherapy and radiotherapy, and less harmful to normal cells (1).

Monoclonal antibodies have emerged as important targeting molecules to improve the selectivity of other types of anti-cancer agents (2). In 1986, the FDA approved the first monoclonal antibody, muromonab-CD3 (Orthoclone OKT3), which prevents acute organ rejection after transplantation by blocking T-cell function. Since then, almost 20 other monoclonal antibodies have been approved, about a half of them for the treatment of cancer (Table 1). The fragment antigen binding (Fab) of a monoclonal antibody, which recognizes and binds to antigens,

is responsible for the highly specific targeting that is possible with such therapies. Monoclonal antibodies exert their anti-cancer effects through a variety of mechanisms: by recruiting host immune functions to attack the target cell; by binding to ligands or receptors, thereby interrupting essential cancer cell processes; or by carrying a lethal payload, such as a radioisotope or toxin, to the target cell (3).

Table 1. Monoclonal antibodies for cancer treatment.

Monoclonal antibody	Target	Antibody type	FDA-approved indications
Rituximab (Rituxan)	CD20	Chimeric	Low-grade B-cell non-Hodgkin lymphoma
Trastuzumab (Herceptin)	HER2/neu	Humanized	Metastatic breast cancer
Gemtuzumab-ozogamicin (Mylotarg)	CD33	Humanized	Acute myeloid leukemia
Bevacizumab (Avastin)	VEGF	Humanized	Metastatic colorectal cancer Head and neck squamous cell carcinoma
Cetuximab (Erbitux)	EGFR	Chimeric	Metastatic colorectal cancer Head and neck squamous cell carcinoma
Alemtuzumab (Campath)	CD52	Humanized	Chronic lymphocytic leukemia
Ibritumomab-tiuxetan-^{90}Y (Zevalin)	CD20	Mouse	Non-Hodgkin lymphoma
Tositumomab/ Tositumomab-^{131}I (Bexxar)	CD20	Mouse	Non-Hodgkin lymphoma

The development of targeted therapies requires the identification of good targets that play a key role in cancer cell growth and survival. The molecular pathways most often targeted in the treatment of solid tumors are those of the EGFR (4), VEGF (5, 6) and HER2/neu (7). Such pathways can be inhibited at multiple levels: by binding and neutralizing ligands; by occupying receptor-binding; by blocking receptor signaling within the cancer cell; or by interfering with downstream intracellular molecules. However, these molecular targets are also present on normal cells at a low level, so novel candidate biomarkers are still required.

Migration-inducing gene 7 (Mig-7) is a cysteine-rich protein found in cell membranes and cytoplasm of carcinoma cells (8, 9, 10). In previous studies, 87% of tumors from breast endometrial, colon, lung, ovary, stomach, kidney, thyroid, cervix, small intestine and prostate (n>200 patients), and blood from untreated metastatic cancer patients possess cells expressing Mig-7 mRNA. However, the Mig-7 mRNA was not detectable in tissues or blood from 25 normal subjects (18). Also, Petty et al has demonstrated that the Mig-7 protein plays an important role in regulating vasculogenic mimicry and facilitating metastatic spread through increased invasion (8). Thus, Mig-7 seems to be a promising cancer cell biomarker for detection, diagnosis, and disease progression.

3. Objectives

This work is based on the production and characterization of a novel monoclonal antibody against Mig-7, a promising cancer cell biomarker for detection, diagnosis, and disease progression. Several themes underline the present proposal:

- Immunize mice with KLH conjugated Mig-7 peptide.
- Produce monoclonal antibodies specific to Mig-7 peptide in hybridoma cells and purify them by using the technique of affinity chromatography.
- Determine the specificity and sensitivity for each antibody with respect to its interaction with Mig-7 peptide. The best antibody will be selected for further analysis.
- Determine cellular localization and cellular specificity of the selected antibody by immunofluorescence microscopy. The Mig-7 specific antibody with high sensitivity will be further developed for using in targeted cancer therapies.

4. Methodology

4.1 Preparation of Mig-7 peptide conjugates

The putative immunogenic residues in the extracellular domain of Mig-7 are the N-terminal amino acids 1-36 of Mig-7 protein sequence. The Mig-7 peptides (Mig-7(1-30) and Mig-7(1-9)) were purchased from GenScript, USA. The carboxylic group of the Mig-7(1-30) was conjugated with Keyhole Limpet Hemocyanin (KLH) according to the standard protocol. KLH-conjugated Mig-7(1-30) was used for generating mouse monoclonal antibodies. The same procedure was performed for conjugation of Mig-7(1-30) and Mig-7(1-9) to OVA and the OVA conjugates were used for screening of Mig-7 specific monoclonal antibodies.

