USM SHORTERM REPORT
(304/PPSP/613218)

IMMUNOHISTOCHEMICAL STUDY OF BREAST CANCER ANTIGENS (PART I)

MAIN RESEARCHER
Dr. Fawwaz Shakir Mahmoud Al-Joudi

OTHERS RESEARCHERS
Dr. Imran Abdul Khalid
A.P. Dr. Mustaffa Musa
A.P. Dr. Hasnan Jaafar
Dr. Hamid Mat Sain

Pusat Pengajian Sains Perubatan
USM

1892 X

Semua laporan kemajuan dan laporan akhir yang dikemukakan kepada Bahagian Penyelidikan dan Pembangunan perlu terlebih dahulu disampaikan untuk penelitian dan perakuan Jawatankuasa Penyelidikan di Pusat Pengajian.

USM R&D/JP-04

LAPORAN AKHIR PROJEK PENYELIDIKAN R&D JANGKA PENDEK

A. MAKLUMAT AM

Tajuk Projek: Immunohistochemical Study of Breast Cancer Antigens

Tajuk Program: _____

Tarikh Mula: 2003

Nama Penyelidik Utama: Dr. Fawwaz Shakir Mahmoud Al-Joudi
(*berserta No. K/P*)

Nama Penyelidik Lain: Rujuk Laporan yang dilampirkan
(*berserta No. K/P*)

B. PENCAPAIAN PROJEK:

(*Sila tandakan [/] pada kotak yang bersesuaian dan terangkan secara ringkas di dalam ruang di bawah ini. Sekiranya perlu, sila gunakan kertas yang berasingan*)

☐ Penemuan asli/peningkatan pengetahuan

In this study, we found that the polyclonal antibodies raised against survivin that were produced gave a good detection signal of survivin protein in immunohistochemical assay.

The preliminary clinical survey indicated that survivin was detected in more than 80% of the breast masses examined.

☐ **Rekaan atau perkembangan produk baru,**
(Sila beri penjelasan/makluman agar mudah dikomputerkan)

(1) _____

_____

(2) _____

_____

(3) _____

_____

☐ **Mengembangkan proses atau teknik baru,**
(Sila beri penjelasan/makluman agar mudah dikomputerkan)

(1) Improved method in detecting of survivin in breast cancer tissues.

_____

(2) *De novo* production of the diagnostic assay.

_____

(3) _____

_____

☐ **Memperbaiki/meningkatkan produk/proses/teknik yang sedia ada**
(Sila beri penjelasan/makluman agar mudah dikomputerkan)

(1) _____

_____

(2) _____

_____

(3) _____

_____

C. PEMINDAHAN TEKNOLOGI

☐ Berjaya memindahkan teknologi.

Nama Klien: (1) _____
(Nyatakan nama penerima pemindahan teknologi ini dan sama ada daripada pihak swasta ataupun sektor awam)
(2) _____

(3) _____

☐ Berpotensi untuk pemindahan teknologi.
(Nyatakan jenis klien yang mungkin berminat)

_____

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_____

D. KOMERSIALISASI

☐ Berjaya dikomersialkan.

Nama Klien: (1) _____

(2) _____

(3) _____

☐ Berpotensi untuk dikomersialkan.
(Nyatakan jenis klien yang mungkin berminat)

_____

_____

_____

_____

E. **PERKHIDMATAN PERUNDINGAN BERBANGKIT DARIPADA PROJEK**

(*Klien dan jenis perundingan*)

(1) _____

(2) _____

(3) _____

(4) _____

F. **PATEN/SIJIL INOVASI UTILITI**

(*Nyatakan nombor dan tarikh pendaftaran paten. Sekiranya paten/sijil inovasi utiliti telah dipohon tetapi masih belum didaftarkan, sila berikan nombor dan tarikh fail paten*).

(1) _____

(2) _____

(3) _____

G. **PENERBITAN HASIL DARIPADA PROJEK**

(i) LAPORAN/KERTAS PERSIDANGAN ATAU SEMINAR

(1) 9th National Conference on Medical Sciences Mei 2004

Medical School of Sciences, USM, Kubang Keriang. (submitted).

(2)Detection of survivin in tissues using rabbit antisera raised against survivin: A CPC presentation (29th January 2004, PPSP-USM)

_____

(3) _____

_____

(4) _____

_____

(5) _____

_____

(ii) **PENERBITAN SAINTIFIK**

(1) Development of antibodies to survivin using two different oligopeptides and testing using two different immunization protocol. (in preparation). Al-Joudi FS[1] , Iskandar ZA[2]

(2) The expression of survivin , BCL-2 and p53 in breast cancer in the East Cost of Malaysia, (in preparation). Fawwaz SA[1], Iskandar ZA[2], Imran AK[3], Hasnan J[4]., Rosli J[4], Hamid MS[5], Kamal Y[5], Mustafa M[6], Ahmad M[7], Zakaria J[8]. [1]School of Dental Sciences, USM, [2]Department of Chemical Pathology, School of Medical Sciences, USM, [3]Seberang Perai Hospital, [4]Department of Pathology, [5]Department of Surgery, [6]Department of Immunology, School of Medical Sciences, USM, [7]Department of Surgery, [8]Department of Pathology, Kuala Terengganu General Hospital.

(3) The differential expression of survivin in embryonic tissues (in preparation)

Iskandar ZA[2] & Fawwaz SA[1]

(4) _____

_____

(5) _____

_____

(6) _____

_____

H. **HUBUNGAN DENGAN PENYELIDIK LAIN**
(*sama ada dengan institusi tempatan ataupun di luar negara*)

(1) Kota Bharu General Hospital – Dr. Imran Abdul Khaleed

_____

(2) Kuala Terengganu General Hospital : Dr. Ahmad Marzuki & Dr. Zakaria Jusoh.

