Investigating the expression and function of the Steroid Receptor RNA Activator Protein (SRAP) in breast cancer

by

Yi Yan

A Thesis submitted to the Faculty of Graduate Studies of
The University of Manitoba
In partial fulfillment of requirements of the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry and Medical Genetics
University of Manitoba
Winnipeg, Manitoba

Copyright @2016 by Yi Yan
TABLE OF CONTENTS

Abstract .................................................................................................................. 6
Acknowledgements .................................................................................................. 7
List of Abbreviations .............................................................................................. 9
List of Figures ......................................................................................................... 11
List of Tables .......................................................................................................... 14
Thesis organization ................................................................................................. 15

CHAPTER I Introduction

I-1. Normal breast and breast cancer .................................................................... 17
   I-1-1 Normal breast .......................................................................................... 17
   I-1-2 Overview of breast cancer ...................................................................... 19
   I-1-3 Breast cancer diagnosis ......................................................................... 19
   I-1-4 Breast cancer treatment ......................................................................... 27
   I-1-5 Etiology of breast cancer ....................................................................... 33
   I-1-6 Breast cancer biomarker ........................................................................ 34
   I-1-7 Further exploring steroid receptor signaling, investigating co-regulators 35

I-2. Steroid Receptor RNA activator (SRA).......................................................... 38
   I-2-1 Discovery of SRA RNA .......................................................................... 38
   I-2-2 Mechanisms of SRA RNA action ......................................................... 40
   I-2-3 Structure of SRA RNA .......................................................................... 45
   I-2-4 Relevance of SRA RNA to breast cancer ............................................. 47

I-3. Steroid Receptor RNA activator protein (SRAP) .......................................... 49
   I-3-1 Discovery of SRAP ................................................................................ 49
   I-3-2 SRA variants and alternative splicing events ....................................... 51
   I-3-3 Potential function of SRAP ................................................................... 53
CHAPTER V Expression of both ER-β1 and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with Estrogen Receptor-alpha (ER-α)-Negative Early Breast Cancer (EBC) _______ 93

V-1 Abstract .................................................................................................................. 95
V-2 Introduction ............................................................................................................ 95
V-3 Materials and Methods ......................................................................................... 97
V-4 Results ................................................................................................................... 98
V-5 Discussion .............................................................................................................. 100
V-6 References ............................................................................................................ 101

CHAPTER VI The Steroid receptor RNA Activator protein (SRAP) controls cancer cell migration/motility ...................................................................................... 104

VI-1 Abstract .............................................................................................................. 106
VI-2 Introduction ......................................................................................................... 106
VI-3 Materials and Methods ....................................................................................... 107
VI-4 Results and Discussion ....................................................................................... 109
VI-5 References ........................................................................................................ 113
VI-6 Supplementary figures ...................................................................................... 117

CHAPTER VII Discussion ............................................................................................. 121

VII-1 Overall summary ............................................................................................... 121
VII-2 What is the real significance of assessing SRAP expression in breast tissue .... 126
VII-3 What are the exact roles played by SRA/SRAP in breast cancer cells .......... 127
VII-4 Conclusion and future Directions ..................................................................... 134
Appendix: list of publications .................................................................................... 141
References .................................................................................................................. 143
Abstract

Seventeen years ago, the Steroid receptor RNA Activator (SRA) was identified as a functional non-coding RNA able to increase the activity of the estrogen receptor (ER), a critical player in mediating the mitogenic role of estradiol in breast cancer. Interestingly, four years later, SRA appeared to be the first ever discovered functional RNA also able to encode a corresponding protein (SRAP). As such, the products of the SRA1 gene delineate a fascinating bi-faceted system, involving both a functional RNA and a protein. Since its discovery, the non-coding aspect of this system has been widely investigated, with multiple groups contributing new information on the structure and related functions of SRA. Overall, the non-coding SRA transcript is thought to act as a broad co-regulator modulating the activity of different transcription factors. Conversely, limited information has been obtained on the coding aspect (SRAP) of this system, even though SRA/SRAP is currently believed to be involved in several mechanisms including tumourigenesis, tumour progression, myogenesis and adipogenesis.

In this body of work, I have attempted to define the clinical relevance of SRAP to breast cancer and extend the understanding of the cellular processes potentially regulated by this protein. I first established that SRAP has the potential to become a new prognostic and predictive factor in specific groups of patients. Indeed, I have demonstrated, using tissue microarray analyses (TMAs), that SRAP expression was up-regulated in some breast tumours, with high levels associated with poor prognosis in estrogen receptor α (ER-α) positive breast cancer patients. Using the same technique, I have further identified a significant high correlation between a positive response to tamoxifen treatment and a high level of SRAP expression in a large cohort of ER-α negative cases. This observation highlights the potential for SRAP to become a new predictive factor of response to endocrine therapy in this specific group of patients. Using RNA-seq to define the
transcriptomes of cervical Hela and breast MDA-MB-231 cancer cells upon depletion or overexpression of this protein, I further identified cellular movement amongst the potential cellular processes affected by changes in SRAP expression. Using classical trans-wells assays as well as an live-cell imaging assays, I have confirmed that SRAP indeed regulates individual cancer cell motility.

Overall, my results provide critical new insights into the potential functions of the protein counterpart of the intriguing SRA/SRAP bi-faceted gene system. SRAP herein appears as a potential biomarker and new therapeutic target in the fight against breast cancer.
Acknowledgements

First and foremost I acknowledge my supervisor Dr. Etienne Leygue for his unselfish and patient guidance in the academic field. I have learned a lot from him, inside and outside of science. He has also provided me with a great working environment. Throughout my whole PhD periods, his motivation, expertise and enthusiasm toward science have always inspired me. I thank him for teaching me the right attitude toward research and how to be a real scientist.

Second, I would like to thank my committee members who have contributed to my thesis work, Dr. Murphy who has provided me with plenty of excellent opportunities to learn and accomplish my goals; Dr. Xie who have guided me with the structure of this thesis and shared his expertise in the RNA aspect of my project; Dr. Myal, who have helped and inspired me during my whole PhD study. Thanks to all for the professional guidance and advices you promulgated during my research;

I wish to mention past and present members of the Leygue's Lab. I was lucky to work with Charlton Cooper, Mohammad Hamedani, Shilpa Chooniedass and Jimin Guo. You are excellent people to work with. In particular, Charlton thanks for all the demonstrations of goodwill and criticisms when I embarked on the research. Your wide knowledge and logical way of thinking have been of great value to me.

I would like also to thank many Collaborators and their trainees, Brent from Dr. McManus’s Lab, Edgar from Dr. Hatch’s Lab, Christine from Dr. Marshall’s Lab and Vasu from Dr. Raouf’s lab for all their advice and fruitful discussions about my project. Thanks to all colleagues in the Department of Biochemistry and MICB for helping hands when I was in all different kinds of “trouble”.

Lastly, I am grateful to my parents and my wife for the mental support. Love brings us together and binds us tightly.
In closing, I also would like to thank funding agencies, which have sustained my Research throughout my PhD. I have been funded by the Manitoba Health Research Council (MHRC) doctoral scholarship and Canada Institute Health Research (CIHR) doctoral scholarship. In addition, I acknowledge the University of Manitoba and the CIHR who provided travel awards that have enabled my research work to be presented at several national and international conferences.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AF-1:</td>
<td>Activation Function 1</td>
</tr>
<tr>
<td>AF-2:</td>
<td>Activation Function 2</td>
</tr>
<tr>
<td>AR:</td>
<td>Androgen Receptor</td>
</tr>
<tr>
<td>ChIP:</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>ChIP-seq:</td>
<td>Chromatin immunoprecipitation sequencing</td>
</tr>
<tr>
<td>CTCF:</td>
<td>CCCTC-Binding Factor (Zinc Finger Protein)</td>
</tr>
<tr>
<td>DBD:</td>
<td>DNA binding domain</td>
</tr>
<tr>
<td>DCIS:</td>
<td>Ductal Carcinoma In-Situ</td>
</tr>
<tr>
<td>ER:</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>ERE:</td>
<td>Estrogen receptor response element</td>
</tr>
<tr>
<td>GATA3:</td>
<td>Trans-acting T-cell-specific transcription factor</td>
</tr>
<tr>
<td>GR:</td>
<td>Glucocorticoid receptor</td>
</tr>
<tr>
<td>HRE:</td>
<td>Hormone responsive element</td>
</tr>
<tr>
<td>HER2:</td>
<td>Human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>IHC:</td>
<td>Immunohistochemistry</td>
</tr>
<tr>
<td>LBD:</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LCIS:</td>
<td>Lobular Carcinoma in Situ</td>
</tr>
<tr>
<td>LCoR:</td>
<td>ligand-dependent nuclear receptor corepressor</td>
</tr>
<tr>
<td>MBD3:</td>
<td>Methyl-CpG binding domain protein 3</td>
</tr>
<tr>
<td>MEF’s:</td>
<td>Mouse embryonic fibroblast cells</td>
</tr>
<tr>
<td>MRI:</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NCoR:</td>
<td>Nuclear receptor co-repressor</td>
</tr>
<tr>
<td>NCoR/SMRT:</td>
<td>Nuclear receptor co-repressor/silencing mediator for retinoid and thyroid-hormone receptors</td>
</tr>
</tbody>
</table>
MAPI3K1: Mitogen-Activated Protein Kinase Kinase Kinase 1, E3 Ubiquitin
PI3K: Phosphatidylinositol-4,5-bisphosphate 3-kinase
PLAU: Urokinase plasminogen activator
PgR: Progesterone Receptor
Pus1p: Pseudouridylate synthase 1
Pus3p: Pseudocuidylate synthase 3
PTM: Post-translation modification
RRM: RNA recognition motif
RID: RNA interacting domain
RISC: RNA-Induced Silencing complex
SERM: Selective Estrogen Receptor Modulators
SDM: Site-directed mutagenesis
SHARP: SMRT/HDAC1 Associated Repressor Protein
SHAPE: S selective 2’-hydroxyl acylation analyzed by primer extension
SLIRP: SRA stem-loop interacting RNA binding protein
SRA: Steroid receptor RNA activator
SRAP: Steroid receptor RNA activator protein
SRC-1: Steroid Receptor Co-activator 1
STR: Secondary structural motif
SWI/SNF: The ATP-dependent chromatin remodeling complexes
TMA: Tissue microarray
TPPCR: Triple Primer PCR
TP 53: Tumour protein p53
TR: Thyroid receptor hormone
YB-1: Y-box binding protein
List of Figures

Chapter I

Figure I-1: Anatomy of normal mammary gland ........................................ 18
Figure I-2: Estrogen signaling pathway and co-regulators ............................ 37
Figure I-3: SRA core sequence ................................................................. 39
Figure I-4: Potential mechanisms underlying non-coding SRA mediated biology __ 42
Figure I-5: Secondary structure of SRA RNA STR7/H13 ................................ 47
Figure I-6: Alignment of human SRAP and several putative SRAP sequences from multiple non-vertebrate species ........................................... 50
Figure I-7: SRA RNAs currently registered in the nr database of NCBI .......... 52
Figure I-8: SRAP 3D structure ................................................................. 57

Chapter III

Figure III-1: Western blot detection of ~30 kDa SRAP in MCF-7 cells .......... 66
Figure III-2: Detection of SRAP in Hela cells by fluorescent immunocytochemistry. ___ 67
Figure III-3: Western blot detection of ~30 kDa, ~25 kDa and ~40kDa SRAP in breast tumour tissues ................................................................. 68
Figure III-4: SRAP Immunohistochemical detection in breast tissue sections ______ 69
Figure III-5: Tissue micro-array analysis of SRAP expression .................. 70
Figure III-6: Distribution of SRAP H-scores in 372 breast cancer cases ________ 71
Figure III-7: Low SRAP is an indicator of better survival for younger patients and patients with ER positive or PR positive tumours .............................. 73
Figure III-8: SRAP as a poor prognostic indicator in younger patients whose tumours are ER positive and node negative ................................. 74
Chapter IV
Figure IV-1: Immunodetection of SRAP related peptide in breast cancer cells  83
Figure IV-2: Immunodetection of SRAP related peptide in breast cancer tissues  86
Figure IV-3: Tissue micro-array analysis of SRAP expression  87
Figure IV-4: Distributions of IHC scores in 170 ER+ breast cancer cases  88
Figure IV-5: Low SRAP is an indicator of better survival for patients with ER positive tumours  89

Chapter V
Figure V-1: Expression of high levels of both ER-β1 and SRAP is predictive of response to tamoxifen in the entire NCIC-CTG-MA12 cohort  98
Figure V-2: Expression of high levels of both ER-β1 and SRAP is predictive of response to tamoxifen only in ER-α-negative breast cancer  99

Chapter VI
Figure VI-1: Silencing SRA1 gene expression and overexpressing "only-SRAP" inversely modifies the expression of 106 genes, twenty of which are linked to cell migration  108
Figure VI-2: Silencing SRA1 expression reduces MDA-MB-231 cell migration as assessed by wound-healing assay  109
Figure VI-3: Live cell imaging of individual cells expressing or not SRA1 gene  110
Figure VI-4: Decrease in motility of individual MDA-MB-231 cells upon SRA Silencing  111
Figure VI-5: Increase in motility of individual Hela cells upon overexpression of SRAP  112
Figure VI-1: SRA RNA and SRAP expression upon different SRA siRNAs silencing

Figure VI-2: Both functional and coding isoforms are similarly decreased by Si-RNA treatment

Figure VI-3: Silencing SRA1 expression reduces MDA-MB-231 cell migration as assessed by Invasion- and trans-well assays

Chapter VII

Figure VII-1: Schematic profile of SRAP antibodies used

Figure VII-2: Secondary structure of SRA STR5

Figure VII-3: Overview of BioID approaches for interactome mapping
### Table list

**Chapter I**

Table I-1: Breast cancer historical subtypes ........................................... 21
Table I-2: Breast cancer molecular subtypes ........................................... 24
Table I-3: Breast cancer stages ................................................................. 26
Table I-4: Proteins forming complexes with non-coding SRA ...................... 43
Table I-5: Proteins forming complexes with or directly binding to SRAP ....... 55

**Chapter III**

Table III-1: Distribution of Low and high SRAP cases in case subgroups ....... 72
Table III-2: Cox Multivariate analysis of Breast Cancer Specific and Recurrence-Free Survivals ................................................................. 75

**Chapter IV**

Table IV-1: Cox analysis of Breast Cancer Specific Survival ...................... 84
Table IV-2: Cox Multivariate analysis of Breast Cancer Specific Survival ...... 89

**Chapter V**

Table V-1: Predictive analysis for ER-β1 and SRAP ................................. 96

**Chapter VII:**

Table VII: Relevance of SRAP to breast cancer survival (summary of TMA study) ................................................................................................. 126
Thesis organization

My PhD thesis is the extension of my MSc thesis where I attempted to define the biological relationship existing between the Steroid receptor RNA activator (SRA) and the estrogen receptor beta (ER-β1). To further this search for SRA/SRAP significance in breast cancer, I have focused my PhD research on SRAP. I have divided this thesis into seven chapters mainly focused on 4 published articles.

CHAPTER I, is a general introduction containing information pertaining to breast cancer as well as to our current knowledge of the SRA1 gene system. As the reader will see, this system consists of both a functional RNA (SRA) and a protein (SRAP). CHAPTER II describes the overview of my thesis rationale and objectives. The subsequent CHAPTERS III and IV present two complementary studies investigating the clinical significance of SRAP using two antibodies previously validated during my MSc training. I have published these results as two articles in separate journals (Breast Cancer Research 2009 and Journal of Cancer Research and Clinical Oncology 2013) as our observations are significant enough to justify the use of two independent antibodies to compare SRAP expression patterns. This has clearly established the validity of the observed associations between SRAP expression and patient's outcome. In Chapter V, the clinical significance of the expression of SRAP and ER-β1, another significant molecule in breast cancer, has been explored. In this study, I have investigated a large cohort of cases from a randomized placebo controlled trial (NCIC CTG MA12) organized nationwide to assess the potential benefits of tamoxifen treatment for pre-menopausal patients with early breast cancer after chemotherapy. The corresponding results have been published in Annals of Oncology (2013). In Chapter VI, the potential cellular functions of SRAP have been investigated and a direct link between SRAP expression and cancer cell motility has been established. These results have been published in FEBS Letters.
(2015). Chapters III-VI are built from my four first author articles; they each contain their own Abstract, Introduction, Materials and Methods, Results and Discussion sections. I have added concise background information and major conclusions at the beginning of each chapter. This should facilitate the overall reading of the thesis and link the independent Chapters as well as possible. Lastly, in Chapter VII, overall observations and limitations of my research are discussed and potential future directions highlighted.
CHAPTER I  Introduction

I-1  Normal breast and breast cancer

I-1-1  Normal breast development

The normal breast sits on the chest muscle and consists mainly of adipose tissue, also referred to as mammary fat pads. In between these pads figures the mature mammary gland, which is composed of only 15-20 lobes, each composed of smaller structure called lobules [1], themselves connected to each other by tiny lactiferous ducts [Fig I-1] [2]. At the cellular level, a typical ductal structure consists of a hollow lumen, enclosed by a layer of epithelial luminal cells that produce milk [Fig I-1]. Outside this ring of epithelial luminal cells stands a layer of myoepithelial cells and a basement membrane [3]. The overall correct morphology of each duct is ultimately maintained through an extracellular matrix, which together with fibroblasts, endothelial cells, macrophages, and adipocytes, constitutes the mammary stroma.