4.2 Immunization of mice with KLH-conjugated Mig-7(1-30)

Antigen used in hybridoma technique is KLH-conjugated Mig-7(1-30). Each of four BALB/c mice was immunized intraperitoneally with 50 μg of the antigen emulsified in complete Freund's adjuvant. The mice were boosted 2 times at 4 weeks-intervals by intraperitoneal injection. After 3-5 times immunization, the mice were bled and monitored levels of Mig-7 specific antibodies in serum. The final boost was given by intravenous injection and the mice were sacrificed. Spleen cells from immunized mice were fused with myeloma cells 3 days after final boost to produce hybridomas.

4.3 Fusion of spleen cells from immunized mice and myeloma cells

Spleen cells from immunized mice were gently mixed with myeloma cells. The mixer was washed with RPMI-1640 and centrifuged at 300x g for 5 minutes at room temperature. The supernatant was removed as completely as possible. The pellet was slowly resuspended in 1.5 ml of pre-warmed PEG-3000. After that, 20 ml of pre-warmed RPMI-1640 were slowly added to

the suspension. The total suspension was centrifuged at 300g for 5 minutes at room temperature and the supernatant was removed. The cells were resuspended in pre-warmed HAT medium to make a cell concentration of 2-4.5×10^5 cells/ml. The suspension was gently mixed and subsequently plated into 96-well cell culture plates. The plates were incubated at 37°C, 5% CO_2. After 7-10 days, the medium was removed, and 75 µl of fresh medium containing feeder cells were added. The plates were further incubated and the medium was changed every 2-3 days. The growth of hybridoma cells was observed under microscope. When the cells grow into about 2-3 mm, the medium from individual well was collected for screening of Mig-7-specific antibodies.

4.4 Screening of Mig-7-positive serum or hybridomas by indirect ELISA

Immunoplates were coated with 2 µg/ml of OVA-conjugated Mig-7(1-30) and OVA-conjugated Mig-7(1-9) and KLH-conjugated Mig-7(1-30) and incubated overnight at 4°C. The plates were washed with PBS containing 0.05% tween (PBST) and blocked with 3% nonfat milk in PBST for 1 hour at 37°C. Fifty microliter of diluted serum or individual culture supernatant were transferred into ELISA plates. The plates were incubated for 30 minutes at 37°C and washed again with PBST. Fifty microliter of HRP conjugated anti-mouse antibody were added to each well and incubated for 30 minutes at 37°C. The plates were washed and 50 µl of TMB substrate were added. After 30 minute incubation in the dark at room temperature, the reaction was stopped by adding 50 µl/well of 0.6 N H_2SO_4 and the absorbance at 450 nm was measured by using a BioTek microplate reader.

4.5 Western blot analysis

RL95 cells expressing Mig-7 protein were trypsinized from culture flasks and collected by centrifugation at 2000*g* and resuspended in CeLyticTM MT cell lysis buffer (Sigma) and a mixture of protease inhibitor cocktail (Sigma). The whole cell extracts were resolved in SDS-polyacrylamide gels and transferred to a nitrocellulose membrane. After blocking with PBS containing 0.05% Tween 20 (PBST) and 3% nonfat milk, the membrane was incubated with the monoclonal antibody at 1:1000 dilution in PBST-1% nonfat milk and washed three times with PBST. The membrane was probed with horseradish peroxidase-conjugated secondary antibody at 1:10000 and washed with PBST three times. The blot was developed using the TMB membrane peroxidase substrate.

4.6 Immunofluorescent microscopy

Mig-7 expressing cells and normal white blood cells were spotted on glass slides. After air-drying, the slides were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100/PBS and blocked with 10% goat serum, 1%BSA in PBS. The slides were then incubated with

the monoclonal antibody and FITC-conjugated anti-mouse IgGAM. The slides were observed under a fluorescence microscope.

4.7 Immunocytochemistry

Mig-7 expressing cells and buccal mucosa cells were spotted on glass slides. After air-drying, the slides were fixed with 4% paraformaldehyde, permeabilized with 0.2% TritonX-100/PBS and blocked with 10% goat serum, 1%BSA in PBS. The slides were then incubated with the monoclonal antibody and horseradish peroxidase-conjugated secondary antibody. The color was developed using the TMB membrane peroxidase substrate. A microscope was used to visualize cells at magnification x4 or x10.