_____

(3) _____

_____

(4) _____

_____

I. **SUMBANGAN KEWANGAN DARI PIHAK LUAR**
(*Nyatakan nama agensi dan nilai atau peralatan yang telah diberi*)

(1) _____

(2) _____

(3) _____

J. **PELAJAR IJAZAH LANJUTAN**
(*Nyatakan jumlah yang telah dilatih di dalam bidang berkaitan dan sama ada diperingkat sarjana atau Ph.D).*

	Nama Pelajar
Sarjana	_____
	_____
	_____
Ph.D	**Iskandar Zulkarnain bin Alias**
	_____
	_____

K. **MAKLUMAT LAIN YANG BERKAITAN**

_____

_____

_____

_____

_____

_____

5-5-2004 — 5-5-04

Tarikh — **Tandatangan**

Professor Zabidi Azhar Mohd. Hussin
Chairman of Research Ethics Committee
School of Medical Sciences
Health Campus
Universiti Sains Malaysia
16150 Kubang Kerian,
KELANTAN, MALAYSIA.

TANDATANGAN PENGERUSI
JAWATANKUASA PENYELIDIKAN
PUSAT PENGAJIAN

Dr. Fawwaz AL-Jundi
PP.SG - USM
H 284505

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INTRODUCTION

Survivin has been characterized as a member of the family of inhibitor of apoptosis proteins (IAP). It is a 16.5 kD protein made up of 142 amino acids. Survivin is expressed in the G2/M phase of the cell cycle. It has been previously shown to be expressed in a large number of cancers, and its expression has been associated with the aggression of tumors, or with the poor survival of cancer patients.

OBJECTIVE: To develop a clinically useful assay for the detection of survivin in breast cancer by immunohistochemistry.

IMPACTS OF THE STUDY

1. Comprehensive training in the production of immunohistochemistry assay system.
2. The results of this present study gave the information on, which was the better oligopeptide from selected oligopeptides to use for antibody production raised against survivin or the mixture, and which the better protocol of immunization between rapid method and conventional method.
3. They also gave the preliminary data on survivin expression in breast cancer among the Malaysian patients who were registered in University of Science of Malaysia Hospital, Kota Bharu General Hospital and Kuala Terengganu General Hospital..
4. The preliminary information of survivin expression in breast cancer could be used for the further study on other types of cancers in the future, in addition to the potential for expanding the breast cancer work.

METHODOLOGY

Peptide selection and design

Two sequences of survivin molecule were selected for peptide synthesis were C-terminal and N-terminal of survivin sequence of human origin.

Peptide I was 12 amino acid sequence (MGAPTLPPAWQP), molecular formula C59 H88 N14 O15 S1 and Peptide II was 21 amino acids sequence (KEFEETAKKVRRAIEQLAAMD), molecular formula C106 H179 N31 O34 S1. Peptide I and Peptide II were purchased from Peptron (Korea), specially purified grade 1

Preparation of antigen for immunization

Survivin is a small antigen with a molecular weight of 16.5 kD. To increase its immunogenicity, selected sequence of amino acids of C-terminal and N-terminal of survivin molecule were conjugated to keyhole limpet homocynine (KLH) using glutaral aldehyde method. Glutaraldehyde (grade 1) will crosslink via free amino groups, i.e. the amino terminal of the peptide and/or the epsilon amino group of lysine.

Animals

Animals (New Zealand white rabbits) were purchased from The Animal House, School of Medical Sciences, University of Sciences of Malaysia. Healthy female New Zealand white rabbits aged 3-4 months (body weight ≈ 3 kg) lived in animal house at least for 3-5 days for adapting to the new circumstance. Females were preferred as they often produce a stronger immune response (Clark et al. 2002).

Polyclonal antibody production

These preparations based on Hu et al. (2002). Sample A : Peptide 200-300 μg was emulsified in PBS with same volume of the Freund's complete adjuvant (3-4 mg/ml) heat –killed *Bacillus tubercle* in lanonin: mineral oil =2:5 [v/v]) in 1 ml total volume. Sample B: Peptide 200-300 μg was emulsified in PBS with same volume of the Freund's incomplete adjuvant (no *Bacillus tubercle*) in 1 ml total volume.

The conventional method: On the first day, 1.5 ml blood was collected by bleeding rabbits from the marginal vein of the ear for preparing the pre-immunized sera as internal negative control. 1.5 ml of blood from the control rabbits (without any immunizations) also was taken as external negative control. Afterwards, 1 ml of sample A was injected subcutaneously on the right flank of the rabbit. Subcutaneous injections was preferred in order to minimize the formation of sterile abscesses (Clark et al. 2002). This method needed several sample B injections in every 2-4 weeks successively. The name of this antibody was SUR12A-CFI.

The rapid method : Exactly same as the conventional method, except there is an additional sample A injection on the 3rd day. On the third day, the injection was repeated once to strengthen the first immunization stimulation. On the 28th day, 1 ml sample B was injected by the same way. On the 35th day, 0.5 ml blood was collected by the same way used before for checking its titer of the antisera.. Then, the antisera was harvested from the marginal vein of the ear. The name of this antibody was SUR12A-RFI.

Immunostaining of antisera against survivin in colon cancer and breast cancer sections.

Briefly, the immunohistochemistry of paraffin-embedden sections from colon cancer and breast cancer was done by using ABC method (DAKO). The dilution of primary antibody SUR12A-CFI was 1: 1280 (after cheuqerboard done) and latter step was according to the manufacturer's direction. Antisera staining was developed by 3'3–diaminobenzidine (DAKO). Slides were counterstained by Eosin complex and Haematoxylin.

Evaluation of IHC results.