Even though normal mammary gland development starts during embryogenesis, it takes several years for the breast to be functional. At the embryonic stage, primitive structures are formed, that are not yet connected to the nipple and therefore not functional at birth [4,5]. The breast grows extremely slow in size until puberty. However, at puberty, it undergoes a spurt of allometric growth and formation of the milk-producing lobules is initiated. The lobules then extend into the mammary fat pad, and the glands become more complex. During each following menstrual cycle, ovarian hormones such as estrogen and progesterone induce the remodeling of budding structures up until the age of 35 [6]. Overall, the most remarkable mammary gland growth and differentiation occurs during pregnancy. As such, the mammary gland of a nulliparous woman significantly differs from
that of a multiparous mother. The glands indeed undergo extensive proliferation during earlier stages of pregnancy, when development of lactiferous ducts also occurs. In the later stages of pregnancy, breast cells undergo hypertrophy rather than proliferation [7,8]. The growth of the mammary gland during pregnancy mainly results from the combined action of both estrogen and progesterone. The massive amounts of these two as well as other hormones leads to the ultimate development of the lobular alveoli and ducts into active

Figure I-1. Anatomy of normal mammary gland.
A) The mature mammary gland is composed of 15-20 lobes, each composed of smaller structure called lobules. Tiny lactiferous ducts connect lobules to each other. The bulk of the breast tissue is adipose tissue interspersed with connective tissue. B) A cross section of image review of a typical structure of duct consisting of a hollow lumen, enclosed by a layer of epithelial luminal cells that produce milk. Outside of epithelial luminal cells is a layer of myoepithelial cell and basement membrane. Maintain the correct morphology of duct requires extracellular matrix which together with fibroblasts, endothelial cells, macrophages, and adipocytes, constitute mammary stroma. Fat fills the spaces between the lobules and ducts. C) Axial plane of review of a typical structure of duct [3].
Figures adapted from Visvader, J.E. et al. Genes & development (2009)
milk secreting structures that will allow the breast to fulfill its biological role. With the cessation of lactation (weaning), the alveoli developed during pregnancy undergo degeneration through apoptosis, in a process also referred to as involution. The mammary gland alveoli and ducts then turn back to their original resting state. Upon the onset of menopause, the decrease in estrogen levels results in a permanent regression of the glandular breast tissue, which is mainly replaced by fat [9]. Overall, the breast is considered as a very dynamic organ whose growth and differentiation remain under a lifelong and tightly hormone regulated control.

I-1-2 Overview of breast cancer

Breast cancer is an extremely complex disease overall resulting from uncontrolled breast cell division and growth [10]. The disease typically starts locally as a tiny mass of cells within ducts or lobules. This original event is followed by the formation of new blood vessels (angiogenesis), which allow the growth of larger tumours and ultimately the spread of the disease and the potential genesis of lethal distant metastases [10].

I-1-3 Breast cancer diagnosis
I-1-3-1 How is breast cancer detected?

Early breast cancer detection remains a critical part of our fight against this disease. Indeed it is clearly established that the earlier the detection, the higher the chances to overcome cancer lethal progression. The majority of breast cancer cases are initially found as small masses detected during self-examination or annual physical checkup by family physicians. Breast lesions can also be discovered by mammogram screening in patients older than 50 [11-13].

The widespread performance of mammographic screening programs has significantly improved breast cancer diagnosis in the last 30 years [11]. Static images produced by
X-ray also allow the detection of the presence of calcifications or solid lesions. Further investigations by other diagnostic modalities such as ultrasound are however required to confirm the breast cancer diagnosis [11]. Ultrasonography, which does not produce radiation, is the most favored modality today [11]. It should be noted that the American Cancer Society now recommends ultrasound for screening women with a higher risk of breast cancer and dense breasts[14]. Additionally, radiologists can to use Magnetic Resonance Imaging (MRI) now to further examine suspicious areas found by a mammogram or ultrasound [14]. If an abnormal area in the breast is found during those exams, this gives the radiologist the opportunity to immediately biopsy the affected region under the guidance of the images obtained [14]. These biopsies consist in fine needle aspiration (FNA) and core needle biopsies. The subsequent cytologic analysis of biopsy remains today the gold standard to diagnose breast cancer as it confirms the differentiation of the cancerous cell from normal breast cells.

I-1-3-2 Breast cancer histological subtypes

When the biopsy specimens arrive in the laboratory, a pathologist first examines them under a microscope to establish what kind of lesions has been detected. The detected lesions can be normal breast tissue or hyperplasia, a term used when there are more cells than usual. However, if the detected lesions contain cancerous cells, further histological classifications are required. Ductal and lobular are two basic histological subtypes of breast malignancy [Table I-1] [15,16]. Ductal carcinomas, which originate from the lining of the ducts, are more common and visible on mammography. On the other side, lobular carcinomas arise within the milk-producing glands (lobules) and are generally less visible on mammography. As such they are often incidentally discovered by biopsy of other abnormalities [17].
Furthermore, each histological subtype of adenocarcinoma can be further divided into in situ and invasive [16]. In situ lesions (Ductal carcinoma in situ, DCIS and lobular carcinoma in situ, LCIS), sometimes referred to as “pre-cancer,” are those showing malignant potential but have not invaded the surrounding breast duct or lobule [16]. They fortunately account for the majority of detected breast disease. Invasive carcinomas on the other side have a tendency to spread outside the membrane of the lobule or duct into the surrounding breast tissue [16]. The most common type of invasive cancers is invasive ductal carcinomas, followed by invasive lobular carcinoma, accounting for around 75% and 10% of total invasive cases, respectively [Table I-1] [18]. Beside the pre-cited in situ and invasive lesions, other rarer forms of breast cancer have been also characterized, including Paget disease (carcinoma involving the nipple) and inflammatory carcinoma.

<table>
<thead>
<tr>
<th>Histological Subtypes</th>
<th>Frequency</th>
<th>5-year Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ductal Carcinoma in-situ (DCIS)</td>
<td>3.6%</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Lobular Carcinoma in-situ (LCIS)</td>
<td>1.6%</td>
<td>&gt;99%</td>
</tr>
<tr>
<td>Infiltrating (Invasive) ductal carcinoma</td>
<td>63.6%</td>
<td>79%</td>
</tr>
<tr>
<td>Infiltrating (Invasive) lobular carcinoma</td>
<td>5.9%</td>
<td>84%</td>
</tr>
<tr>
<td>Medullary Carcinoma</td>
<td>2.8%</td>
<td>82%</td>
</tr>
<tr>
<td>Inflammatory breast cancer</td>
<td>1.0%</td>
<td>79%</td>
</tr>
<tr>
<td>Mucinous breast cancer</td>
<td>2.1%</td>
<td>95%</td>
</tr>
<tr>
<td>Others</td>
<td>19.4%</td>
<td>62%</td>
</tr>
</tbody>
</table>

Table I-1. Major breast cancer histological subtypes and their characteristics.  
Adapted from http://p53.free.fr/our_work/breast.html
Inflammatory breast cancer is highly aggressive and only account for 1-3% of all breast cancer [19]. This type of tumour usually spreads into the lymph vessels and can cause a blockage of the lymphatic drainage [13]. The affected breast may appear to be thick, pitted and orange-peel like [13].

Despite the use of histological category that have routinely been used for breast cancer therapy decision making, and helped significantly improving overall survival, 25% to 35% breast cancer patients still develop resistance to these drugs [20]. As such, breast cancer remains the second leading cause of cancer-related deaths among women after lung cancer with more than 500,000 reported deaths per year around the world [21]. Sadly, one in 29 Canadian women will still pass away due to breast cancer [21]. It is believed that exact molecular subtypes of each detected cancer will allow the identification of new therapeutic targets and the design of more effective treatments.

Molecular subtypes of breast cancer

High throughput sequencing technology has allowed researchers to group breast cancers according to their global gene expression profile. At least five distinct molecular subtypes of breast cancer have now been identified. These are luminal A, luminal B, HER2-enriched, basal-like and normal breast-like groups [22-24]. These subtypes were originally categorized based on different molecular make-ups from unsupervised hierarchical clustering analysis of gene expression microarray data. They drastically differ from the histopathologic subtypes previously described and are thus referred to as molecular subtypes [25]. Each molecular subtype shows significant difference in overall and recurrence free survival, underscoring the significant prognostic value of the molecular markers identified [25]. Among them, Luminal A is the most common type that accounts for approximately 55-60% of all breast cancer cases. This subtype, which has the most favorable prognosis, is characterized by an expression of the estrogen
receptor (ER-α) and the progesterone receptor (PR) [Table I-3]. Similar to Luminal A subtype, patients with Luminal B subtype also express ER-α and PR. However, compared to Luminal A, Luminal B subtype has a higher cellular proliferative rate as determined by the high expression of proliferative biomarker Ki67 [26] [Table I-3]. Therefore, Luminal B tumours have a tendency to be more resistant to SERMs treatment and thus generally have a greater risk of recurrence when compared to Luminal A.

Molecular HER2 positive tumours are characterized by high expression of the HER2 and other genes associated with HER2 pathway. Molecular HER2 positive tumours are usually highly proliferating and thus morphologically are of a higher tumour grade. It should be noted that tumours belonging to this molecular HER2 subtype do not always present with an aforementioned immunohistochemical HER2 positive profile. Indeed, only 70% of molecular HER2 subtype has high level of expression of HER2 protein [Table I-3].

Another molecular subtype is the basal-like tumour, characterized by high level of proteins usually expressed in “basal” cells of the normal breast epithelium, as opposed to myoepithelial and luminal cells [27]. The characteristics of basal-like tumour are lacking of ER, PR, and HER2 expression in conjunction with expression of CK5/6 and/or epidermal growth factor receptor (EGFR) [Table I-3] [28]. CK5/6 is one of the high-molecular-weight basal cytokeratins associated with the intermediate phenotype of cells undergoing metastasis [28,29]. The high-level expression of epidermal growth factor receptor (EGFR) found in basal-like subtype can trigger downstream signaling pathways that promote cell growth, proliferation and cell survival [28]. Thus, basal-like patients have a worse prognosis and a higher recurrence rate when compared to other subtype tumours. In addition, basal-like tumours have been shown to be associated with p53 and BRCA1 germ-line mutations [28]. “Triple negative” tumours are another classification of breast cancer without expression of ER, PR and HER2 and 70% of triple negative tumours are the basal-like tumours [28].
Normal breast-like represents a small portion of breast cancers. Gene expression profile is mainly similar to normal breast tissue control. Unlike the other molecular subtypes, normal subtype is poorly characterized. They neither express ER, PR or HER2 nor respond to neo-adjuvant chemotherapy. The normal breast-like subtype has variable prognosis. It is hypothesized that this subtype may be due to a high amount of normal tissue contamination. Therefore it is controversial whether normal breast-like has been mistakenly categorized or indeed defines a separate molecular subtype [25].

<table>
<thead>
<tr>
<th>Molecular Subtypes</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal A</td>
<td>ER + PR+ Ki67-</td>
</tr>
<tr>
<td>Luminal B</td>
<td>ER+ PR+ Ki67+</td>
</tr>
<tr>
<td>HER2-enriched</td>
<td>ER- PR- HER 2+</td>
</tr>
<tr>
<td>Basal-like</td>
<td>ER- PR- HER2- EGFR+ and/or Cytokeratin 5/6+</td>
</tr>
<tr>
<td>normal breast-like</td>
<td>Expression of genes characteristic of adipose tissue</td>
</tr>
</tbody>
</table>

Table I-2. Main breast cancer molecular subtypes and their characteristics.

Additional molecular subtypes have been identified in more recent microarray analyses. For example, claudin-low is a newly identified subtype based on overexpression of a set of 40 genes related to immune response [30]. Claudin-low tumour has a high infiltration rate of immune cells with a down-regulation of *E-cadherin* gene. E-cadherin inhibits the strength of cellular adhesion within a tissue. As such, a low E-cadherin expression results in an increase in cellular motility [30]. Even though, for the past ten years, significant advances have been made using gene expression profiling to discern the molecular drivers behind breast cancer, these new classifications need to be fully
standardized. Much work remains needed to apply this knowledge to implement new clinical practices [31].

I-1-3-4 Breast cancer grading and staging

Cytologic analysis from core needle biopsy also allows the pathologists to assess the degree of variation between normal cells and observed tumour cells; it provides extremely valuable clinical information to establish a valid prognosis of the detected disease [32]. Grading depends on the evaluation of glandular formation, nuclear morphology and mitotic counts, each scored from 1 to 3 [32]. The resulting values are combined and categorized into three groups: grade I (score 3–5), grade II (scores 6 and 7), and grade III (scores 8 and 9) [13,32]. Generally, the patients diagnosed with higher grade have a worse clinical outcome. This simple grading system is however not accurate enough. Indeed, other factors such as the size of tumours or any signs of spreading have also to be considered. Generally, two main staging classifications, not limited to breast cancer, are currently used: the tumour node metastases (TNM) system and the International Union Against Cancer (UICC) system [13,32]. Staging based on the TNM classification system evaluates tumour size (“T”), presence of cancer cells within local lymph nodes (“N”) and tumour metastasis (“M”) [Table I-2] [33]. Usually the bigger the tumour size, the worst the prognosis is. However, a small cancer can also be aggressive while a larger cancer may not. The presence of cancer cells in lymph nodes suggests an early spreading of the disease as the lymph nodes are believed to catch and trap cancer cells before they eventually drain into the bloodstream. The more lymph nodes involved, the more advanced the cancer is suspected to be. The UICC further groups the TNM data into a scale of 0 through IV listed in Table I-2 [34]. According to the National Cancer Data Base, the 5-year survival rate of a patient decreases with increasing breast cancer stage. Stage 0 tumours describes non-invasive carcinomas that remain within their original location and
usually have best clinical outcome, while stage IV tumours describes invasive cancers that have metastasized distally to other organs of the body and often with worst prognosis. Overall, the goal of grading and staging systems is to better understand the nature and stage of the disease to further establish the best treatment plan possible.

<table>
<thead>
<tr>
<th>Stage</th>
<th>TNM Classification</th>
<th>Legend</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 0</td>
<td>Tis, N0, M0</td>
<td>Tis</td>
<td>Carcinoma in situ</td>
</tr>
<tr>
<td>Stage IA</td>
<td>T1, N0, M0</td>
<td>T0</td>
<td>No evidence of primary tumor</td>
</tr>
<tr>
<td>Stage IB</td>
<td>T0,N1,M0 or T1,N1,M0</td>
<td>T1</td>
<td>Tumor is less than 20mm in greatest dimension</td>
</tr>
<tr>
<td>Stage IIA</td>
<td>T0,N1,M0;T1,N1,M0 or T2,N0,M0</td>
<td>T2</td>
<td>Tumor is more than 20mm but less than 50mm in greatest dimension</td>
</tr>
<tr>
<td>Stage IIB</td>
<td>T2,N1,M0 or T3,N0,M0</td>
<td>T3</td>
<td>Tumor is more than 50mm in greatest dimension</td>
</tr>
<tr>
<td>Stage IIIA</td>
<td>T0/T1/T2,N2,M0 or T3,N1/N2,M0</td>
<td>N0</td>
<td>No regional lymph node metastasis</td>
</tr>
<tr>
<td>Stage IIIB</td>
<td>T4,N0/N1/N2,M0</td>
<td>N1</td>
<td>Metastases to movable ipsilateral level I, II axillary lymph nodes</td>
</tr>
<tr>
<td>Stage IIIC</td>
<td>Any T, N3, M0</td>
<td>N2</td>
<td>Metastases in ipsilateral level I, II axillary lymph nodes; or ipsilateral internal mammary nodes in the absence of clinically evident axillary lymph node metastasis</td>
</tr>
<tr>
<td>Stage IV</td>
<td>Any T, any N, M1</td>
<td>N3</td>
<td>Metastases in ipsilateral infraclavicular (level III axillary) lymph nodes with or without level I, II axillary lymph node involvement; or in clinically detected ipsilateral internal mammary lymph nodes with clinically evident level I, II axillary lymph node metastases; or metastases in ipsilateral supraclavicular lymph nodes</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M0</td>
<td>No clinical or radiographic evidence of distant metastases</td>
</tr>
<tr>
<td></td>
<td></td>
<td>M1</td>
<td>Distant detectable metastases as determined by classic clinical and radiographic means and 1 or histologically proven larger than 0.2mm</td>
</tr>
</tbody>
</table>

Table I-3. Breast cancer stages. 
Adapted from:http://www.cancer.ca/en/cancer-information/cancer-type/breast/staging/?region=on
I-1-4 **Breast cancer treatments**

Treatments of breast cancer mainly consist of two complementary lines of therapies: primary surgery and adjuvant/neoadjuvant therapies. The exact treatment options are here usually based upon different clinical parameters such as tumour stages, and specific tumour characteristics such as hormonal status and patient’s general health [13,35].