4.8 MTT assay

The cytotoxic activity of the monoclonal antibody on MCF7, HT29 and normal fibroblast cells was analyzed by a 3-(4,5-dimethylthiazole-2-yl)-2,5-biphenyl tetrazolium bromide (MTT) assay (13). The cells were seeded on a 96 well microplate and precultured overnight. Then, 100ul of the monoclonal antibody in the concentration range of 0-50 μM were added. Cells were incubated with the monoclonal antibody for 72 h. 20 μL of MTT solution (5 mg/mL) was added to each well and incubated for an additional 3 h. The colored formazan crystal produced from the MTT was dissolved in 100 μL of dimethyl sulfoxide (DMSO). The optical density (OD) values of the solutions were measured at 540 nm using a BioTek microplate reader.

5. Results and Discussion

Mig-7, a promising cancer cell biomarker for detection, diagnosis, and disease progression, is a cysteine-rich protein found in cell membranes and cytoplasm of carcinoma cells. The putative immunogenic residues in the extracellular domain of Mig-7 are the N-terminal amino acids 1-36 of Mig-7 protein sequence. Therefore, the KLH-conjugated Mig-7(1-30) was used to generate the monoclonal antibody. BALB/c mice were immunized with the KLH-conjugated Mig-7(1-30), and blood was collected from the mice after multiple injections. Antibody titers were tested by ELISA on plates coated with the KLH-conjugated Mig-7(1-30) and OVA-conjugated Mig-7(1-30) (data not shown). The two mice with the highest titers were sacrificed and spleens from both mice were fused to myeloma cells following standard procedures. Individual hybridomas was grown and 375 hybridomas were further characterized. Supernatant from the growing hybridoma clones was screened with ELISA. Screening was performed on plates coated with KLH-conjugated Mig-7(1-30), OVA-conjugated Mig-7(1-30) and OVA-conjugated Mig-7(1-9) to determine antibody specificity. A total of 2 hybridomas (12H11F9 and 10E1C12) reacted selectively with the OVA-conjugated Mig-7(1-30) and none binds to OVA-conjugated Mig-7(1-9). Isotype analysis revealed that 12H11F9 was of IgM, kappa subtype and 10E1C12 was of IgG2a, kappa subtype.

The immunoreactivities of the 12H11F9 and 10E1C12 mAbs with RL95 cells expressing Mig-7 were shown in Figure 1, both of which specifically recognized approximately 28 and 56 kDa protein bands which corresponded to the Mig-7 protein. Immunofluorescence and immunocytochemistry assays demonstrated that only 12H11F9 could specifically recognize Mig-7 expressed on the cell surface of RL95, TK6, HT29, A431, A549, HepG2, Hela, MCF7 carcinoma cell lines (Figure 2A and 3A). The 12H11F9 did not bind to normal cells, human white blood cells and buccal mucosa cells (Figure 2B and 3B), indicating the specificity of 12H11F9 against cancer cells.

Antibody cytotoxicity against MCF7, HT29 and normal fibroblast cells was measured by MTT assay 72 h after 12H11F9 treatment. As shown in Figure 4, the 12H11F9 show toxicity on MCF7 and HT29 cancer cell lines but not on normal fibroblast cells. The percentage of cancer cell viability decreases when increasing the concentration of antibody. The MCF7 and HT29 cell lines showed IC50 of ~ 16 and 50 ug/mL of 12H11F9 monoclonal antibody, respectively. Our results suggested that the 12H11F9 monoclonal antibody can be possibly used in applications of cancer therapeutics.

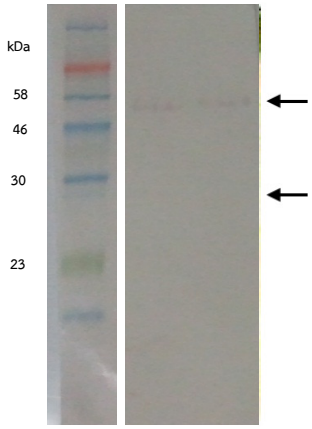

Figure 1. Western blot analysis of 12H11F9 (left) and 10E1C12 (right) monoclonal antibodies with RL95 cells. Arrows indicate the protein bands of Mig-7 protein.