The mean percentage of positive tumor cells was determined in at least five areas at 400-fold magnification and assigned one of the following five categories: 0, <5%; 1, 5-25%; 2, 25-50%; 3, 50-75%; and 4, >75%. The intensity of survivin immunostaining was scored as follows: 1+, weak; 2+, moderate; and 3+, intense. Because tumors showed heterogenous staining, the dominant pattern was used for scoring.

RESULTS

Table 1 Determination of optimal titers for indirect immunoperoxidase method

(Chequerboard titration for SUR12A-RFI antibody)

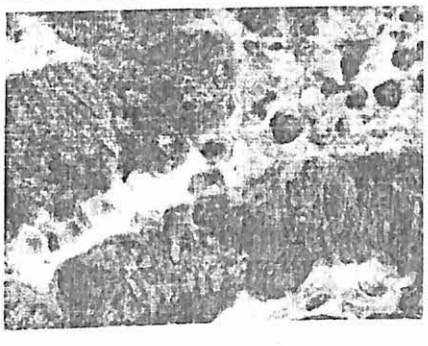
	Dilutions of primary antibody SUR12A-RFI →						
Dilutions of *secondary* antibody (conjugate) ↓		1/5	1/20	1/80	1/320	1/1280	Negative *control*
	1/40	+++ (+++)	+++ (+++)	+++ (+++)	+++ (+++)	+++ (++)	0 0
	1/80	+++ (+++)	+++ (+++)	+++ (+++)	+++ (++)	+++ (++)	0 0
	1/160	+++ (+++)	+++ (+++)	+++ (++)	+++ (++)	+++ (++)	0 0
	Negative control (primary antibody was omitted, and was replaced by Bovine serum albumin (BSA) or normal serum or preimmunized serum). *Intensiy of specific staining is indicated as scale 0 to +++, nonspecific* background given in the same scale in parentheses (0 to +++) *Eg. (+++) indicates strong specific staining with moderate background* (+)						

Table 2 . The plates of chequerboard titration of primary antibody SUR12A-RFI in fixed dilution of secondary antibody (1:160).

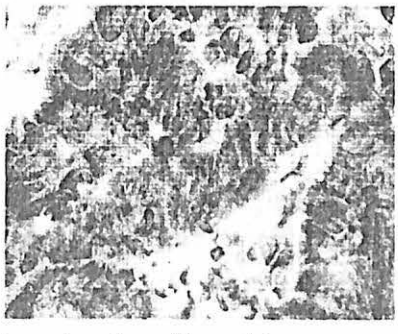
Intensity of specific staining +++
Nonspecific background (+++)
Magnification *X 400*
Dilution : 1/5 (1/160)

Intensity of specific staining +++
Nonspecific background (+++)
Magnification *X400*
Dilution : 1/20 (1/160)

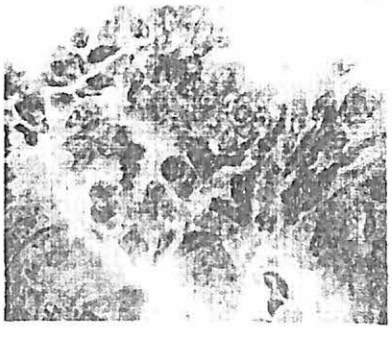
Intensity of specific staining +++
Nonspecific background (++)
Magnification *X 400*
Dilution : 1/80 (1/160)



Intensity of specific staining +++
Nonspecific background (++)
Magnification *X400*
Dilution : 1/320 (1/160)



Intensity of specific staining +++
Nonspecific background (++)
Magnification *X 400*
1/1280 (1/160)



Intensity of specific staining (0)
Nonspecific background (0)
Magnification *X 400*
Negative control : without primary Ab or normal rabbit antiserum

Figure 1: The chequerboard titration of primary Ab SUR12A-RFI in fixed dilution of secondary Ab (1:40)

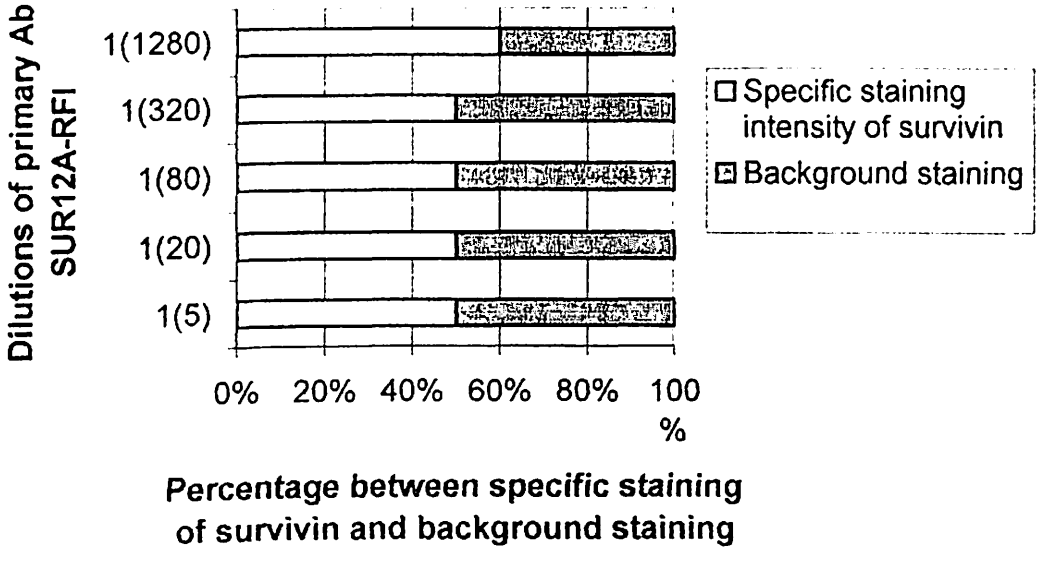


Figure 2 :The cheqeurboard titration of primary Ab SUR12A-RFI in fixed dilution of secondary Ab (1: 80)