I-1-4-1 **Surgery**

Lumpectomy and mastectomy are two major types of surgery for the treatment of breast cancer [35]. Mastectomy is a surgical procedure consisting in removing part of or whole affected breast. This approach includes radical mastectomy, modified radical mastectomy and simple mastectomy, which are named after the progressively decreasing amount of tissue taken [35]. Recent focus has been put on more breast-conserving strategies. Lumpectomy procedures, that involve removal of only the breast tumour and some surrounding tissue (margin) is now the first treatment option for women with early-stage breast cancer [36]. The patients with late-stage tumour usually undergo more aggressive surgery such as radical mastectomy.

I-1-4-2 **Adjuvant/Neoadjuvant therapy**

The second line of treatment is performed either after (adjuvant) or before (neoadjuvant) surgery [35]. Adjuvant therapy is widely used to prevent recurrence of the disease by targeting any potential remaining tumour tissues [13]. The true effectiveness of adjuvant treatment, however, cannot be adequately assessed in individual patients since there is no remaining tumour to monitor after surgical removal. In contrast, neoadjuvant treatments allow the assessment of the direct effects on the cancer tissue. This approach, which also reduces the size of larger tumours, favors the use of breast-conserving
surgeries rather than that of mastectomy. Adjuvant/Neoadjuvant therapies consist of radiation, chemotherapy, immunotherapy and hormonal therapy [13].

I-1-4-2-1 Radiation Therapy

Radiation therapy uses high-energy x-ray or other radioactive particles [37]. Radiation therapy destroys cancer cells by damaging their DNA [38]. The current guidelines suggest that all breast cancer patients are supposed to receive radiotherapy after surgery to reduce the risk of recurrence [39]. Axillary nodal radiation may be recommended if nodal involvement has been observed [37]. Radiotherapy was found to improve breast cancer-free survival rates at 15 years by over 20% after operation compared to patients without radiotherapy [40]. Side effects are minor including unilateral arm lymphoedema and skin telangiectasia, as well as small dilated veins near the surface of the skin [41,42]. With modern machinery and limited exposure to the skin, dose of radiotherapy is also minimized [42].

I-1-4-2-2 Chemotherapy

Chemotherapy is often used in conjunction with radiation therapy as adjuvant therapy post-surgery. Traditional chemotherapy includes cyclophosphamide, methotrexate and fluorouracil (CMF) [43]. These agents are cytotoxic; in other words, they tend to kill the cells that grow rapidly, one of the representative features of malignant cells. The mechanisms of action of these drugs differ. Cyclophosphamide, which adds an alkyl group to DNA, interferes with DNA replication by allowing the formation of intra- and inter-strand DNA crosslinks. Methotrexate (MTX), on the other hand, is an antimetabolite that inhibits an enzyme called dihydrofolate reductase involved in the synthesis of folic acid, a compound needed for the de novo synthesis of thymidine. Lastly, Fluorouracil acts by blocking the action of the enzyme thymidylate synthase, resulting in an imbalance of
deoxynucleotides and subsequent DNA damage. Recently, it was reported that the use of modern regimes including natural compounds and their derivatives such as the anthracyclines and taxanes improves treatment benefits, when compared with traditional CMF combinations [44]. Anthracyclines prevent the replication of cancer cells by inhibiting DNA synthesis in dividing cells through insertion between base pairs of DNA strand. Anthracyclines can also function by inhibiting the enzyme topoisomerase II or generating reactive oxygen species[45]. Taxanes, on the other side, destroy the functions of microtubule and thus inhibit cell division [46]. Combination of taxanes and anthracyclines further improves overall survival in breast cancer patients with node positive lesions [44].

Unfortunately, chemotherapy remains broadly cytotoxic and also kills normal rapidly dividing cells such as bone marrow and hair follicles cells. Side effect of chemotherapy includes hematological toxicity particularly neutropenia, alopecia (hair loss), lethargy and oral mucositis [13]. Due to the high discomfort of these side effects and the extensive damage to normal cells, more advanced targeted therapy regimens are needed, that would attack cancer cells specifically based on their biological characteristics. Two strategies are currently used, immunotherapy and hormonal therapy.

**I-1-4-2-3 Immunotherapy**

Because deregulation of cell growth is a critical characteristic of cancer cells, targeting growth factor receptors located on their membrane remains an efficient strategy to slow down their multiplication. HER2 is a transmembrane growth factor receptor, over-expressed in approximately 20% of breast cancers patients. Once activated, this receptor stimulates down-stream signaling pathways that ultimately promote cell growth and proliferation. Immunotherapy drugs that interfere with HER2 signaling pathways have therefore the potential to be effective in treating this specific sub-group of breast cancer.
patients [47]. Herceptin (also known as Trastuzumab) is a humanized monoclonal antibody directed against the external domain of the HER2 receptor [47]. Malignant cells treated with Herceptin undergo mitotic arrest as their proliferation is inhibited [48]. Herceptin has therefore now become a common component of adjuvant therapy programs for women whose tumours overexpress HER2, as detected by immunohistochemistry [48]. For HER2 negative patients, the clinicians have the option to exploit therapies targeted on other biological features of the tumours, such as their potential dependence upon the act of hormones to grow.

I-1-4-2-4 Hormonal therapy

The link between risk of breast cancer and hormone exposure has long been suspected. As early as 1700, Dr. Bernardino Ramazzini, the father of occupational medicine, reported that nuns were more likely to be afflicted by breast cancer than any other women [49]. He was the first to document the risk of contracting the disease associated to nulliparity. It was later observed that women who took the anti-miscarriage drug diethylstilbestrol (DES) – a potent form of estrogen prescribed for pregnant women from 1938 until 1971, had a higher risk of being afflicted by breast cancer [50]. It has also been found that obese postmenopausal women also develop more breast cancers than leaner counterpart, a phenomenon attributable to the elevated level of estrogen secreted by fat components [50]. In 2003, the million women study, a multi-center population-based prospective cohort of more than a million British women aged 50–64 years showed that the use of hormone-replacement therapy (HRT) increases the incidence as well as mortality of breast cancer [51]. This study also showed that the effect of the combined (estrogen-progestogen) HRT is substantially greater than for estrogen-only HRT [51]. Besides this largest study, multiple follow-up studies have evaluated and further confirmed this significant association between hormonal factors and the risk of breast cancer [52-54]. It has also
been also observed that females who experience an early menstruation, late menopause, or non-pregnancy have higher risk of developing breast cancer [55]. Overall it appeared that the longer exposure to reproductive hormones, mainly estrogen, the greater risk to be affected by breast cancer. This observation in part stems from the fact that estrogen can act as a potent mitogen, stimulating the growth and proliferation of normal as well as cancer breast epithelial cells. From these observation, estrogen has been suspected to participate in breast tumour growth promotion and cancer progression [56]. In order to reduce estrogen level in women at risk, clinicians first developed a strategy referred to as ovarian ablation, where ovaries were surgically removed, ultimately suppressing the main source of estrogen production [57]. However, having ovaries removed was obviously extremely distressing to young breast cancer patients planning on having children. Researchers therefore widely explored the mechanisms behind estrogen signaling and attempted to treat breast cancer by inhibiting its action. Estrogen has long been thought to act primarily through a single estrogen receptor cloned in 1986, and now referred to as ESRI or ER-α [58,59]. This receptor belongs to the steroid/thyroid/retinoic acid receptors superfamily and primarily acts as a ligand-dependent transcription factor [60]. However, ER status may have to be re-defined as a second estrogen receptor, estrogen receptor β (ER-β) has recently been discovered [59]. ER-β, which also belongs to the steroid/thyroid/retinoic acid receptors superfamily, shares ER-α structural/functional organization of ER-α [60,61]. Several ER-β isoforms, products of alternative splicing, have been identified [62]. Among them, ER-β1 is major isoform, able to bind estradiol and form dimers (homodimers or heterodimers) with ER-α [63]. Most of, but not all, studies suggest that higher levels of ER-β1 proteins in the presence of ER in human breast cancer are associated with a better prognosis and the increased likelihood of responsiveness to anti-estrogen therapies [62,64]. Anti-estrogen treatment options have mainly consisted in using selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, which compete with estrogen to
bind ERs [65]. Tamoxifen has been used to treat early stage breast cancer patients for over 30 years and have to date, saved thousands of patients lives [66]. A recent clinical trial aTTom (adjuvant tamoxifen Treatment offers more) involving 6800 breast cancer patients has clearly revealed that 10 years tamoxifen treatment is more effective than 5 years treatment [67]. More importantly, it was found in 1998 that the administration of tamoxifen to women at risk of contracting the disease had a protective effect; outlining for the first time the possibility of preventing the disease by using antiestrogenic drugs [68]. Raloxifene, a second generation SERM for endocrine therapy, appeared to have similar efficacy but with fewer toxic side effects [69]. Recently, Other SERMs such as fulvestrant has been clinically used in addition to tamoxifen and raloxifene [70]. Fulvestrant leads to an ER conformational change after binding and ultimately induces ER degradation [70].

Similar to ovarian ablation, an alternative method to counteract estrogen action is to reduce its overall amount of production by interfering with its synthesis. Aromatase inhibitors (such as letrozole) act by blocking the activity of the enzyme that converts androgens into estrogen [71]. Because breast fat is a site of synthesis of estrogen, such treatment has the power to reduce the local production of this mitogenic hormone. Side effects of these inhibitors tend to be less than SERMs and thereby are becoming more commonly used in post-menopausal women [71]. Also Aromatase inhibitors seem to have a favored clinical outcome compared to tamoxifen [72], especially in lobular cancer as shown by the Breast International Group (BIG) 1-98 study, a randomized, double-blind trial that compared five years of treatment with various adjuvant endocrine therapy regimens [73]. One of the drawback of this approach remains however that it is mainly prescribed to treat post-menopausal patients since it is less effective to block estrogen synthesis in pre-menopausal women mostly as a result of the presence of relatively high circulating estrogens level in this population [74,75].
I-1-5  Etiology of breast cancer

The majority of breast cancers is suspected to result from genetic anomalies. Indeed, 90% of these lesions are caused by a sporadic accumulation of genetic aberrations [76,77]. These aberrations consist in an intrinsic interplay between gene mutations, chromosomal gains and losses as well as clonal expansions. They generally occur at different time points and can leave a permanent record indicated by genome sequence changes [78].

The most common mutations observed in cancer are somatic mutations, which are acquired by a non-germ cell during the course of cell division occurring after conception. These “driver” mutations play a key role in transforming normal cells into cancerous cells by altering the activity/amount of specific proteins. For simplicity, we can separate mutations that can either lead to an inhibition of tumour suppressor genes or an activation of oncogenes [79]. Recently, large tumour genomic database TCGA containing thousands of breast tumours samples sequenced by high-throughput sequencing platforms reported that most frequently somatic mutated genes in breast cancer were TP53, PI3K and GATA3 [79-81]. TP53, which functions as a tumour suppressor, is crucial in multicellular organisms as it prevents cancer formation by repairing DNA damage [82]. PI3K, on the other side, can function as an oncogene, through an overactivation of its kinase activity and a decoupling of important signaling pathways directly controlling cell growth [83]. Conversely, GATA3 is involved in regulating luminal epithelial cell differentiation in the mammary gland [84]. Aside from these frequently mutated genes, other "rarely" mutated genes are also cited as potential breast cancer driver genes. These include the pre-cited HER2 and ER-α genes. HER2 is mutated in around 1.5% of all breast cancers and ER-α is only mutated in 0.6% of ER-α positive primary breast tumour according to the TCGA database [81]. Interestingly, the mutations of these two genes are often associated with drug resistance [85].
While most of breast cancer cases are associated with a sporadic accumulation of somatic mutations, less than 10% of breast tumours can arise from inherited genetic anomalies or germline mutations [86]. Mutations in two genes, breast cancer gene 1 and 2 (BRCA1 and BRCA2) contribute to the majority of inherited breast cancer [87]. BRCA1 and BRCA2 genes, which are considered to be tumour suppressors, are located on chromosome 17 and 13, respectively, and are responsible for DNA damage repair [87,88]. The penetrance of these mutations is however extremely high with a life-time risk for affected women of more than 75% of contracting breast cancer [87,89]. As BRCA1 is involved in DNA repair, it is believed that this high risk of contracting breast cancer results from the elevated genomic instability [88]. Complex DNA copy number abnormalities such as loss of 12q, 4q and 5q are indeed often observed in BRCA1 familial breast cancer upon cytogenetic studies [90].

Overall, even though some common features can be pinpointed, breast cancers genetically differ from one another. The main challenge remains therefore to find molecular markers that would allow establishing quickly after disease detection which patient will respond to what treatments. Currently, huge efforts are being undertaken to find new molecular markers that would help clinicians find the right treatment for each patient.

I-1-6 Breast tumour biomarker

Tumour biomarkers are defined as substances that are produced by cancer or by other cells of the body in response to cancer conditions. Tumour markers have been widely used for prognosis, prediction and treatment selection [91]. Markers including previously mentioned estrogen receptor-α (ER-α), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), remain currently the most important and prevalent biomarkers for breast cancer classification. The progesterone receptor (PR), a known
target gene of ER-α, is activated by progesterone, another steroid hormone [92]. The presence of both receptors in a given tumour, assessed today by immunohistochemistry (ER-α positive/PR positive tumour), appears therefore to be a good indicator of a functional estrogen signaling and a dependence on estrogen to grow [93]. Patients diagnosed with such tumours are predicted to benefit more from an "anti-estrogen" endocrine therapy than those bearing ER-α/PR negative patients [94]. Also, patient with HER2 positive status are likely to benefit from anti-HER2 antibody immunotherapy. In addition, Mucin 1 (Muc1) has emerged as an important biomarker due to up-regulated expression over the entire cell surface in breast cancer [95]. Lately, more breast-specific biomarkers such as the detection of mammaglobin protein were shown to be clinically useful for primary, metastatic and occult breast cancer [96].

These advances have nevertheless drastically increased our understanding of tumour biology at large and of the potential signaling pathways involved in breast tumour formation, progression and invasion [81]. The ultimate goal is that new molecular subtype classifications may help find new potential therapeutic target and allow clinicians to offer personalized 100% effective treatment [25,97]. In the meantime, basic scientists all around the globe continue investigating the mechanisms behind breast cancer cell growth and motility as it is understood that knowledge of these mechanisms will also provide new targets to develop new therapies.

I-1-7 Further exploring steroid receptor signaling, investigating co-regulators

The fact that hormone-therapy effectively saved patients and even in some specific cases prevented the manifestation of the disease led many researchers to further focus their studies on the mechanisms controlling steroid action. Classical female steroid hormones include estrogens, progesterone, glucocorticoids and mineralocorticoids [98]. It has been established that steroid receptors primarily act as ligand-dependent transcription factors
The classical signaling pathway of steroid receptor action has been widely studied for past 20 years since its discovery [98,99].

Once bound to the ligands, activated receptors undergo conformational changes, are released from heat shock protein containing complexes and translocate into the nucleus [Fig I-2] [98,99]. Dimers of receptors are subsequently recruited on hormone responsive elements (HRE), cis-acting sequences located upstream of target genes [100]. Receptor interaction with co-activators leads to the remodelling of local chromatin as well as the assembly of a transcriptional pre-initiation complex that will ultimately switch on gene transcription. Inversely, interaction with co-repressor has the potential to switch off gene expression. Numerous studies have focused on those co-regulators, as they ultimately control the actions of specific hormones [101]. Unlike DNA binding steroid receptors, co-regulators do not directly interact with genomic DNA but are recruited by nuclear receptors to regulatory regions as part of large multiprotein complexes [101]. Transcription regulation by co-regulators is a high dynamic process, which requires collaboration between multiple molecules in a timely fashion. Co-regulators overall provide a critical “fine tuning” of hormone action [102,103].

In 1995, the first co-regulator, the steroid receptor co-activator 1 (SRC-1) also known as NCOA1 was successfully discovered using the Progesterone receptor (PR) activation function domain AF-2/ ligand binding domain as bait [104]. Twenty years later, more than 450 co-regulators have been reported in the literature [105]. Overall, they can be divided into co-activators and co-repressors[101]. Co-activators refer to factors physically associating with agonist-bound receptors and promoting ligand dependent gene transcription activation [101]. Co-activator containing complexes can be categorized into different functional groups: ATP-dependent chromatin remodelers such as BRG1 associated factors (BAF), Histone modifiers such as acetyltransferase (CBP/P300, P/CAF), as well as other mediators and adaptors [101][Fig I-2]. The overall role of these
co-activators is to locally open the chromatin region and allow access to other factors responsible for the transcription per se. As opposed to co-activator, co-repressors inhibit gene transcription through interacting with antagonist-bound or unliganded steroid receptors [101] [Fig I-2]. For instance, NCoR/SMRT (nuclear receptor co-repressor/silencing mediator for retinoid and thyroid-hormone receptors) in a co-repressor complex that physically interacts with ER and can recruit histone deacetylase (HDAC) activity. NuRD/Mi2: The Mi-2/nucleosome remodeling and deacetylase (NuRD) complex is unique in that it couples histone deacetylation and chromatin remodeling ATPase activities in the same complex. SWI/SNF: is a yeast nucleosome remodeling complex composed of several proteins products of the SWI and SNF genes (SW11, SW12/SNF2, SW13, SW15, SW16). It possesses a DNA-stimulated ATPase activity and can destabilize histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner.