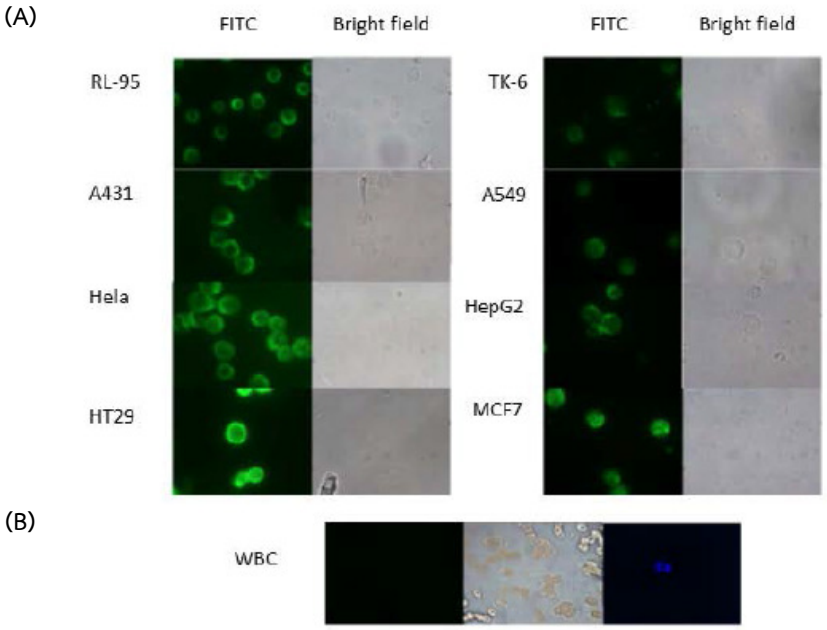

Figure 2. Immunofluorescence staining of 12H11F9 monoclonal antibody with cancer cell lines (A) and human white blood cell (WBC, negative control) (B). RL95 endometrial cancer; HepG2 liver cancer; MCF7 breast cancer; TK6 lymphoblastoid cell; A431 skin cancer; A549 lung cancer; HT29 colon cancer; Hela cervical cancer.

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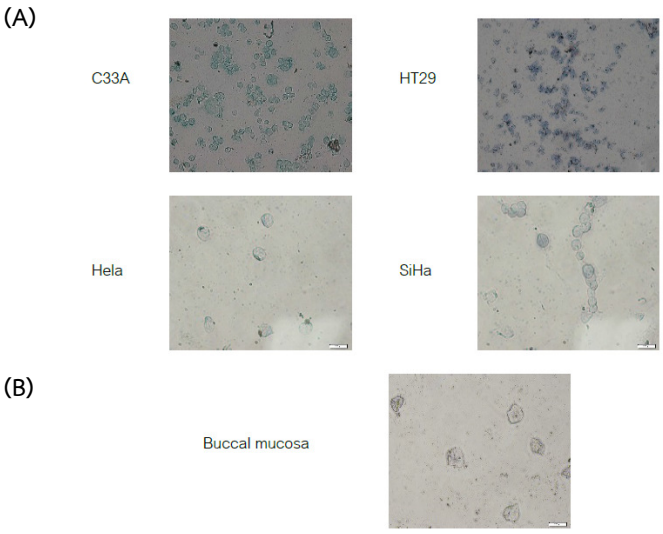

Figure 3. Immunofluorescence staining of 12H11F9 monoclonal antibody with cancer cell lines (A) and buccal mucosa (negative control) (B). C33A cervical cancer; SiHa cervical cancer; Hela cervical cancer; HT29 colon cancer.

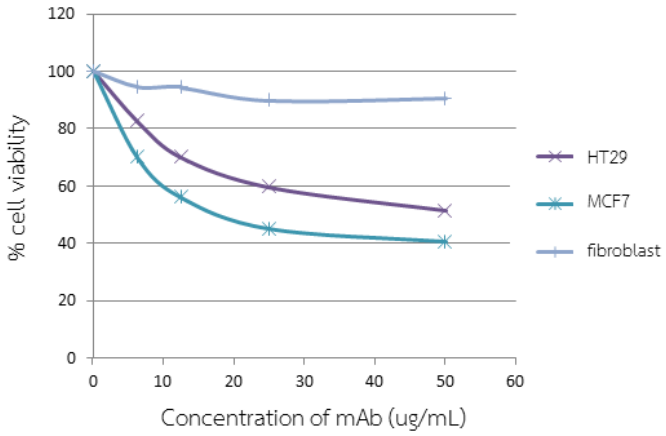

Figure 4. Cytotoxicity of 12H11F9 monoclonal antibody against HT29, MCF7 and normal fibroblast cells.

6. Conclusions and Recommendations

In summary, we generated a specific monoclonal antibody directed against the Mig-7 N-terminal. The 12H11F9 monoclonal antibody exhibited the best performance in a variety of assays including immunoblotting, immunofluorescence stainning, immunocytochemical staining, and MTT. Specific monoclonal antibody may provide an ideal reagent for further investigation of the function of Mig-7 protein and could be useful in applications for cancer diagnosis and targeted therapy.

7. Outputs

- Monoclonal antibody specific for Mig7(1-30) and use thereof. Patent application number 1401000630, dated 4 Feb 2014.
- Poster presentation: "The 21st International Molecular Medicine Tri-Conference 2014", February 9-14, 2014, San Francisco, USA.
- An academic paper for international publication is in progress.

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