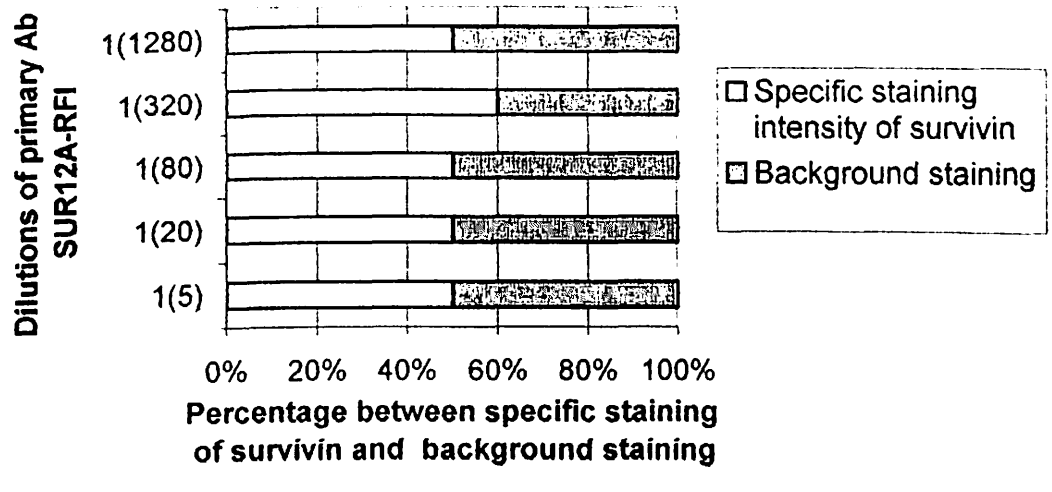


Figure 3: The chequerboard titration of primary Ab SUR12A-RFI in fixed dilution of secondary Ab (1:160)

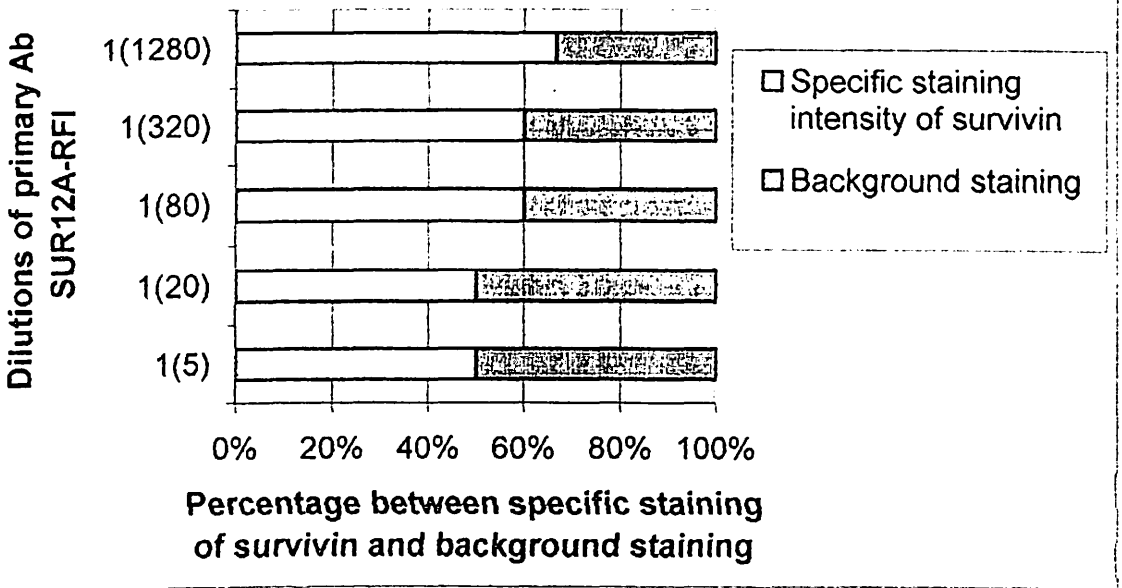


Table 3 Determination of optimal titers for indirect immunoperoxidase method

(Chequerboard titration for SUR12A-CFI)

	Dilutions of primary antibody SUR12A-CFI →						
Dilutions of secondary antibody (conjugate) ↓		*1/5*	*1/20*	*1/80*	*1/320*	*1/1280*	*Negative control*
	1/40	+++ (+++)	+++ (+++)	++ (+++)	++ (+++)	+++ (++)	0 0
	1/80	+++ (++)	+++ (++)	+++ (+++)	+++ (++)	+++ (++)	0 0
	1/160	+++ (+++)	+++ (+++)	+++ (++)	+++ (++)	+++ (+)	0 0
	Negative control (primary antibody was omitted, and was replaced by bovine serum albumin (BSA) or normal serum or preimmunized serum). Intensiy of specific staining is indicated as scale 0 to +++, nonspecific *background given in the same scale in parentheses (0 to +++)* Eg. (+++) indicates strong specific staining with moderate background (+)						

Table 4: . The plates of cheqeurboard titration of primary antibody SUR12A-CFI in fixed dilution of secondary antibody (1:160)