Figure I-2. Steroid receptor signaling pathway and co-regulators.
Once bound to the ligands, receptors undergo conformational changes, dissociate from heat shock proteins (HSP) and translocate into the nucleus, dimerize, and then specifically recognize hormone responsive elements (ERE). Activated receptors, through dynamic interplays with additional co-regulators, direct the assembly and stabilize a pre-initiation complex that ultimately conducts gene transcription. (Co-activator complexes and their mechanisms of action are shown on the top; co-repressor complexes are shown on the bottom. Complexes cited here are briefly described: CBP/P300 : CREB binding protein/E1A binding protein p300 are two closely related proteins able to co-activate various transcription factors. pCAF: p300/CBP associated factor binds to p300 and CBP. It has histone acetyltransferase activity. NCoR/SMRT: nuclear receptor co-repressor/silencing mediator for retinoid and thyroid-hormone receptors in a co-repressor complex that physically interacts with ER and can recruit histone deacetylase (HDAC) activity. NuRD/Mi2: The Mi-2/nucleosome remodeling and deacetylase (NuRD) complex is unique in that it couples histone deacetylation and chromatin remodeling ATPase activities in the same complex. SWI/SNF: is a yeast nucleosome remodeling complex composed of several proteins products of the SWI and SNF genes (SW11, SW12/SNF2, SW13, SW15, SW16). It possesses a DNA-stimulated ATPase activity and can destabilize histone-DNA interactions in reconstituted nucleosomes in an ATP-dependent manner.
co-repressor/silencing mediator for retinoid and thyroid-hormone receptors) is recruited in a co-repressor complex containing antagonist-bound receptors and histone deacetylase (HDAC) activity [103] [Fig I-2].

Other co-repressors complex such as LCoR (ligand-dependent nuclear receptor co-repressor), can interact with estrogen receptor and compete with co-activators by replacing them under certain antagonist treatment [103]. Interestingly, some co-regulators, such like SWI/SNF (the ATP-dependent chromatin remodeling complexes) could be recruited to modulate both gene activation and repression [103] [Fig I-2]. Indeed, it is believed that transcription regulation is a highly dynamic process and that the function of a given co-regulator depends on different ligands or promoter considered [103] [Fig I-2]. A direct participation of altered levels of co-regulators is suspected to contribute to the mechanisms underlying breast tumourigenesis, cancer progression as well as acquisition of resistance to hormonal therapy [106] [107]. Increasing co-activation as well as decreasing co-repression of the activity of steroid receptors in breast cancer can ultimately lead to resistance to hormonal therapy [107]. These observations further suggest that regulation of co-regulators and downstream activity in tumours is crucial in predicting response or resistance to therapy in a given breast cancer patient. Thus, identifying novel co-regulators of steroid receptors still remains a valid approach to discover potential targets to develop new therapeutic options to overcome hormone resistance.

I-2  Steroid receptor RNA activator

I-2-1  Discovery of SRA RNA

In order to identify novel co-regulators, Lanz et al. in 1999 performed a yeast-two hybrid assay using progesterone receptor (PR) activation function domain 1 (AF-1) as bait. A positive clone was isolated from a human B-lymphocyte library that they called steroid receptor RNA activator or SRA. Interestingly, SRA sequence from the Gal/SRA fusion
clone obtained in this yeast two hybrid screening had an in frame stop codon terminating prematurely the translation of gal/SRA fusion product at the N-terminal end. Even though this sequence was not able to code for a fusion protein, it still led to a positive clone in the yeast two-hybrid screening. The author speculated that this positive selection of the SRA containing clone could be due to a transcriptional activation of PR AF-1 domain by SRA RNA transcript, although the potential RNA and protein interaction might have required a yeast protein with transcription mediator-like function. The authors subsequently identified three different human SRA cDNAs from skeletal muscles, heart and Hela cell line cDNA libraries, which shared an identical central core sequence consisting of exon2 to exon5, but differed in their 5’ and 3’ extremities [Fig I-3]. Interestingly, all three identified SRA isoforms lacked an initiation codon and were not able in vitro to encode any recombinant SRA proteins when fused upstream of GST or GAL4 sequences. In contrast, fusing SRA downstream of Gal4 and GST produced the expected translation products. Mouse monoclonal antibodies targeting the peptide encoded by the C-terminus of the putative SRA open reading frame however failed to detect any endogenous SRA

![Figure I-3. SRA RNA core sequences.](image)

Three original SRA RNA transcripts (I, II and III) were shown by Lanz et al. with different 5’ and 3’ extremities, but sharing a same central core sequence indicated by light blue. Among three isoforms, one sequence AF092038 registered with NCBI nucleotide database was aligned with chromosome 5q31.3 genomic region.
protein in multiple cell extracts and other lysates investigated in this study. The authors therefore concluded that endogenous SRA cDNAs were not able to produce any peptides.

Using transient transfection of Hela cells and classical Chloramphenicol-acetyltransferase reporter assays, Lanz et al. (2002) showed that SRA sequences co-activated in a hormone dependent manner not only the progesterone receptor activity but also that of estrogen receptor, glucocorticoid receptor and thyroid receptor. Interestingly in this study, SRA was not able to co-activate other receptors such as thyroid receptor β, retinoic acid receptor, retinoid X receptors or peroxisome proliferator receptor γ. Thus SRA appeared as this time to be a co-activator of steroid receptors only rather than of all nuclear receptors.

The observation that this activation function seemed associated to the presence of a RNA rather than a protein, was a first in the field of co-activators of steroid receptors. This led the researchers to thoroughly investigate the existence of any potential peptide linked effect. They first showed that SRA sequence was able to co-activate PR in an open reading frame independent manner. Indeed, all three potential SRA open reading frame generated products that were equally able to co-activate the PR transcriptional activity. Authors also reported that adding point mutations to change any reading frames or introducing translation stop codons did not affect SRA ability to co-activate PR transaction.

Using fractionation on Superose 6 column, co-immunoprecipitation and Xenopus oocytes expression system, Lanz et al. (1999) also showed that SRA was contained in a ribonucleoprotein complex containing SRC-1 and recruited specifically by ligand bound steroid receptors.

I-2-2 Mechanism of SRA RNA action

Since its discovery, several groups have further investigated SRA RNA mechanisms of action. First, using luciferase reporter assays, SRA RNA co-activation upon ligand
binding has been expanded to a variety of other nuclear receptors including but not limited to thyroid receptor β [108,109], retinoic acid receptor (RAR) [110,111], peroxisome proliferator receptor γ (PPARγ) [112] [Table I-4]. This apparent discrepancy compared to the original findings of Lanz et al. (1999) may result from differences in cell-type and reporter systems used. Co-activation of SRA RNA on different receptors may also require other regulatory regulators in promoter dependent manner. Indeed, SRA RNA is also able to bind to and synergizes with p68/p72, a DEAD-box RNA binding helicases as well as SRC2/TIF2 to co-activate ER-α activity in the presence of ligand [113]. Further studies have implicated broader functions of SRA RNA in transcriptional regulation of the myogenic differentiation factor (MyoD), a protein that plays a major role in regulating muscle differentiation [114,115]. In addition, both Steroidogenic factor 1 (SF1) and dosage-sensitive sex reversal-adrenal hypoplasia congenital critical region on X chromosome gene 1 (Dax1) have been shown to interact with SRA RNA in adipocytes to modulate expression of downstream adrenal genes. Therefore, SRA appears involved in adrenal and reproductive function [116]. Two recent publications have also revealed co-activation by SRA RNA of other transcription factors involved in Notch signaling [117,118]. Jung et al. (2005) first showed that, in a murine pre T-cell model, SRA RNA up-regulates notch signaling through direct interaction with the recombining binding protein suppressor of hairless (RBP-J), an important regulator in embryonic and postnatal development. Wongtrakoongate et al. (2015) further demonstrated that the transcription factor NANOG directly interacted with SRA RNA to form regulatory complexes of polycomb repressive complex 2 (PRC2) and trithorax group complexes (TrxG). These complexes are critical transcriptional regulators involved in the maintenance of the pluripotent state [118]. These authors concluded that SRA RNA is not only required for maintaining the stem cell state, but also for reprogramming human fibroblasts to achieve the pluripotent state [118]. Interestingly, a common transcription regulator DEAD box
Figure I-4. Potential mechanisms underlying SRA RNA mediated biology.

SRA1 (black stem loop structure) was initially thought to form a complex with the coactivator SRC-1 in the presence of hormone. SRA RNA was also found to associate with p68/p72 proteins. In addition, SRA RNA can be recruited in co-repressor complexes such as the SMRT/HDAC1 associated repressor protein (SHARP) and SRA stem-loop interacting RNA binding protein (SLIRP) to repress downstream gene transcription, potentially through interacting with HDAC. The alternative repressive model of SRA RNA has been proposed through interacting with other repressive complex containing HP1γ, LSD1, HDAC1/2. Interestingly, this SRA RNA containing repressive complex is already formed prior to ligand addition through the interacting with unliganded PR.

In addition to nuclear receptor co-regulation, SRA RNA has now been shown to be a co-regulator for other transcription factors, including SF1/Dax1, MyoD and RBP-J/NICD through interactions with p68. SRA RNA has been shown to regulate adipogenesis in adipocytes through interacting with SF1/Dax and promote myogenesis/ muscle differentiation through interacting with MyoD. In addition, transcription factor NANOG directly interacted with SRA RNA to form regulatory complexes of PRC2 and TrxG, which are involved in the maintenance of the induced pluripotent stem (iPS) cells state. More recently the implication of SRA RNA in chromosome looping or pairing has been recently suggested. SRA RNA is an essential component of chromatin binding insulator complexes with CTCF, dead box protein P68. Non-coding SRA has been shown to mediate CTCF/p68 complex to recruit the cohesion complex to insulator sites.

In the cytoplasm SRA acts as a scaffold molecule in RNA-Induced Silencing (RISC) complex and mediates miRNA processing. Interestingly SLIRP has been shown predominantly expressed in mitochondria membrane. Both non-coding SRA mediated RISC and SLIRP complex are able to translocate to nucleus and load at target gene promoter region to regulate hormone-responsive gene expression.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Dir. Inter.</th>
<th>Complex</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>PR</td>
<td>x</td>
<td>x</td>
<td>Lanz et al. 1999; Vicent et al. 2013</td>
</tr>
<tr>
<td>AR</td>
<td>x</td>
<td></td>
<td>Lanz et al. 1999</td>
</tr>
<tr>
<td>ESR1</td>
<td>x</td>
<td></td>
<td>Watanabe et al. 2001</td>
</tr>
<tr>
<td>RAR</td>
<td>x</td>
<td></td>
<td>Zhao et al. 2004</td>
</tr>
<tr>
<td>MyoD</td>
<td>x</td>
<td></td>
<td>Caretti et al. 2006</td>
</tr>
<tr>
<td>Pus1p</td>
<td>x</td>
<td></td>
<td>Zhao et al. 2004, Huet et al. 2014</td>
</tr>
<tr>
<td>Pus3p</td>
<td>x</td>
<td></td>
<td>Zhao et al. 2006</td>
</tr>
<tr>
<td>SRC-1</td>
<td>x</td>
<td></td>
<td>Lanz et al. 1999, Watanabe et al. 2001</td>
</tr>
<tr>
<td>TIF2</td>
<td>x</td>
<td></td>
<td>Watanabe et al. 2001</td>
</tr>
<tr>
<td>YB1</td>
<td>x</td>
<td></td>
<td>Honig et al. 2002</td>
</tr>
<tr>
<td>TR-α-β</td>
<td>x</td>
<td>x</td>
<td>Xu 2004, 2005</td>
</tr>
<tr>
<td>p68</td>
<td>x</td>
<td>x</td>
<td>Watanabe et al. 2001, Caretti et al. 2006</td>
</tr>
<tr>
<td>p72</td>
<td>x</td>
<td>x</td>
<td>Watanabe et al. 2001, Caretti et al. 2006</td>
</tr>
<tr>
<td>Sharp</td>
<td>x</td>
<td>x</td>
<td>Shi et al. 2001, Arieti et al. 2014</td>
</tr>
<tr>
<td>SLIRP</td>
<td>x</td>
<td>x</td>
<td>Hatchell et al. 2006</td>
</tr>
<tr>
<td>Dax-1 and SF-1</td>
<td>x</td>
<td>x</td>
<td>Xu et al, 2009</td>
</tr>
<tr>
<td>Chicken insulator HS4</td>
<td></td>
<td>x</td>
<td>Fujita et al, 2011</td>
</tr>
<tr>
<td>CCTF, CCCTC binding factor</td>
<td></td>
<td>x</td>
<td>Yao et al, 2010</td>
</tr>
<tr>
<td>HP1/LSD1/uPR Rep. complex</td>
<td></td>
<td>x</td>
<td>Vicent et al. 2013</td>
</tr>
<tr>
<td>NANOG</td>
<td>x</td>
<td></td>
<td>Wongtrakoonangate et al. 2015</td>
</tr>
<tr>
<td>EZH2, EED, SUZ12 PRC2 complex</td>
<td>x</td>
<td>x</td>
<td>Wongtrakoonangate et al. 2015</td>
</tr>
<tr>
<td>TrxG complex WDR5, RBBP5, ASH2L</td>
<td>x</td>
<td>x</td>
<td>Wongtrakoonangate et al. 2015</td>
</tr>
</tbody>
</table>

**Table I-4.** Proteins forming complexes with SRA RNA.
RNA helicase p68 is found in all previously mentioned SRA RNA-protein regulatory complexes involving a variety of transcription factors such as MyoD, RBP-J/NICD, PRC2 and TrxG complexes [114,117-119][Fig I-4][Table I-4].

Interestingly, SRA RNA co-activation function can be controlled through two pseudo-uridylases, a Pus1p and Pus3p, which themselves have also been considered as co-regulators for variety of nuclear receptors [110]. The pseudo-uridylases specifically modify uridine residues at U206 of SRA RNA and are believed to consolidate the secondary structures and promote proper folding, leading to synergized co-activation function. However, hyper-pseudo-uridinylation of SRA RNA caused by U206A mutation can switch off co-activation activities [110]. Therefore, it appears that SRA RNA can co-activate hormone-dependent gene activation as part of different complexes under different cellular context [Fig I-4].

Besides co-activation function, several studies have also demonstrated that non-coding SRA can also be recruited in co-repressor complexes containing molecules such as SRA stem-loop interacting RNA binding protein (SLIRP) and the SMRT/HDAC1 associated repressor protein (SHARP). These complexes are believed to repress target gene transcription, potentially through the specific interaction with histone de-acetylase (HDAC)[120,121]. SHARP specifically binds to SRA RNA through a RNA recognition motif (RRM), and interacts with co-repressors through its repression domain (RD) [120,122]. Similarly, the co-repressor SLIRP specifically interacts with SRA STR-7 (secondary structural motif-7), one of the important functional/structural domains of SRA involved in SRA action (see Chapter 2.4). This interaction is believed to inhibit SRA-mediated co-activation activity of ER-α [121,123]. An alternative repressive model has been proposed where SRA interacts with other repressive complexes containing Lysine-specific histone demethylase-1 (LSD1), HDAC and heterochromatin protein γ (HP1γ), hence participating in transcriptional repression [124]. Vicent et al. (2009) indeed
showed that in order to maintain hormone-inducible genes silenced before ligand addition, un-liganded PR binds to specific genomic sites and targets SRA RNA-containing repressive complex by heterochromatin formation. As such, SRA containing repressive complex appeared in this model to be already formed prior to hormone treatment [124]. It has therefore been proposed that SRA RNA serves as an anchor for certain co-repressor complexes which contribute to maintain very low basal activity of target genes in the absence of ligand [Fig I-4].

Interestingly, the model function of SRA RNA on gene regulation has now expanded to a larger context of epigenetic regulation. Using mass spectrophotometric analysis to identify proteins associated with the known chromatin insulator CCCTC-binding factor (CTCF), Yao et al. (2010) identified the pre-cited RNA helicase p68. They found that the interaction between CTCF and p68 was mediated through SRA RNA [125]. Depletion of SRA RNA was indeed shown to disrupt the association of CTCF complex and p68 leading to a reduced binding of the cohesion complex to insulator sites in both human cervical Hela and primary mouse embryonic fibroblasts [126][Fig I-4]. This suggested for the first time that SRA RNA could also contribute to chromosome looping or pairing.

In addition to SRA nuclear functions, cytoplasmic signaling functions have been proposed as well. In the cytoplasm SRA RNA is indeed suspected to also act as a binding scaffold for the RNA-induced Silencing complex (mi-RISC). These complexes would translocate to the nucleus and load on specific target gene promoter regions to regulate hormone-responsive gene expression in MCF-7, MDA-MB-468 and HeLa cells [127]. [Fig I-4].

Overall, SRA RNA is believed to serve as scaffold in many molecular protein complexes in both the cytoplasm and the nucleus. Those regulatory complexes rely on physical interactions, which are believed to depend upon specific secondary SRA structures, a concept developed by Lanz et al. in 2002 [128].
Structure of SRA RNA

Through the use of low-resolution structure modeling software (MFOLD), Lanz et al. (2002) first predicted the presence of twelve putative secondary structure elements (STRs) conserved between mouse and human [128]. Among them, six STRs (STR1,7,9,10,11 and 12) appeared as critical for SRA RNA co-activation. The silent mutations throughout these six STRs sequence damaged RNA binding/structure, and its ability to co-activate PR by luciferase reporter assays [128]. Ten years later, Novikova et al. (2014) experimentally established SRA RNA secondary structures [129]. These authors used four complementary biochemical techniques, including RNase V1 digestion, DMS probing, in-line probing and SHAPE (S selective 2′ -hydroxyl acylation analyzed by primer extension), to confirm the existence of specific structures within SRA RNA. Full length human SRA RNA (870 nucleotides) contains 4 sub-domains and 25 helices (H1–H25) [129]. These domains globally overlap with the previously proposed “stem-loop regions” mentioned as secondary structure elements (STRs). Among them, domain II contains H13, which is highly associated with the previously described STR7 region investigated by several groups [129]. STR7 region is referred to as a “hotspot” of protein-RNA interactions [Fig I-5]. At least three proteins including previously mentioned SLIRP (stem-loop interacting RNA binding protein); SHARP (SMRT/HDAC1 associated repressor protein) and un-liganded PR have been experimentally shown to physically interact with this region [115,122-124,129]. Several other regions, highly conserved in all chordata, have also been identified, exact relevance of which remains to be investigated [129]. Overall, SRA is thought, through specific secondary structures, to act as a broad co-regulator of transcription and to potentially contribute to some biological events that remain to be characterized.
I-2-4  Relevance of SRA RNA to breast cancer

The relevance of this novel functional RNA to breast cancer has been studied by several research groups. SRA RNA expression was increased in a variety of cancers including breast, uterus and ovarian tumours compared to the corresponding normal tissue [130-132]. It has been proposed that different level of SRA RNA expression might characterize particular subtypes of lesions. For example, serous ovarian tumours expressed higher levels of SRA RNA than granulosa cell tumours [132]. In breast cancer tissues,

![Secondary Structure of SRA STR7/H13](image)

**Figure 1-5. Secondary structure of SRA STR7/H13.**

SRA RNA domain II contains H13, which is highly associated with previously described STR7 region. STR region is referred to as a “hotspot” of protein-SRA interaction. At least four proteins including SRAP itself, SLIRP (stem-loop interacting RNA binding protein), SHARP (SMRT/HDAC1 associated repressor protein) and un-liganded PR have been experimentally shown to physically interact with this region. Graph was generated by The Vienna RNA website (http://rna.tbi.univie.ac.at/cgi-bin/RNAfold.cgi.)

higher levels of SRA RNA than granulosa cell tumours [132]. In breast cancer tissues,
particular SRA RNA levels also seemed to be expressed in specific subgroups of breast tumours differing in their level of ER-\(\alpha\) and PR. Indeed, ER-\(\alpha\)-positive/PR-negative breast tumours expressed more SRA RNA than ER-\(\alpha\)-positive/PR-positive breast tumours whereas ER-\(\alpha\)-negative/PR-negative tumour subgroups expressed less SRA RNA than ER-\(\alpha\)-negative/PR-positive lesions [130]. Whether or not SRA RNA participates in the mechanisms underlying the responsiveness to hormonal therapy remains an opened question. Interestingly, similar levels of SRA RNA were detected in tamoxifen sensitive and resistant breast tumours, suggesting that SRA might not directly contribute to tamoxifen resistance [131]. Also, a SRA isoform missing exon 3 was detected in breast tumour tissues. Expression of this specific isoform but not that of the full-length SRA appeared positively associated with high tumour grade [130]. This isoform lacks a portion of SRA core sequence and therefore should be unable to co-activate estrogen receptor function. This suggests that the tumours highly expressing this alternatively spliced isoform tend to be more aggressive possibly due to disrupted estrogen receptor activities. Furthermore, both SRA genetic mutations and single nucleotide polymorphisms (SNPs) were also shown to be associated with breast cancer [133,134]. A study by Xiao et al. (2014) revealed that a germline mutation C91T of SRA is present in the genome of blood cells in BRCA1 + familial breast cancer family [133]. It is proposed that this SRA mutation may result from the genomic instability caused by the loss of BRCA1 function [133]. A more recent study demonstrated that two SRA polymorphism located in intron 2 were positively associated with estrogen receptor (ER) status, and that patients with those two polymorphisms have a significantly greater risk of developing breast cancer compared to individuals with wild-type SRA [134]. The exact mechanism behind this observation remains to be elucidated.

*In vivo* study of SRA transgenic mice revealed that over-expression of the core SRA sequences in the mammary glands led to pre-neoplastic lesions [135]. It should however
be stressed that it did not appear to be sufficient to generate fully malignant breast tumours [135]. SRA RNA is suspected to contribute to cell tumour proliferation. Using gene ontology (GO) analysis on microarray dataset, Vicent et al. (2013) revealed that genes down-regulated upon SRA1 silencing were significantly enriched in the functional group of the cell proliferation. Depletion of SRA1 gene reduced PR-induced proliferation in T47D breast cancer cells [124]. Vicent et al. (2013) also showed that down-regulation of SRA expression attenuates apoptosis, which is consistent with the previous conclusion from in vivo mouse studies where overexpressing SRA in mammary gland also increased apoptosis [135]. Lastly, knocking down SRA expression in MCF-7 cells altered the expression of several genes involved in invasion/metastasis [136]. This effect of SRA RNA on tumour invasiveness has been confirmed by functional invasion assay in MDA-MB-231 invasive breast cells with silenced SRA expression [136].

Taken together, this suggested that SRA RNA, even though not sufficient per se to lead to tumour formation, might participate in molecular mechanisms underlying proliferation and breast tumour invasion. The molecular mechanism of SRA RNA action in breast cancer still remains to be fully elucidated.

I-3 Steroid Receptor RNA activator protein (SRAP)

I-3-1 Discovery of SRAP

Shortly after the initial report describing no evidence of a translatable product corresponding to the SRA1 gene, our laboratory reported the isolation of three SRA alternative variants (Genbank accession number: AF293024, AF293025, AF293026). Unlike the variants cloned by Lanz et al. (1999) these isoforms contained two putative initiating methionine codons in their 5' extended sequence [137]. These transcripts, that were translatable in vitro in reticulocyte translation assays, were also able to code for 236/237 amino acids long product detectable by western blot in transfected cells.
Interestingly, alignment of the putative SRAP sequences obtained in multiple Chordata, revealed the existence of two highly conserved domains (CD1 and CD2), corresponding to 15-52 amino acid and 135-204 amino acid of the human SRAP sequence [138].

The existence of the human SRAP protein is now clearly demonstrated by Mass spectrometric analysis using anti-SRAP antibody in human Hela and MCF7 cell lines [139]. The expression of SRAP in other species such as amphioxus is now also experimentally evidenced by Sun et al. (2014) [140]. The two conserved domains CD1
and CD2 also corresponded to the regions showing the highest degree of homology between human and species associated to lower taxa [Fig I-6]. Conservation of these same domains in species such as trichoplax adhaerens suggests this protein might have played a specific role very early in animal evolution.

Importantly, this was the first time a gene was described as encoding a functional transcript also able to code for a protein. Since then other examples have been cited such as Oskar, a bi-functional RNA identified in Drosophila for its ability to be translated into a protein induced in oocytes [141]; and VegT, a maternal RNA localized in Xenopus oocytes and able to code for a T-box transcription factor involved in mesoderm induction [142].

I-3-2 SRA variants and alternative splicing events

Since the respective discovery of both non-coding and coding SRA RNAs, multiple human transcripts have been registered in the non-redundant (nr) database of the National Center for Biotechnology Information (NCBI). Indeed, data mining of the human genome using 262 expressed sequence tags (ESTs) and 9 SRA mRNA sequences resulted in the classification of 20 SRA transcripts, of which 11 variants should not be coding and 9 coding.

As shown in Fig I-7 coding isoforms possess an extended exon-1 containing two translation initiating methionine codons. They also contain an intact core region consisting of exons 2 through 5. Non-coding isoforms are predominantly the result of alternative intron-1 splicing, leading to either a reading frame shift or introduction of a premature stop codon [Fig I-7][138]. These alternative splicing transcripts include full length, partial intron-1 retention, or the concomitant use of an alternative 5’ donor site in exon-1 with the same 3’ acceptor site as Partial intron-1 retention isoform [138]. Non-coding transcripts also include those with exon-3 deletion. It should be stressed that both fully and
Alternatively spliced SRA transcripts are expected to retain their properties as a functional RNA due to the presence of the core sequence (exon 2 – 5), which was found to be necessary and sufficient SRA RNA activity [128]. The exception to this is exon-3 deleted transcripts that are not expected to behave like SRA RNA, although this has yet to be experimentally verified. Both non-coding and coding SRA transcripts co-exist in breast cells with different relative levels of coding/non-coding SRA transcripts. Interestingly, the
three most invasive cell lines (MDA-MB-231, MDA-MB-468, and BT-20) expressed the highest, whereas the "closest to normal" MCF-10A1 breast cells expressed the lowest, relative levels of non-coding SRA intron-1 RNA [143]. This suggests that the balance between non-coding and coding SRA RNA might be associated with breast cancer invasion. This remains to be further explored.

I-3-3 Potential SRAP function

Compared to many studies investigating the functional SRA RNA, overall little is known about SRAP function. A potential co-activation function of SRAP on nuclear receptors was first described by Kawashima et al. [144]. Using luciferase reporter assays and a construct expressing a truncated rat SRAP lacking the evolutionary conserved N-terminal domain (CD-1), these authors observed ligand-dependent co-activation of several nuclear receptors, including androgen receptor (AR), peroxisome proliferator receptor γ (PPARγ), and glucocorticoid receptor (GR). Notably, the SRA construct used started at exon3 and may not be fully active at either RNA or protein level since the resulting SRA RNA lacks exon-2 core sequence and the corresponding protein is N-terminally truncated. Subsequently, two reports have consistently found that SRAP protein independently of its RNA counterpart increased the activation of ER-α by estradiol treatment [145,146]. In the first study, through silent mutation of nucleotides located in SRA functional loop structures, a construct was generated to encode for SRAP but devoid of RNA function. Using luciferase reporter assays, Chooniedass et al. (2010) showed that SRAP itself acts as a positive transcriptional regulator enhancing ER-α activity in a ligand and response-element dependent manner [145]. Borth et al. (2009) also showed that SRAP is capable of co-activating ER-α AF1 domain in HEK-293 cells using different SRA construct via introduction of premature stop codons [146]. Interestingly, in this later report, it was further suggested that CT441, a chlamydial protease can sequester SRAP in the
cytoplasm and consequently partially inhibits SRAP co-activation activity of ER-α in the nucleus [146]. This suggests that different cellular localization of SRAP might have different functional significances. In addition to increasing steroid receptor action, Hube et al. (2010) showed that SRAP co-activated the activity of transcription factors such as ETS2 (v-ets avian erythroblastosis virus e26 oncogene homolog2), by enhancing ETS2 responsive element driving luciferase reporter activity in a dose dependent manner [115]. Overall, a growing body of evidence is pointing toward SRAP, independently of its RNA counterpart, being able to act as a transcriptional activator.

In apparent contrast with this co-activator function, SRAP has also been found, in independent studies, to have additional intrinsic repressive transcriptional potential [147,148]. In the first study, in stably expressing SRAP MCF-7 breast cancer cells, a vitellogenin estrogen responsive-element (ERE) driven reporter gene had a decreased response to estrogen. It was therefore proposed that overexpression of SRAP may inhibit the activity of ER-α [148]. In the second study, hybrid-protein constructs expressing either full-length SRAP, N-terminal or C-terminal regions were artificially physically recruited to a luciferase-reporter promoter driven by the strong VP16 transcriptional activator. All constructs significantly inhibited VP16 dependent luciferase activity suggesting that both conserved regions had the ability to repress transcription were recruited to promoters [147]. It was however found that the C-terminal domain of SRAP had a stronger inhibitory effect than the N-terminal region of SRAP on VP16 transcription activity. Further treatment with Trichostatin-A (TSA), an inhibitor of HDAC activity, fully abolished the repressive activity of N-terminal SRAP but only partially blocked the inhibitory effect of C-terminal SRAP [147]. This suggests that different activities noted between C- and N-terminal SRAP on transcriptional repression possibly result from different interacting partners.

Altogether, data point toward SRAP potentially playing a role in regulating gene transcription. This hypothesis appeared to be also partially supported by mass
spectrometric analysis of nuclear Hela cell proteins co-immunoprecipitating with SRAP. Jung et al (2005) indeed reported that endogenous SRAP could form complexes with a wide range of transcriptional regulators including SMARCC2 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily c, member 2), a member of the SWI/SNF chromatin remodeling complex); MEF2A (myocyte enhancer factor 2A), a DNA binding transcriptional regulatory proteins originally found to regulate muscle gene expression, as well as YB-1 (Y box binding protein 1), a critical modulator of estrogen receptor function in breast cancer cells [139] [Table I-5]. It should however be stressed that none of these putative interactions have been confirmed nor validated using other techniques.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Direct interaction</th>
<th>Complex formation</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclear receptor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AR</td>
<td>X</td>
<td>Kurisu et al 2006, Kawashima et al. 2003</td>
<td></td>
</tr>
<tr>
<td>ERα</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>ERβ</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>GR</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>Transcriptional factors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>P68</td>
<td>X</td>
<td>Jung et al 2005</td>
<td></td>
</tr>
<tr>
<td>ETS2</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>HAND1</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>others</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HDAC2</td>
<td>X</td>
<td>Chooniedass-Kothari et al. 2010 b</td>
<td></td>
</tr>
<tr>
<td>CT441</td>
<td>X</td>
<td>Borth et al. 2010</td>
<td></td>
</tr>
<tr>
<td>SRA RNA**</td>
<td>X</td>
<td>Hube et al. 2010</td>
<td></td>
</tr>
</tbody>
</table>

Table I-5. Proteins forming complexes with or directly binding to SRAP.

Interestingly, it has also been proposed that SRAP could interact with its own RNA [115]. Hube et al. (2013) proposed that SRAP could modulate SRA RNA-mediated
transcriptional activation of MyoD through direct binding to SRA RNA. Physical interactions between SRAP and SRA has however not been confirmed [122,123]. In order to decipher SRAP potential roles, several independent groups have investigated its crystal structure.

I-3-4 Structure of SRAP

The crystal structure of the mouse SRAP (2yru) has been first deposited in the Protein Data Bank (PDB) site by Nameki et al. (2009) [149]. The method of torsion angle dynamics was used to refine the structure from aas 87-216 of mouse SRAP with a 2.2-Å resolution. Using homology modeling by threading the crystallized mouse SRAP structure we were able to predict a 3D human SRAP structure [Fig I-9][149]. The N-terminal of SRAP has a S/T proline-rich region (P-rich), which could not be crystalized by multiple methods and thus has been characterized as intrinsically disordered protein [150] [Fig I-8]. Both mouse and predicted human SRAP conserved C-terminal demonstrate extremely identical organization consisting of a five-helix up-down bundle structure, in which helix cross with an angle of ~110°. RNA recognition motif (RRM) and nuclear localization signal (NLS) motifs were predicted in the third helix localized on the surface of the 3D structure [Fig I-8]. Interestingly, we noted that the yeast-splicing factor PRP18 had a very similar fold pattern [Fig I-8][149]. The Yeast PRP 18 is a splicing regulator, which plays a role in splicing component associated with the U5 snRNP through interacting with many other splicing factors such as SLU7 [151]. Structure similarity between crystalized C-terminal of SRAP and PRP18 suggests that SRAP may be involved in the splicing regulation through protein-protein interacting. In 2014, two experimental crystal structures of C-terminal human SRAP (2MGX, 4NBO) have been refined by two groups with different methods [122,123]. (4NBO solution NMR model; 2MGX: X-ray diffraction). Both articles by McKay et al. (2014) and Bilinovich et al. (2014) confirmed the structural
organization previously predicted, but no RNA recognition motif (RRM) was found in either model. McKay et al. (2014) further showed that in HEK293t cells, pull-down of SRAP from cell lysates failed to carry any significant amount of SRA RNA [122], therefore refuting the idea that SRAP might physically interact with its own RNA. Overall, SRAP function remains largely unknown.