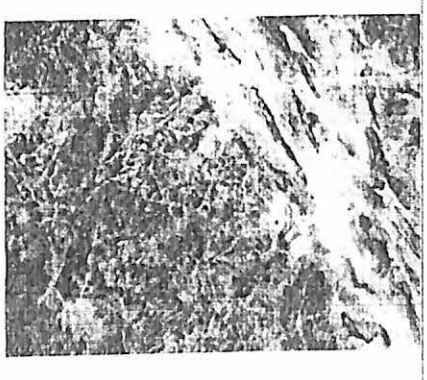
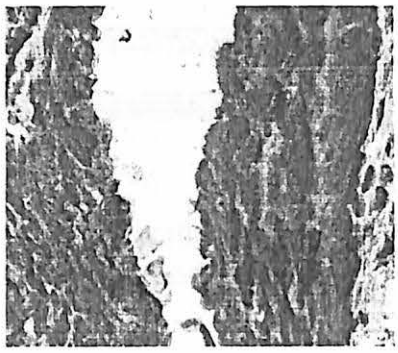
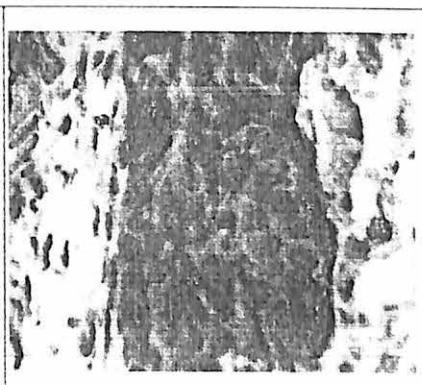
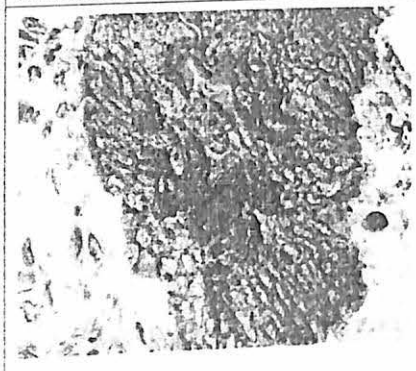
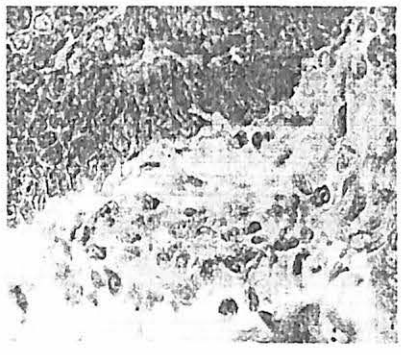
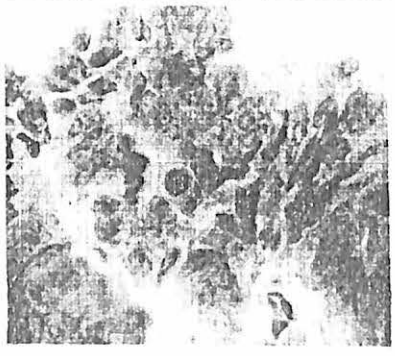
 Intensity of specific staining +++ Non-specific background (+++) Magnification *X 400* *Dilution : 1/5 (1/160)*	 Intensity of specific staining +++ Non-specific background (+++) Magnification *X 400* *Dilution : 1/20 (1/160)*	 Intensity of specific staining +++ Non-specific background (++) Magnification *X400* *Dilution : 1/80 (1/160)*
 Intensity of specific staining +++ Non-specific background (++) Magnification *X400* *Dilution : 1/320 (1/160)*	 Intensity of specific staining +++ Non-specific background (+) Magnification *X 400* *1/1280 (1/160)*	 Intensity of specific staining (0) Non-specific background (0) Magnification *X 400* *Negative control : without primary Ab/ normal rabbit antiserum*

Figure 4: The cheqeurboard titration of primary Ab SUR12A-CFI in fixed dilution of secondary Ab (1:40)

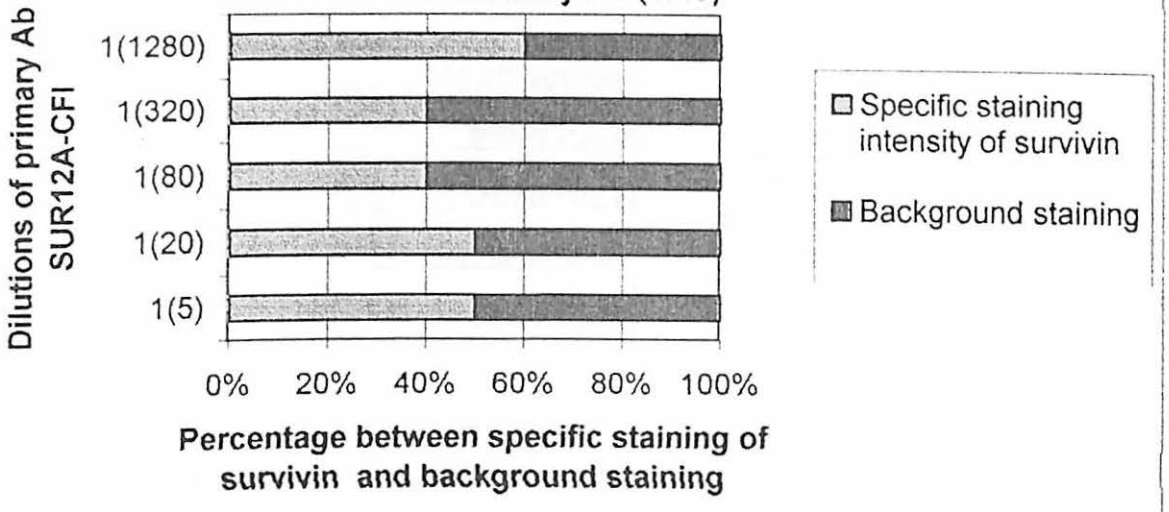


Figure 5: The chequerboard titration of primary Ab SUR12A-CFI in fixed dilution of secondary Ab (1:80)