**Figure 1-8. SRAP 3D structure.**

A) Ribbon cartoon representation of the experimentally determined Mouse SRAP structure (mSRAP, PDB: 2yru) is shown in the left panel. Human SRAP 3D structure (hSRAP) has been predicted by threading using homology modeling (Swiss-model http://swissmodel.expasy.org/). SRAP protein from both species shows a similar structure where the protein core consists in a five-helix X bundle structure, where helices α3-5 (α3: green, α4: grey, α5: pink) are anti-parallel and located on the same side of the α1-α2 (α1: blue, α2: red) plane. B) The conserved RRM motif (pink), L-X-X-A-L motif (red) and NLS motif (yellow) are highlighted on the surface cartoon representation of both the mouse (left panel) and human (right panel) SRAP 3D structure. C) Ribbon cartoon representation of the experimentally determined 3D structure (PDB: 1dyk) from PRP18, a yeast splicing factor, is shown in the left panel. The predicted human SRAP structure is shown in the middle panel. Superimposed image generated using PyMOL reveals an extensive overlapping of the five helices bundle structure.
I-3-5  Relevance of SRAP to breast cancer

Endogenous SRAP expression in breast cancer was originally detected by Western blot using an "in-house" rabbit polyclonal antibody recognizing amino-acids 20-35 [148,152]. This antibody, beside the expected 30kDa signal also specifically detected a lower 25kDa band [148]. Interestingly, it was found in a small cohort of ER-α positive breast cancer cases treated with tamoxifen that patients were less likely to die from the disease if they expressed higher levels of 30kDa SRAP detected by western blotting in their primary tumour [148]. This suggested that the presence of the protein could characterize a less aggressive tumour phenotype. However, this potential prognostic value still required to be validated by different detection methods in larger breast cancer cohorts.
CHAPTER II Thesis rationale, hypothesis and objectives

II-1 Overall rationale

The steroid receptor RNA activator gene (SRA1) produces a particularly intriguing genetic system consisting of both a functional RNA (SRA) and a protein (SRAP). However, very few studies have tried to define the function and relevance of the protein. Using a custom-made rabbit polyclonal antibody for SRAP, Chooniedass-Kothari et al analyzed a small cohort of ER-α positive breast tumours by Western blotting [148]. Several bands were detected. Interestingly, the detection of a band corresponding to the expected 30kDa SRAP-peptide, was associated with a longer survival. Because of the potential important implication of this observation for breast cancer patients, it was necessary and critical to further investigate the expression and potential roles of SRAP in breast cancer.

II-2 Hypothesis and Objectives

Hypothesis: SRAP expression is associated with specific tumor characteristics and plays a critical role in breast cancer

My project focuses on two main objectives:

Objective 1 To assess potential associations between the expression of SRAP and SRAP-like peptides and tumour/patient characteristics.

In Chapter III, IV and V, I validated multiple anti-SRAP antibodies for immunohistochemistry. Using these selective anti-SRAP antibodies, I have assessed SRAP expression in multiple tumours in tissue microarrays (TMAs) obtained from the Manitoba Breast Tumour Bank (MBTB) and the National Cancer Institute of Canada
(NCIC-CTG MA) clinical trial). In each individual cohort, I wanted to interrogate whether the detection of SRAP expression was associated with a particular tumour phenotype and with patient survival.

Objective 2  To identify the cellular processes potentially controlled by SRAP

In Chapter VI, I wanted to analyze the transcriptome of Hela and MDA-MB-231 cells following both “loss” and “gain” of SRAP function to identify the impact on gene expression. Effect of SRA expression on cell motility and invasion was further investigated using both classical migration assay as well as a new live imaging approach.
CHAPTER III Steroid Receptor RNA Activator Protein (SRAP): a potential new prognostic marker for estrogen receptor-positive/node-negative/younger breast cancer patients

To address the potential prognostic value of SRAP in breast cancer, I initially used tissue micro-arrays (TMAs) to investigate its expression in 372 cases. Possible associations with a wide range of clinical parameters (such as hormonal, node statuses, tumour grades, sizes and patient ages) were investigated. Survival analyses were also performed to establish the link between SRAP expression and clinical outcome. I discovered that higher expression of SRAP was associated with poor prognosis in patients presenting with tumours containing estrogen receptor and received tamoxifen treatment within a large cohort of invasive breast tumour. The data therefore suggests that higher levels of SRAP in primary tumours characterize a particular set of patients less likely to respond to this otherwise successful therapy. Identification of such patients could help clinicians considering alternative or additional treatment options at earlier stages. This important observation was published on Sept 9 2009 in *Breast cancer research; 11(5): R67* entitled “Steroid Receptor RNA Activator Protein (SRAP): a Potential New Prognostic Marker for Estrogen Receptor-positive / Node-Negative / Younger Breast Cancer Patients”. Because of the potentially significant clinical implication, this article was also featured and highlighted in *Nature Review of Clinical Oncology 7,(4)* on Jan 4, 2010.

My contributions to this article
I contributed to this work by performing all the antibody validations and TMA. I also performed the statistical analysis and drafted the manuscript.
Research Highlight


Subject Category: Screening

SRAP—new prognostic marker
Vessela Vassileva

Expression of steroid receptor RNA activator protein (SRAP) could potentially be used as a predictive marker for the clinical outcome of patients with breast cancer, according to a new study.

Breast cancer is one of the most frequently diagnosed malignancies and the second cause of cancer-related deaths among women worldwide. Previous studies have suggested that SRAP plays a part in breast cancer progression. SRAP is expressed in multiple cancer cell lines, and an increase in its expression has been shown during the development and progression of breast, ovarian and uterine cancer.

Researchers from Canada analyzed expression of SRAP by tissue microarray analysis in 372 breast tumor samples obtained from the Manitoba Breast Tumor Bank to investigate whether SRAP levels in patients with breast cancer are associated with clinical parameters, such as steroid receptor status, node status and outcome.

Expression of SRAP was found to be significantly elevated in tumors of patients aged >64 years and in estrogen and progesterone receptor-positive tumors. Additionally, SRAP expression was negatively associated with breast cancer-specific survival in patients aged >64 years with estrogen and progesterone receptor-positive tumors. In patients with estrogen receptor-positive cancers, high expression of SRAP significantly correlated with a higher rate of breast cancer mortality and recurrence compared with patients who had estrogen receptor-negative tumors. In patients aged ≤64 years with estrogen receptor-positive and node-negative tumors, high SRAP expression was also indicative of poor prognosis.

SRAP expression might be predictive of outcome in a specific set of patients with otherwise good prognosis when only considering clinical parameters.

References and links

ORIGINAL RESEARCH PAPERS


© Previous article | PubMed | ChemPort

© Next article | Subscribe
Research article

Steroid Receptor RNA Activator Protein (SRAP): a potential new prognostic marker for estrogen receptor-positive/node-negative/younger breast cancer patients

Yi Yan1,2*, George P Skliris1,2*, Carla Penner2, Shilpa Chooniedass-Kothari1,2, Charlton Cooper2, Zoann Nugent1, Anne Blanchard3, Peter H Watson4, Yvonne Myal3, Leigh C Murphy1,2 and Etienne Leygue1,2

1Manitoba Institute of Cell Biology, 675 McDermot Avenue, R3E0V9, Winnipeg, Manitoba, Canada
2Department of Biochemistry and Medical Genetics, University of Manitoba, 770 Bannatyne Avenue, R3E0W3, Winnipeg, Manitoba, Canada
3Department of Physiology, University of Manitoba, 770 Bannatyne Avenue, R3E0W3, Winnipeg, Manitoba, Canada
4BC Cancer Agency's Trev & Joyce Deely Research Centre, 2410 Lee Avenue, V8R 6V5, Victoria, BC, Canada

* Contributed equally

Corresponding author: Etienne Leygue, eleygue@cc.umanitoba.ca

Received: 24 Jun 2009  Revisions requested: 20 Jul 2009  Revisions received: 6 Aug 2009  Accepted: 9 Sep 2009  Published: 9 Sep 2009

This article is online at: http://breast-cancer-research.com/content/11/5/R67
© 2009 Yan et al.; licensee BioMed Central Ltd.
This is an open access article distributed under the terms of the Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0), which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

Introduction The steroid receptor RNA activator is a functional RNA suspected to participate in the mechanisms underlying breast tumor progression. This RNA is also able to encode for a protein, Steroid Receptor RNA Activator Protein (SRAP), whose exact function remains to be determined. Our aim was to assess, in a large breast cancer cohort, whether levels of this protein could be associated with outcome or established clinical parameters.

Methods Following antibody validation, SRAP expression was assessed by tissue-microarray (TMA) analysis of 372 breast tumors. Clinical follow-up and parameters such as steroid receptor and node status were available for all the corresponding cases. Immunohistochemical scores were independently determined by three investigators and averaged. Statistical analyses were performed using standard univariate and multivariate tests.

Results SRAP levels were significantly (Mann-Whitney rank sum test, \( P < 0.05 \)) higher in estrogen receptor-alpha positive (ER+, \( n = 271 \)), in progesterone receptor positive (PR+, \( n = 257 \)) and in older patients (age > 64 years, \( n = 182 \)). When considering ER+ tumors, PR+ tumors, or younger patients (≤64 years), cases with high SRAP expression had a significantly (Mantel-Cox test, \( P < 0.05 \)) worse breast cancer specific survival (BCSS) than those with low SRAP levels. SRAP also appeared as a very powerful indicator of poor prognostic for BCSS in the subset of ER+, node negative and young breast cancer patients (Cox regression analysis, \( n = 60 \), BCSS Hazard Ratio = 8.61, \( P < 0.006 \)).

Conclusions Our data suggest that SRAP levels might provide additional information on potential risk of recurrence and negative outcome in a specific set of patients with otherwise good prognosis when considering only estrogen receptor and nodal status.

Introduction

Breast cancer remains the second leading cause of cancer-related deaths in women worldwide and is one of the most frequently diagnosed cancers with an estimated 1,000,000 new cases detected each year worldwide [1]. Following diagnosis, several critical prognostic and predictive markers are assessed in order to determine, for each patient, the most appropriate treatment to be administered. Estrogen receptor (ER) status, progesterone receptor (PR) status, nodal status, tumor size, and grade of malignancy are the classical parameters used to date by clinicians to narrow down prognoses and weight treatment options [2]. More recently, human epidermal

BCSS: Breast Cancer Specific Survival; DMEM: Dulbecco's modified Eagle's medium; ER: estrogen receptor alpha; FBS: fetal bovine serum; HER: human epidermal growth factor receptor; HR: hazard ratio; LBA: ligand binding assay; PBS: phosphate-buffered saline; PR: progesterone receptor; RFS: recurrence-free survival; SRA: steroid receptor RNA activator; SRAP: steroid receptor RNA activator protein; SRC1: steroid receptor co-activator 1; TMA: tissue micro-arrays.
growth receptor (HER)-2, which is over-expressed in about 25% of breast cancers and is associated with a more aggressive disease and a poorer outcome, has also been used as a prognostic and predictive marker [3]. Recent approaches such as gene profiling and tissue micro-arrays (TMAs) have increased our ability not only to identify new potential markers, but also to rapidly test their potential validity [4,5]. The more such molecules are identified, the higher becomes the odds of finding the optimal combination of markers allowing the determination of an 'ideal' treatment for any given patient [6].

The steroid receptor RNA activator (SRA) was originally identified as a functional non-coding RNA increasing the transcriptional activity of ligand-bound steroid receptors [7]. It is currently believed that this action is mediated by the formation, at the promoter of target genes, of regulatory complexes containing steroid receptors, SRA, and both positive and negative protein regulators [8-10]. SRA RNA is over-expressed during breast, ovarian and uterine tumorigenesis and tumor progression [11-14]. It has therefore been suggested that by increasing the activity of the ER, SRA could participate in the mechanisms underlying these events [7,12].

It has now been confirmed that coding SRA transcripts coexist in breast cancer cells, with the previously described non-coding transcripts [15-17]. The corresponding endogenous protein, steroid receptor RNA activator protein (SRAP), has been detected by western blot in multiple cell lines as well as in muscle and breast tissue [15,17-19]. It has been suggested that, as its RNA counterpart, the protein might also regulate the activity of estrogen and androgen receptors [10,17-19]. This hypothesis is further corroborated by the identification of the RNA helicase p68, a well-characterized regulator of ER activity [20], in nuclear complexes co-immunoprecipitating with endogenous SRAP [21].

Overall, accumulated data raise the intriguing possibility that SRAP levels could be associated with ER activity and/or expression, and could also potentially reflect on the response of breast cancer patients to endocrine therapy. It has recently been reported that the relative proportion of coding and non-coding SRA transcripts varies from one breast tumor to another and might characterize particular tumor subgroups [22]. Altogether, this suggests that SRAP expression could also differ between cases and potentially be a prognostic and/or predictive indicator in breast cancer. To address this issue, we have herein investigated the use of TMAs for the expression of SRAP in 372 cases with a wide range of clinical parameters.

Materials and methods

Cell culture

Hela and Michigan Cancer Foundation (MCF)-7 cells (Cedarlane Laboratories Ltd., Burlington, ON, Canada) cells were cultured in DMEM (Gibco, Grand Island, NY, USA) medium supplemented with 5% FBS (Cansera, Rexdale, ON, Canada), penicillin (100 units/ml), streptomycin (100 μg/ml) (Gibco, Grand Island, NY, USA), and 0.3% glucose. Cells were grown in a 37°C humidified incubator with 5% carbon dioxide. Cells were transfected with empty vector or plasmids containing either the full SRA coding sequence and leading to the production of a SRAP-V5 tagged protein [16], or a pSuper.retro-SRA construct expressing a SRA-Interfering RNA (SRA-RNAi) SRA-Interfering RNA sequence [15], as previously described [15,16].

Western blot

Total proteins were extracted from cells pellets as previously outlined [17]. Frozen breast tumor sections were lysed and sonicated for 30 seconds in ice-cold Sodium-dodecyl-sulfate-Isolation-Buffer (SIB) containing 60 mM α-glycerophosphate, 1% SDS and a mini-protease inhibitor cocktail tablet (Boehringer Mannheim, Indianapolis, IN, USA) per 10 ml extraction buffer. Samples were then centrifuged at 13,000 g for 20 minutes at 4°C and stored at -20°C until use. Protein concentration was determined using BCA kit (Pierce Company, Rockford, IL, USA). Samples containing 75 μg of total protein were subsequently analyzed by western blot as described previously [17], using a primary anti-SRAP rabbit polyclonal antibody (cat # A300-743A, Bethyl Laboratories, Montgomery, TX, USA) raised against the C-terminal extremity of SRAP (peptide 180-237 aa). Signal detection and documentation was performed using the ChemiDoc imaging system (Bio-Rad, Mississauga, ON, Canada).

Antibody neutralization experiments

For neutralization experiments, 2 μg of SRAP-180-237 aa blocking peptide (cat # BP300-743 Bethyl Laboratories, Montgomery, TX, USA) was pre-incubated with 1 μg of 743A antibody for two hours at room temperature. A peptide, corresponding to 69 to 89 aa from the Small Breast Epithelial Mucin [23], was used as a non-specific blocking peptide.

Immunofluorescence

Hela cells cultured on cover-slips were transfected with control empty vector or plasmid expressing V5-Tagged-SRAP or SRA-RNAi (see above). Twenty-four hours post-transfection, coverslips were washed with PBS and cells fixed with 4% formaldehyde (Sigma, Oakville, ON, Canada) and 4% sucrose (Sigma, Oakville, ON, Canada) for 15 minutes at room temperature. Fixed cells were then rinsed with PBS and permeabilized with 0.25% Triton-X100 (Sigma, Oakville, ON, Canada) in PBS for five minutes. After rinsing with PBS twice, non-specific binding sites were blocked with 10% BSA (Sigma, Oakville, ON, Canada) in PBS for 30 minutes at 37°C. Cells were then incubated overnight at 4°C with 743A Anti-SRAP antibody diluted at 1/200 in 3% BSA/PBS. After washing with PBS for 15 minutes, cells were incubated with anti-rabbit secondary antibody-Cy3 conjugate (Jackson, West Grove, PA, USA) at a 1/1000 dilution in 3% BSA/PBS for one hour at
room temperature. Cell nuclei were stained with 1 μg/ml Hoechst (Invitrogen, Burlington, ON, Canada), and coverslips were mounted onto microscopy slides with FluorSave™ Reagent (Calbiochem, La Jolla, CA, USA). Fluorescent images were captured and visualized with a Nikon Eclipse E1000 epi-fluorescent microscope at wavelengths of 552 to 620 nm (CY3), 440 to 450 nm (Hoechst) using ACT-1 software (Nikon, Mississauga, ON, Canada).

Breast samples and tissue micro-arrays
All invasive breast cancer and normal breast samples used in the current study were obtained from the Manitoba Breast Tumor Bank, which operates with the approval of the Faculty of Medicine, University of Manitoba, Research Ethics Board [24]. The research reported in this manuscript has been performed with the approval of the Bannatyne Campus, University of Manitoba, Research Ethics Boards. As described previously, all tissues accrued to the bank from cases at multiple centers within Manitoba are frozen at -70°C immediately after surgical removal. A portion of the frozen tissue from each case is then processed to create matched formalin-fixed paraffin-embedded and frozen tissue blocks. The histopathology of all Manitoba Breast Tumor Bank cases has been previously assessed and entered into a computerized database to enable selection based on composition of the tissue as well as clinico-pathological parameters.

Matched frozen and paraffin-embedded sections corresponding to 20 invasive breast tumors and 6 reduction mammoplasties were first selected and evaluated for protein expression by western blot and immunohistochemistry, respectively. Tumor ER and PR levels (determined by ligand binding assay (LBA)) ranged from 2.3 to 180 fmol/mg protein and 4.5 to 42 fmol/mg protein, respectively. The age of patients ranged between 58 and 78 years, and tumor size varied from 15 to 50 mm.