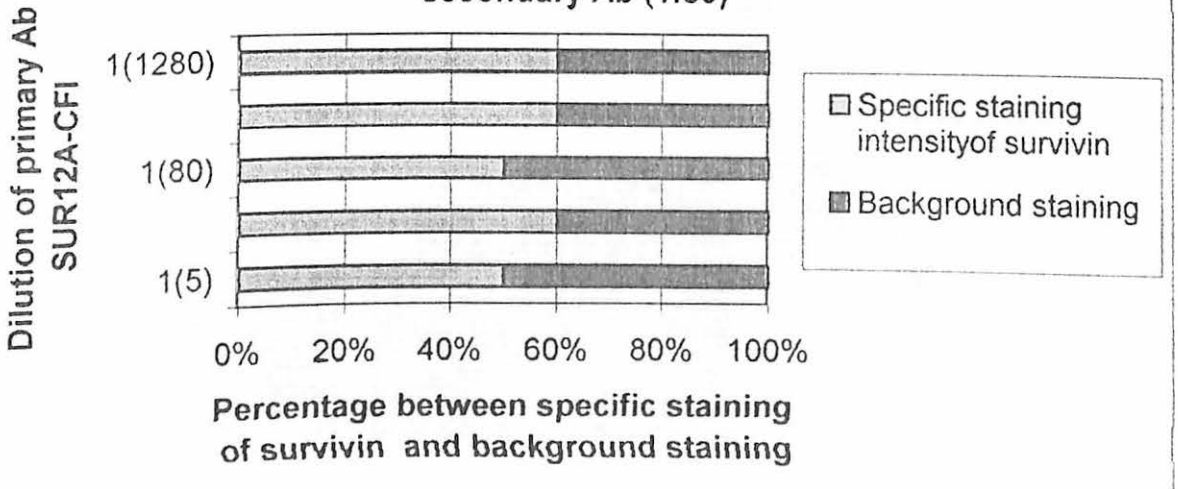


Figure 6: The chequerboard titration of primary Ab SUR12A-CFI in fixed dilution of secondary Ab (1:160)

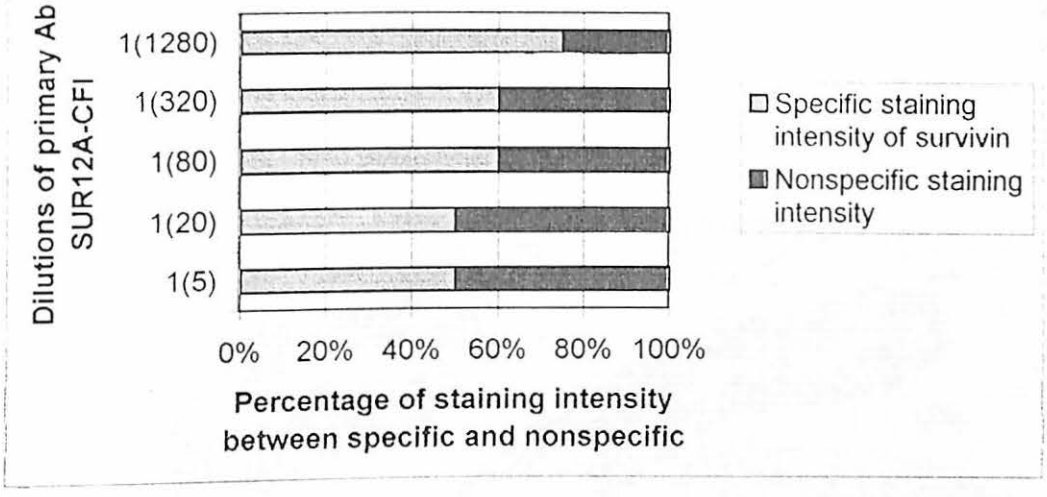


Table 5 : Immunostaining of antisera against survivin in the colon cancer tissue. In this section, the immunostaining was restricted to the cytoplasm of the colon cancer cells.

Preimmunized antisera (Negative control)	Immunized antisera	
	Rapid SUR12A-RFI	Conventional SUR12A-CFI
		
Magnification x400 Specific staining (0) Nonspecific background staining (0) Dilutions 1:1280 (1:160)	Magnification x400 Specific staining (+++) Nonspecific background staining (++) Dilutions 1:1280 (1:160)	Magnification x400 Specific staining (+++) Nonspecific background staining (+) Dilutions 1:1280(1:160)

Immunohistochemical analysis of breast cancer sections (preliminary results)

The plates below (Figure 7.1 and Figure 7.2) shows the positive immunostaining of survivin in breast cancer tissues using SUR12A-CFI antibody with different magnification.