TMAs corresponding to 450 ER positive (LBA > 3 fmol/mg total protein) and 255 ER negative (LBA ≤ 3 fmol/mg total protein) cases were constructed from primary invasive breast carcinomas, as described before [25,26]. Duplicate core tissue samples (0.6 mm diameter) were taken from selected areas of maximum cellularity for each tumor. Two controls, corresponding to cases positive and negative for SRAP expression, respectively, were included in each TMA run to check for run to run reproducibility. From our studies, cases with lost follow-up, unknown grade or nodal status, or data on SRAP expression (loss of a core during processing) were removed. All characteristics were ultimately available for 372 breast cases. Tumors corresponded to 271 cases associated with ER-positive status (ER > 3 fmol/mg total protein, as assessed by LBA) that were treated by surgery and then tamoxifen endocrine therapy or chemotherapy. The whole cohort has ER levels ranging 0 to 331 fmol/mg protein (median 23 fmol/mg protein) and spanned a wide range of PR levels 0 to 1591 fmol/mg protein (median 17.95 fmol/mg protein). Nottingham grade [27] was also known for all 372 tumors, which were assigned to low (n = 79, scores 3 to 5), moderate (n = 205, scores 6 to 7) or high (n = 88, scores 8 to 9) categories. Age of patients ranged from 25 to 92 years old (median 64 years). The clinical follow-up ranged from 1 to 179 months (median 85 months). Out of 372 cases, 172 had a recurrence of the disease and 141 died from the disease.

Breast tumor tissue sections and TMAs were stained with an anti-SRAP antibody (cat # A300-743A, Bethyl Laboratories, Montgomery, TX, USA) using an automated tissue immunostainer (Discovery Staining Module, Ventana Medical Systems, Tucson, AZ, USA) at a dilution of 1:250, as described previously [25,26]. Slides were viewed and scored using standard light microscopy.

Quantification and SRAP staining cut-off selection
SRAP protein expression was assessed using a previously described semi-quantitative scoring consisting of an assessment of both staining intensity (scale 0 to 3) and the percentage of positive cells (0 to 100%), which, when multiplied, generate an H-score ranging from 0 to 300 [25,26]. TMA slides were independently scored by three investigators (GPS, CCP and YY). Average of values scored by the three investigators were calculated. There is no relevant clinical cut-off point reported for SRAP. The median H-score value of 76.67 was arbitrarily set as cut-point. Breast cancers were therefore considered high SRAP expressers when their average score exceeded 76.67 and low SRAP expressers when their H-score was lower than or equal to 76.67. Cohen kappa coefficient for the semi-quantitative H scores were 0.43, 0.50 and 0.65, indicating a moderate to substantial inter-rater agreement between the three readers [28].

Statistical analysis
Differences between SRAP expression in different subgroups (such as ER positive versus ER negative or node positive versus node negative, for example) were tested using the Mann-Whitney rank sum test, two sided. Box and whiskers representation was performed with boxes at 25 to 75% and whiskers at 10 to 90%. Potential distributions of low and high SRAP cases in other clinical-pathological variables were tested using contingency methods (Fisher’s exact test). Survival analyses were performed using the Mantel-Cox log-rank test to generate Kaplan-Meier curves. Breast Cancer Specific Survival (BCSS) was defined as the time from initial surgery to the date of death attributable to breast cancer only. Recurrence-free survival (RFS) was defined as the time from initial surgery to the date of clinically documented local or distant disease recurrence or death attributed to breast cancer. Deaths caused by other known diseases were censored. Statistical
analyses were carried out using GraphPad Prism 5 (GraphPad, San Diego, CA, USA) and PASW Statistics 17 (SPSS Inc. Chicago, IL, USA).

Cox proportional hazards model was used to test the statistical independence and significance of the different predictors on BCSS (events modeled: deaths attributable to breast cancer only) and RFS (events modeled consisting of recurrences or deaths attributable to breast cancer). The predictors considered in the model were: age at diagnosis (age ≤ median age 64, age > median age 64); ER-alpha status (ER ≤ 3 considered negative, ER > 3 are positive); node status (positive, negative); SRAP (low SRAP ≤ 76.67, high SRAP > 76.67); all two, three and four-way interactions involving SRAP.

Results

Anti-SRAP antibody validation

The ability of the 743A anti-SRAP antibody (Bethyl, Montgomery, TX, USA) to specifically recognize SRAP was first assessed by western blot analysis of total protein extracted from MCF-7 breast cancer cells, previously shown to express this protein [15-17]. A doublet, migrating at the expected apparent size of about 30 kDa, is specifically detected (Figure 1, left panel). As anticipated, the signal is decreased when the antibody is pre-incubated with an excess of corresponding blocking (743A + BP) but not unrelated (743A + Unr-P) peptide. The specificity of the signal recognized by 743A anti-SRAP antibody is further demonstrated by its decrease when cells are expressing SRA RNAi or by the appearance of an additional band in cells expressing a V5-Tagged-SRAP construct (Figure 1, right panel).

To further validate the use of 743A anti-SRAP antibody for in situ analyses, similar experiments were performed by immunofluorescence in Hela cells. As shown Figure 2, SRAP signal is detected in the nucleus and the cytoplasm of Hela cells (743A panels). As expected, this signal is decreased when the primary antibody is pre-incubated with the blocking peptide (743A + BP panels) or when cells expressed SRA RNAi (SRA-RNAi panel). Inversely, cells expressing exogenous V5-tagged-SRAP showed an increased signal detected with the 743A antibody (SRAP-V5 panels).

Differential SRAP expression in breast tissues

SRAP expression was subsequently assessed by western blot in a small series of 20 breast tumors and 6 normal breast samples as detailed in the Materials and Methods section. A signal, which varied in intensity between tumor samples and corresponding to SRAP of 30 kDa was detected in most cases (Figure 3, red arrow). Overall, this signal appears stronger in tumors than in normal tissues. It should however be emphasized that SRAP of 30 kDa is not detectable in all tumors (see Tumors 3 and 4) nor normal tissues (see Normal 1). Interestingly, two additional bands migrating at apparent sizes of 40 kDa (see Tumors 1, 9, 10) and 25 kDa (see Tumors 1, 2, 10) are also detected in some tumor cases but not in normal tissues. All these bands are specifically recognized by 743A anti-SRAP antibody, as shown by extinction of the signals in neutralization experiments (Figure 3, middle panel). It should also be noted, that in MCF-7 but not in Hela cells, overexposure of the blot led to the detection of the 40 kDa band. Immunohistochemical analysis performed on matched paraffin sections confirms that SRAP is detectable in epithelial cells within normal ducts (Figure 4a). A wide range of staining can be observed in breast cancers with some tumors showing a weak (Figures 4b, c) and others an intense staining (Figure 4d). The specificity of the staining is demonstrated by its decrease when the primary antibody is pre-incubated with the corresponding blocking peptide (Figures 4e, f) but not with a non-specific/irrelevant peptide (data not shown).
Tissue micro-array analysis of SRAP expression in 372 breast cancer cases

We have investigated SRAP expression in TMAs corresponding to a large cohort of breast cancer cases with different established clinical parameters. Staining and scoring were performed as described in the Materials and Methods section. Examples of staining and corresponding H-scores are shown in Figure 5. SRAP staining varies greatly from one sample to another, with H-scores ranging from 0 to 196.67 (n = 372, median = 76.67, average = 81.27). SRAP expression, ER/PR/node status, Nottingham grade [27], size of the tumor, patient age at surgery and clinical follow-up were available for 372 patients.

As illustrated in Figure 6a, significant (Mann-Whitney rank sum test, two-sided, $P < 0.0001$) H-score values are higher in ER-positive than in ER-negative tumors (ER positive, n = 271, median = 81.67 versus ER negative, n = 101, median = 58.33). Similarly, SRAP staining is significantly stronger in PR-positive than in PR-negative cases (PR positive, n = 256, median = 80 versus PR negative, n = 116, median = 66.67). Tumors with higher Nottingham grade had a significantly lower SRAP staining than both low and medium grade lesions (high grade, n = 88, median = 60 versus low grade, n = 79, median = 85 and medium grade, n = 205, median = 79.17). When cases were divided into two groups using the median age at surgery (median H-score 64), older patients had a significantly higher SRAP expression detected in their tumors than younger patients (older patients, n = 183, median = 80.83 versus younger patients, n = 189, median = 73.83). No difference in SRAP staining was observed between node-positive and node-negative tumors or between small and bigger lesions.

To further investigate potential non-random distributions of SRAP H-score staining in different tumor subgroups, we have arbitrarily divided the cohort into low and high SRAP...
expressors by selecting the median H-score value as cut-off point (low SRAP, H-score lower or equal to 76.67, n = 190; high SRAP, H-scores higher than 76.67, n = 182; Figure 6b).

Contingency table analyses showed that low SRAP cases were significantly (Fisher’s exact test) over-represented in ER-negative, PR-positive and lower grade tumors compared with ER-positive, PR-positive and higher grade tumors, respectively (Table 1). Similarly, a higher proportion of low SRAP expressors was found in lesions from younger patients. Identical proportions of low and high SRAP expressors were found in node-positive and node-negative tumors, and in small and large lesions.

Low SRAP expression is associated with longer survival among ER-positive, PR-positive and younger breast cancer patients

When analyzing the cohort as a whole and most of the case subgroups detailed in Table 1, no difference was found in BCSS between high and low SRAP expressors (data not shown). Interestingly, patients whose tumors were ER positive had a significantly longer BCSS and RFS (Figures 7a, b) when their primary tumor expressed lower levels of SRAP (Mantel-Cox, \( P = 0.0065 \) and \( P = 0.0212 \), respectively). When considering PR-positive cases, patients whose primary tumors expressed low SRAP also had a better BCSS and RFS (Figures 7a, b; Mantel-Cox, \( P = 0.0052 \) and \( P = 0.0041 \), respectively). In younger patients (age ≤ 64 years old, corresponding to values lower and equal to median age at surgery) low SRAP levels were similarly associated with a longer BCSS and RFS (Figures 7a, b; Mantel-Cox, \( P = 0.0117 \) and \( P = 0.002 \), respectively). No significant differences between low SRAP and high SRAP were found in the whole cohort or any other subgroups defined in Table 1.

Lower SRAP level is a significant predictor of survival in younger patients whose tumors are ER positive and node negative

In multivariate regression analyses of the whole cohort, age, ER status and nodal status were, as expected, associated with poor prognosis (Table 2). SRAP, however, did not act as a proportional predictor in either BCSS or RFS analysis. In the ER-negative group, neither SRAP nor any interaction was a predictor of survival. In the ER-positive group (n = 271), High SRAP was associated with a higher rate of death due to breast cancer (hazard ratio (HR) = 4.213, \( P = 0.007 \)) and higher rate of recurrence (HR = 3.392, \( P = 0.005 \)). In this subgroup, interaction between SRAP and age approached and reached significance, when considering BCSS and RFS, respectively (\( P = 0.060 \) and \( P = 0.030 \)). Looked at in isolation, the interaction becomes clear (Figure 8). Within the ER-positive subgroup, SRAP is as a poor prognostic marker for BCSS and RFS in the subset of node-negative and young breast cancer patients (n = 60, HR = 8.616, \( P = 0.006 \) and HR = 3.566, \( P = 0.011 \), respectively), but not in older or node positive patients (Figures 8a, b).

To further establish the different prognostic value of high SRAP levels in different age subgroups, patients with ER-positive/node-negative tumors (n = 130) have been grouped according to their age (corresponding to quartiles: 0 to 25%, 26 to 50%, 51 to 75%, 76 to 100%). Patients whose age fell in the first and second quartiles (24 to 55 and 56 to 64 years old), but not older patients, had a significant decrease in survival when their primary tumors expressed high SRAP levels (Figure 8c).
SRAP immunohistochemical detection in breast tissue sections. Steroid receptor RNA activator protein (SRAP) expression was assessed in (a) normal and (b to f) tumor paraffin-embedded tissue sections using 743A anti-SRAP antibody as described in the Materials and Methods section. (f) A serial section adjacent to section E has been treated with 743A pre-incubated with specific blocking peptide. Bar = 50 μm.
Figure 5

Tissue micro-array analysis of SRAP expression. Immunohistochemical detection of steroid receptor RNA activator protein (SRAP) was performed using 743A polyclonal anti-SRAP antibody on tissue micro-arrays corresponding to a large cohort of breast tumors. For each case, SRAP staining has been assessed and an H-score determined as detailed in the Material and Methods section. Examples of three cores with different H-scores are provided. Bar = 100 μm.
Discussion

In the present study, we have established that several SRAP-like peptides were detectable in breast tumor tissues. Interestingly, in ER positive, and more predominantly within node negative and younger patients, higher levels of the corresponding signal were associated with poor survival.

No data reporting specific detection of SRAP employing the antibody used in our experiments (cat # A300-743A) has been published. It was therefore critical to first validate the use of this antibody for western blot and immunohistochemistry analyses. A doublet migrating at the apparent size of about 30 kDa is detected by western blot in MCF-7 as well as in multiple other cell lines (Figure 1 and data not shown). This size corresponds to the size previously observed using different SRAP antibodies [15,17,19]. The signal decreases upon peptide neutralization and during SRA RNAi experiments. An additional band, also recognized by anti-V5 antibody (data not shown), appears when cells are transfected with a construct encoding an exogenous V5-tagged SRAP. Altogether, these observations strongly suggest that 743A antibody specifically recognizes SRAP.

The endogenous SRAP signal is detected mainly in the cytoplasm of cancer cells in vitro. In some cases, a speckled staining can also be seen in the nucleus. This is in agreement with both localizations previously observed for exogenous tagged SRAP [16]. This also corroborates data from Jung and colleagues [21], who co-immunoprecipitated endogenous SRAP from both nuclear and cytoplasmic protein extracts from Hela cells [10,21].

Figure 6

Distribution of SRAP H-scores in 372 breast cancer cases. (a) Box and whiskers representation showing the distribution of steroid receptor RNA activator protein (SRAP) H-scores in the whole cohort (Total) and in different subgroups. For each group the median is depicted by the thick horizontal bar, the box delineates 25th to 75th percentiles and the whiskers 10 to 90 percentiles. (b) Frequency distribution of SRAP H-scores in the whole cohort (Total). Median H-score of 76.67 delineates Low SRAP and a High SRAP subgroups.
Western blot analysis of breast tumors revealed the presence in some tumors of two additional bands migrating at an apparent size of about 25 kDa and about 40 kDa. Both signals disappear when the antibody is pre-incubated with the corresponding blocking peptide. This suggests that as well as the 30 kDa SRAP-like proteins, these proteins are also specifically recognized by 743A antibody. For example, Ensembl transcript [Ensembl: ENSESTT00000035630] corresponding to the last 162 amino acids of SRAP and with a predicted molecular mass of 18 kDa. As well, other alternative splicing events, such as intron-1 retention conjugated with exon-3 deletion [17], predicts a 220 amino acid residue protein (molecular weight 24.68 kDa) identical to SRAP in its N- and C-terminal extremity but differing in the central sequence.

Further studies are urgently needed to establish the nature of the different splicing events potentially involved in the generation of the different SRA transcripts and their potential protein products. This is critical if we consider that SRA was originally described as a functional RNA whose activity depended on the integrity of the core sequence determined by exon-2 to exon-5 [29]. It has recently been shown that the balance between fully spliced SRA and transcripts still containing intron-1 varied between breast tumors and characterized particular tumor subgroups [30]. Interestingly, alteration of this balance led to a change in breast cancer cell growth [30]. Taken together with size differences between SRA transcripts detectable by Northern blot in breast cancer cells and cells from other origins [7], this suggests that alternative splicing events, leading to different SRA RNAs and potentially to different SRAP-like proteins, might participate in the development of different breast cancer cell phenotypes.

Alternative post-translational modifications might also be responsible for the apparent differences in SRAP molecular mass. For example, a lysine residue at position 21 of the SRAP human sequence is conserved in all vertebrates and is predicted to be sumoylated (data not shown). Sumoylation of this residue could potentially participate to the shift in migration from 30 kDa to 40 kDa [31,32]. Further studies are warranted to address this possibility.

Although only six normal breast cases were analyzed, they all expressed only 30 kDa SRAP at a level lower than that seen in most tumors. This is in agreement with the previous observation that SRA RNA is overall expressed at lower levels in normal than in tumor cells [14]. This also raises the possibility that mechanisms responsible for the generation of the 25 kDa and 40 kDa SRAP-like peptides are less frequent in normal than in tumor cells.