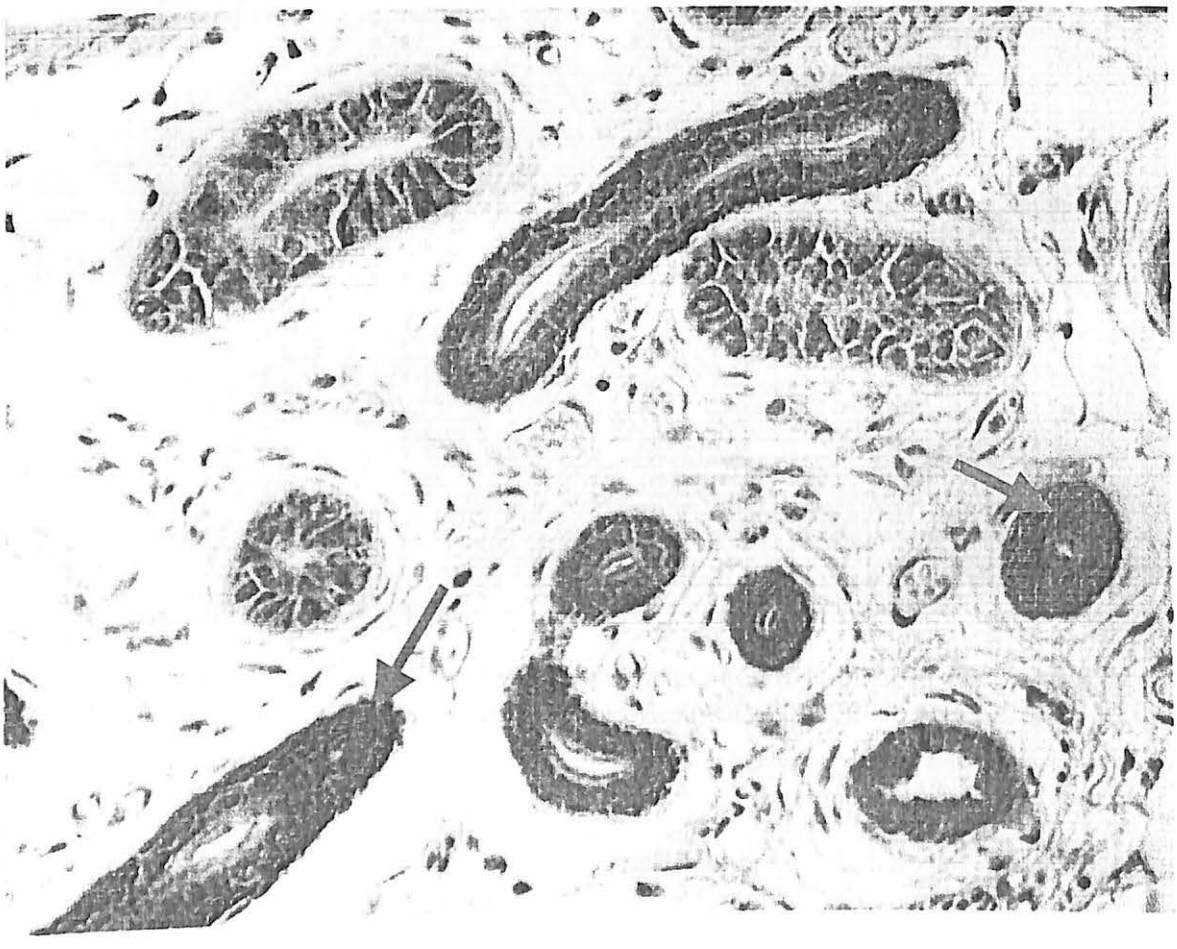


Figure 7.1 Survivin staining in breast cancer showing strong (+3) cytoplasmic and nuclear positivity (arrows) in tumor cells (Magnification x 100) using SUR12A-CFI Ab dilution 1:1280

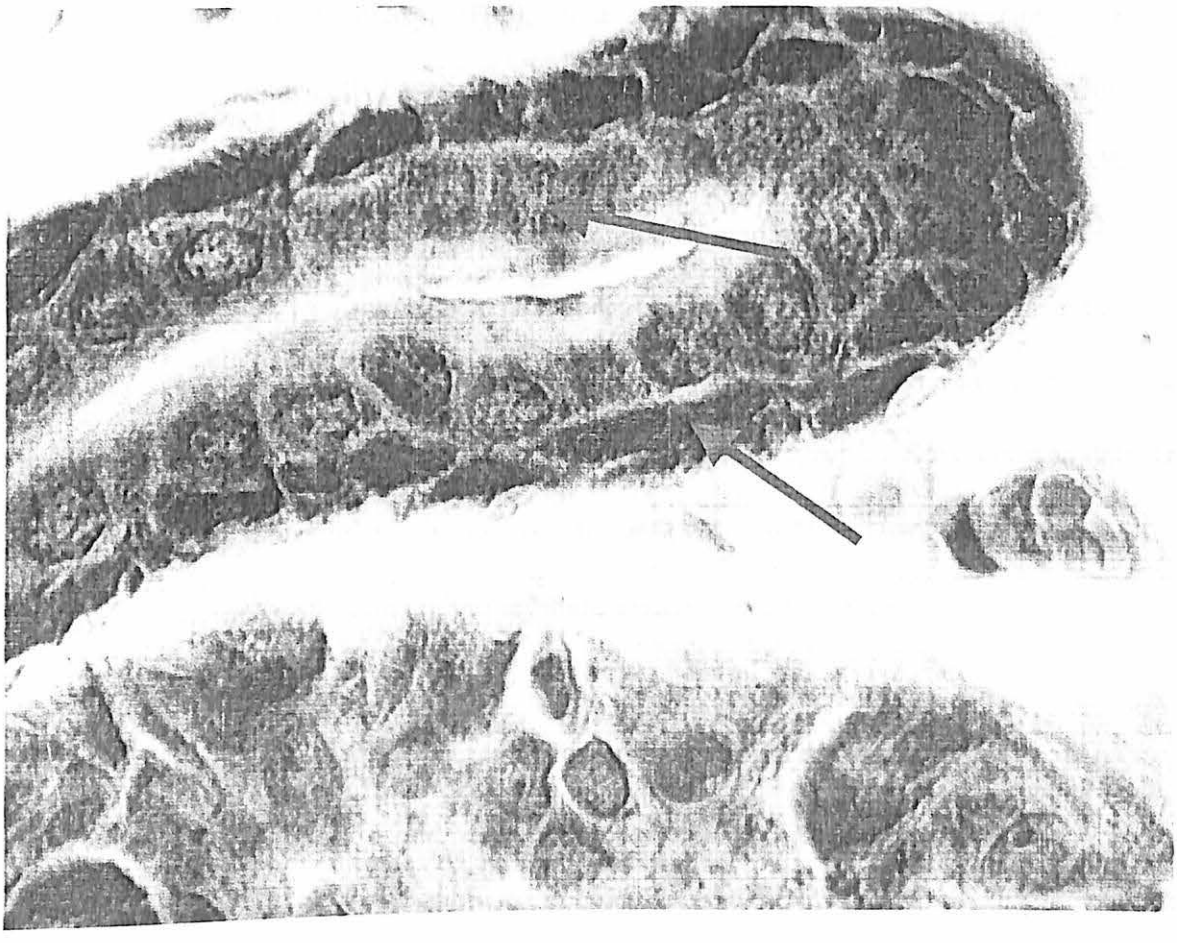


Figure 7.2 Survivin staining (arrows) in breast cancer showing strong (+3) cytoplasmic and nuclear positivity in tumor cells (Magnification x 400) using SUR12A-CFI Ab dilution 1: 1280

DISCUSSION

Immunohistochemistry (IHC), is a method utilized for localizing specific antigens in tissues or cells based on antigen-antibody reaction. According to Taylor et al. (2002), IHC is a sensitive detection systems, that is why we have chosen this method to detect the survivin protein in the tissues or cells, instead of other methods. Based on this, the antibodies SUR12A-CFI and SUR12A-RFI were produced. They were raised against selected oligopeptides. These antibodies were further used to construct an immunohistochemical assay system for the detection of survivin. Survivin was detected successfully in those assay in positive tissues. Moreover, a stringent standardization protocol utilizing professional chequerboard assays, were performed

Determination of optimal titers for indirect immunoperoxidase method

Chequerboard titration for SUR12A-CFI and SUR12A-RFI were performed to investigate the optimal dilutions for those antibodies for using in IHC to detect survivin in colon cancer and breast cancer tissues. This selection of positive controls was based on previous findings stating that colon cancer displays a high rate of expression of survivin (Sarela et al. 2001). Consequently colon cancer was used in this work for the initial testing of the assays, and for the standardization in the chequerboard assay, performed.

It was found that the optimal dilution for SUR12A-CFI was 1:1280 and 1:160 for the secondary antibody whilst for SUR12A-RFI was 1:1280 and secondary antibody 1:160. Even though the optimal dilution for those antibodies were the same but the

results were based on the contrast of the intensity of specific staining of survivin and the intensity of nonspecific background staining .(Refer Table 2 and Table 4). As a results, SUR12A-CFI gave more specific positive staining compared to SUR12A-RFI. This intensity was more contrast compared to SUR12A-CFI (refer Table 5). Evidently, survivin detected efficiently in breast cancer in the following stages of the work.

According to Taylor et al. (2002), the optimal dilution for use of an antibody in immunohistology is defined as that dilution at which the greatest contrast is achieved between the desired (specific) positive staining and any unwanted (nonspecific) background staining Based on this definition, in this study, again, it was found that the SUR12A-CFI antibody produced by the conventional method of immunization protocols gave a better positive staining of survivin and lesser nonspecific background staining compared to the SUR12A-RFI antibody produced by the rapid method of immunization protocols (Table 2 and Table 4)

Quality control

Quality control as define by the College of American Pathologist is "the aggregate of processes and techniques so derived to detect, reduce and correct deficiencies in analytical process". As it pertains to IHC, quality control standards address and define each step of the "total IHC test", including tissue procurement, fixation, processing, sectioning, staining, and finally, the interpretation and reporting of the results (Taylor et al. 2002). In this study, based on the definition above, the rules were abided by, during the production stages, as well as the testing stages.

After finishing the chequerboard titration of those antibodies using selected colon cancer tissues, we chose to use SUR12A-CFI antibody to detect survivin protein in breast cancer tissues which was the main objective of the study. In this study, we have tested the performance parameters including the sensitivity, specificity, precision, accuracy, and reproducibility of the results. We used multitissues control blocks containing known positive (which is known to contain the survivin) which, was selected colon cancer tissues and known negative normal which was adjacent normal breast tissues (internal controls) and tumor tissues which was breast cancer tissues.

According to Taylor et al. (2002), the specificity of antibody staining is shown by the expected absence of staining in certain cells (there was some cells not stained even though other types of cell stained in the same slide) of survivin positive tissues, tissues (normal breast tissues; absence of staining), and tumors (not all tumor shows survivin positive staining) with the multitissue control blocks Precision , in contrast , attest to the validity of the entire procedure and it shown by the presence of both positive and negative elements, as expected on the same control slide (same with there was some cells not stained even though other types of cell stained in the same sections of survivin positive tissues). A sensitive test is one , that detects a small amount of survivin antigen. It was found that a few slides detected a small amount of survivin antigen which the percentage of positivity of the cells less than 25%. Accuracy is determined by the evaluation of nonspecific background staining . A negative reagent control was used (substitution of the primary antibody with antibody diluent (buffer plus bovine serum albumin (BSA) carrier protein) or normal rabbit serum or preimmunized rabbit serum)

instead of SUR12A-CFI. It was found that there was no run-to-run variations in the results obtained, which indicated the high reproducibility of the assay systems developed.

CONCLUSIONS

The polyclonal antibodies raised against survivin that were produced in the present study gave a good detection signal of survivin molecule in immunohistochemical assay.

(THIS IS A PRELIMINARY REPORT FOR PHASE I AND WE ARE WAITING FOR THE RELEASE OF THE USM SHORTERM GRANT PART 2 TO CONTINUE THE PHASE II OF THE STUDY. WE WILL GIVE A COMPLETE REPORT AFTER FINISHING THE PHASE II OF THE STUDY INCLUDING, HOPEFULLY, THE ANTICIPATED PUBLICATION. THANK YOU).

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