Using 743A antibody, significant higher levels of SRAP are found in ER-positive tumors, in PR-positive tumors, and in lower grade tumors. This fits with the pattern of expression of a known activator of steroid receptor, the steroid receptor co-activator 1 (SRC1). Indeed, high SRC1 protein levels have recently been shown to positively correlate with ER, PR levels and low grade in a large cohort of 426 breast cancer patients [33]. In their study, Green and colleagues reported that patients whose tumors expressed higher SRC1 levels had an
Low SRAP is an indicator of better survival for younger patients and patients with ER positive or PR positive tumors. Kaplan-Meier breast cancer-specific (a) death survival and (b) recurrence-free survival plots are shown for patients with ER+ tumors (n = 271), PR+ tumors (n = 256), and for patients younger than 65 years old (n = 189). P values correspond to Mantel-Cox log-rank test. ER = estrogen receptor; PR = progesterone receptor; SRAP = steroid receptor RNA activator protein; + = positive; - = negative.
SRAP as a poor prognostic indicator in younger patients whose tumors are ER positive and node negative. Cox multivariate analyses have been performed as described in the Materials and Methods section. The events modeled were (a) death attributable to breast cancer, or (b) recurrence or death attributed to breast cancer. Hazard ratios within different estrogen receptor positive (ER+) subgroups are shown as squares and 95% confidence intervals as error bars. (c) Kaplan-Meier breast cancer specific death survival (BCSS) plots are shown for patients with ER+/node negative tumors (n = 130). Panels correspond to the four different age quartiles: 0 to 25%, age 25 to 55 years old patients; 26 to 50%, age 56 to 64 years old patients; 51 to 75%, age 65 to 71 years old; 76 to 100%, age 72 to 82 years old patients. P values correspond to Mantel-Cox log-rank test. SRAP = steroid receptor RNA activator protein.
overall longer survival and a lower recurrence rate [33]. In contrast, we found that high SRAP expression is a strong indicator of poor survival in ER-positive patients. It should be noted that a similar finding was obtained when using a training set, validation set method and X-tile software [34] (data not shown).

All 271 women whose tumors were ER positive had been treated with tamoxifen following surgery. Further analyses are needed to establish whether SRAP should be considered as a prognostic factor, a predictive factor, or both.

Interestingly, the potential indicator power of SRAP appears limited to younger patients whose tumors are ER positive and node negative. It should be stressed that the age cut-point of 64 years (corresponding to median age at time of surgery) has been chosen to divide the cohorts in subgroups with comparable number of cases. A cut-point of 51 years, average age for spontaneous menopause [35,36], would have been more informative to establish the potential link between menopausal status and SRAP prognostic value. The number of cases younger than 51 was, however, too low to perform meaningful statistical analyses. For example, only 21 out of the 271 ER-positive patients were younger than 51 years. Menopause age follows a Gaussian distribution ranging roughly from 40 to 60 years [35,36]. As patients’ menopausal status is not registered in the Manitoba Breast Tumor Database, women younger than 51 years could also potentially be menopausal. It is therefore not possible to extrapolate whether SRAP prognostic value is directly linked to menopausal status. Further studies, performed on a cohort consisting of comparable numbers of known pre- and post-menopausal women should be initiated to clarify this issue.

The particular group of younger patients identified in this cohort (i.e ER positive, node negative) usually benefits from better prognosis and is considered as having an increased likelihood of responding to tamoxifen therapy. Our data, however, suggest that among this group, women with higher levels of SRAP might need additional treatment compared with women with lower SRAP levels.

We have previously reported that the detection in breast tumor extracts of 30 kDa SRAP by western blot, but not 25 kDa SRAP, might correspond to a better outcome for ER-positive/node-negative patients [17]. There is therefore an apparent disagreement with the present TMA results that show that higher SRAP is an indicator of shorter survival in ER-positive/node-negative patients. This apparent divergence could be due a variety of reasons. It may potentially result from differences in the cohorts studied. Our previous cohort was indeed smaller (n = 74 versus n = 130), the median ER levels was higher (45.5 fmol/mg versus 37 fmol/mg), the number of

---

**Table 2**

<table>
<thead>
<tr>
<th>Cases</th>
<th>Predictor</th>
<th>Haz Rat</th>
<th>95% CI</th>
<th>P</th>
<th>Haz Rat</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Breast cancer specific survival</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Whole cohort n = 372</td>
<td>SRAP</td>
<td>1.580</td>
<td>0.35-7.01</td>
<td>0.547</td>
<td>0.356</td>
<td>0.29-5.63</td>
<td>0.729</td>
</tr>
<tr>
<td>n = 372</td>
<td>Age</td>
<td>2.576</td>
<td>1.50-4.40</td>
<td>&lt;0.001</td>
<td>1.507</td>
<td>1.39-3.60</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>n = 372</td>
<td>ER</td>
<td>0.213</td>
<td>0.12-0.36</td>
<td>&lt;0.001</td>
<td>0.124</td>
<td>0.24-0.64</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>n = 372</td>
<td>Node</td>
<td>3.214</td>
<td>1.8-5.61</td>
<td>&lt;0.001</td>
<td>1.84</td>
<td>1.67-4.39</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td><strong>ER- n = 101</strong></td>
<td>SRAP</td>
<td>1.489</td>
<td>0.31-6.99</td>
<td>0.614</td>
<td>1.387</td>
<td>0.29-6.43</td>
<td>0.676</td>
</tr>
<tr>
<td>Age</td>
<td>2.556</td>
<td>1.30-5.00</td>
<td>0.006</td>
<td>2.515</td>
<td>1.29-4.88</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Node</td>
<td>2.788</td>
<td>1.33-5.82</td>
<td>0.006</td>
<td>2.547</td>
<td>1.25-5.18</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>SRAP × age</td>
<td>0.216</td>
<td>0.01-2.61</td>
<td>0.228</td>
<td>0.231</td>
<td>0.01-2.79</td>
<td>0.249</td>
<td></td>
</tr>
<tr>
<td>SRAP × node</td>
<td>1.421</td>
<td>0.31-6.99</td>
<td>0.688</td>
<td>1.422</td>
<td>0.29-6.43</td>
<td>0.686</td>
<td></td>
</tr>
<tr>
<td><strong>ER+ n = 271</strong></td>
<td>SRAP</td>
<td>4.213</td>
<td>1.47-12.0</td>
<td>0.007</td>
<td>3.392</td>
<td>1.46-7.87</td>
<td>0.005</td>
</tr>
<tr>
<td>Age</td>
<td>1.637</td>
<td>0.75-3.54</td>
<td>0.210</td>
<td>1.656</td>
<td>0.88-3.09</td>
<td>0.113</td>
<td></td>
</tr>
<tr>
<td>Node</td>
<td>3.466</td>
<td>1.45-8.26</td>
<td>0.005</td>
<td>2.721</td>
<td>1.40-5.27</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>SRAP × age</td>
<td>0.316</td>
<td>0.09-1.05</td>
<td>0.060</td>
<td>0.337</td>
<td>0.12-0.89</td>
<td>0.030</td>
<td></td>
</tr>
<tr>
<td>SRAP × node</td>
<td>0.474</td>
<td>1.47-12.0</td>
<td>0.216</td>
<td>0.539</td>
<td>1.46-7.87</td>
<td>0.210</td>
<td></td>
</tr>
</tbody>
</table>

CI = confidence interval; ER = estrogen receptor; Haz Rat = hazard ratio; SRAP = steroid receptor RNA activator protein; + = positive; - = negative.

*P* values correspond to cox regression model. Significant *P* values are bolded.
events was different (n = 7 deaths versus n = 26) and patients’ ages were not available. Further, in the previous western blot analysis, which was performed as outlined earlier with an antibody recognizing the N-terminal region of SRAP, 30 kDa SRAP was detected in only 24 of 74 cases [17]. Using 743A, we have in the present study detected SRAP signal in all but 2 out of 130 ER-positive/node-negative patients. It should also be stressed that, as mentioned above, SRAP staining assayed in the present TMAs corresponds to multiple SRAP-like peptides (25 kDa, 30 kDa and 40 kDa SRAP) whereas only the 30 kDa SRAP was considered in the first study. This also raises the possibility that different peptides might have different prognostic or predictive values.

Conclusions
Altogether, we have found that several SRAP-like peptides are expressed in breast tumors and that their detection by immunohistochemistry could be used as a new prognostic/predictive marker in younger patients with ER-positive/node-negative breast cancer. Additional studies, performed with other antibodies are warranted to confirm this observation and further establish whether specific SRAP-like peptides have additional predictor values.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
YY and GPS equally contributed to this work by performing antibody validation and TMA. CP scored the slides. ZN performed the statistical analysis. SC-K, CC and AB performed western blot analysis and immunofluorescent experiments. PHW, YM, LCM and EL conceived, designed and coordinated the study. All authors drafted, criticized and approved the manuscript.

Acknowledgements
This work was supported by grants from the Canadian Institute of Health Research (CIHR), the CancerCare Manitoba Foundation (CCMF), the Manitoba Health Research Council (MHRC) and the Canadian Breast Cancer Foundation (CBCF). YY received a MHRC Graduate Student Research (CIHR), the CancerCare Manitoba Foundation (CCMF), the Cancer Foundation (CBCF). YY received a MHRC Graduate Student Research (CIHR), the CancerCare Manitoba Foundation (CCMF), the Cancer Foundation (CBCF).

References


Conclusions of Chapter III

1. I validated the use of anti-SRAP antibody 743A (recognizing the C-terminal region of SRAP) to investigate SRAP expression in breast cancer cell line and breast tumor samples. The endogenous SRAP signal is detected mainly in the cytoplasm of cancer cells in vitro. In some cases, a speckled staining can also be seen in the nucleus. Interestingly, Western blot analyses revealed higher expression levels of SRAP detected in breast tumour samples compared to normal mammary gland sample. Three immuno-reactive bands migrating at ~25 kDa, ~30kDa, and ~40 kDa were seen in mulitple tumour samples whereas only one ~30kDa band was seen in normal mammary samples.

2. Tissue-microarray (TMA) analysis of a cohort of 372 breast tumors from the Manitoba Breast Tumor Bank (MBTB) revealed that expression of SRAP was significantly elevated in ER, PR positive and node positive tumors and in tumors of patients aged >64.

3. When considering ER positive tumors, the higher expression of SRAP was associated with a significantly (Mantel-Cox test, P < 0.05) worse breast cancer specific survival (BCSS) than those with lower SRAP expression.

4. In a sub-group of younger patient with estrogen receptor positive, node negative and age less than 64, SRAP appeared as a very powerful indicator of poor prognostic for breast cancer specific survival (BCSS). This subgroup usually benefits from better prognosis and is considered as having an increased likelihood of responding to tamoxifen therapy. Our data, however, suggest that among this particular group, women with higher levels of SRAP might need additional treatment compared with women with lower SRAP levels.
CHAPTER IV Steroid receptor RNA activator protein (SRAP) expression as a prognostic factor in ER+ human breast tumours

As discussed in Chapter III, using tissue microarray, the global expression of SRAP like peptides was found associated with poor breast cancer survival in ER-α positive patients. Paradoxically, we have previously reported that the detection in breast tumour samples of 30 kDa SRAP by Western blotting may be associated with a better clinical outcome for ER-α-positive/ node-negative patients [148]. These apparent conflicting observations may result from the use of antibodies recognizing different epitopes and/or different tumour cohorts used. In this chapter. We first investigated the suitability of two anti-SRAP antibodies targeting the N- and C- terminal extremity, respectively, for their use in Western blot and IHC [Fig IV-1].

![Figure IV-1. Schematic profile of SRAP antibodies used.](image)
The two evolutionary conserved domains, CD1 and CD2, in are highlighted in green and pink color. Proline-rich area is also labelled in yellow. Different SRAP epitopes are recognized by the commercially available anti-SRAP rabbit polyclonal antibodies from Bethyl Laboratories A300-742A (742) targeting at N-terminus and A300-743A (743) targeting at C-terminus.

Western blot analyses of breast tumour tissues revealed the differential detection of SRAP-like peptides by these two antibodies. Even though the two antibodies differentially recognize SRAP-like peptides, individual Tissue micro-array (TMA) results are consistent with each other. They both show that lower expression of SRAP peptides is associated with a better outcome in estrogen receptor positive cases. When both scores are combined, the benefit of expressing low SRAP level is even more apparent. Results were published
on Aug 2, 2013 in *J Cancer Res Clin Oncol*, 139(10):1637-47 entitled “Steroid Receptor RNA Activator Protein (SRAP) expression as a prognostic factor in ER+ human Breast Tumour”. This article further confirmed the results we obtained in Chapter III. However, the potential predictive value of individual SRAP peptides was not addressed in these two articles.

**My contributions to this article**

I contributed to this work by performing antibody validation and TMA. I also scored the TMA slides and performed the statistical analysis. I drafted the manuscript.
Steroid receptor RNA activator protein (SRAP) expression as a prognostic factor in ER+ human breast tumors

Yi Yan · Carla C. Penner · George P. Skliris · Charlton Cooper · Zoann Nugent · Anne Blanchard · Mohammad K. Hamedani · Xuemei Wang · Yvonne Myal · Leigh C. Murphy · Etienne Leygue

Received: 14 July 2013 / Accepted: 22 July 2013 © Springer-Verlag Berlin Heidelberg 2013

Abstract

Background The steroid receptor RNA activator protein (SRAP) is a newly described protein modulating the activity of multiple transcription factors including the estrogen receptor (ER). We have recently reported the immunodetection by Western blot of multiple SRAP peptides in breast tissue. High expression of these peptides, assessed by tissue micro-array (TMA) analysis, was associated with poor prognosis in patients whose primary tumors were ER positive (ER+). In such studies, it is recognized that intensity as well as specificity of the signal detected directly depends upon the antibody used as well as the position of the epitope recognized. To confirm the potential relevance of SRAP as a new prognostic factor, it is critical to establish whether similar results are obtained with independent antibodies.

Methods Two commercial anti-SRAP antibodies (742A and 743A), respectively, recognizing the N- and C-terminal extremity of the protein, were first used to analyze by Western blot SRAP expression in protein extracts from frozen breast tumor tissue sections. These antibodies were further used to investigate by immunohistochemistry (IHC) SRAP location in paraffin-embedded breast tumors. Comparative TMA analysis of 170 ER+ tumors was eventually performed in order to establish the potential associations existing between SRAP expression and clinical outcome.

Results Multiple SRAP peptides were differentially detected by Western blot. Both antibodies led to similar nuclear and cytoplasmic staining in breast tissue section. A solid correlation was found (Spearman $r = 0.46$, Electronic supplementary material The online version of this article (doi:10.1007/s00432-013-1485-2) contains supplementary material, which is available to authorized users.

Y. Yan · C. C. Penner · G. P. Skliris · C. Cooper · Z. Nugent · A. Blanchard · M. K. Hamedani · X. Wang · Y. Myal · L. C. Murphy · E. Leygue
Manitoba Institute of Cell Biology, 675 McDermot Ave., Winnipeg, MB R3E0V9, Canada
e-mail: umyan9@cc.umanitoba.ca

C. C. Penner
e-mail: crpenner@exchange.hsc.mb.ca

G. P. Skliris
e-mail: skliris@cc.umanitoba.ca

C. Cooper
e-mail: charlton_cooper@hotmail.com

Z. Nugent
e-mail: Zoann.Nugent@cancercare.mb.ca

A. Blanchard
e-mail: ablanch@cc.umanitoba.ca

M. K. Hamedani
e-mail: mkhamedani@gmail.com

Published online: 02 August 2013
P < 0.001) between 742A and 743A IHC scores. Results from both antibodies independently showed that dividing expression levels into lower 25 percentile, 26–75 percentile, and highest 25 percentile demonstrated a hazard ratio (HR) of 1.82 (P = 0.0042) for 742A antibody and 1.35 (P = 0.14) for 743A antibody. When both scores are combined, double high expressor (by 742A and 743A) was associated with a poor prognosis of breast-cancer-specific survival (Mantel–Cox: P = 0.005, HR = 2.24).

**Conclusion** Overall, our data suggest the existence in breast tumor tissue of multiple SRAP-like peptides. Assessing their expression in primary breast tumors can predict clinical outcome in ER+ breast cancer patients.

**Keywords** SRAP · ER+ · Prognostic marker · Breast cancer

**Background**

Breast cancer remains one of the most frequently diagnosed cancers with an estimated 1,000,000 new cases detected each year worldwide. This year, approximately 250,000 American women will be diagnosed with breast cancer and 40,000 will succumb to this disease (American cancer society 2010). Since 1988, breast cancer incidence rates have risen by 10 %. Despite such striking statistics, breast-cancer-related death rates have dropped by 20 %, as continuing research has led to both earlier detection and increased treatment options for patients.

Breast cancer is an extremely heterogeneous disease, both at the cellular and at the molecular level (Perou et al. 2000). Consequently, breast cancer patient with apparent identical clinical presentations often does not uniformly respond to a given treatment. Currently, only a handful of markers, including estrogen receptor (ER) status, progesterone receptor (PR) status, and Her2 levels are used by clinicians to narrow down prognoses and decide upon specific treatment options (Ross et al. 2003). The presence of ER in the primary tumor, for example, initiates a treatment option with tamoxifen (a competitive inhibitor of estrogen) or aromatase inhibitors (inhibiting estrogen synthesis). The presence of Her2, on the other side, can prompt a treatment with trastuzumab (humanized anti-Her2 antibody). Although thousands of women are saved with such treatments, many become resistant in ER+ (Ring and Dowsett 2004) as well as Her2+ patients (Pohlmann et al. 2009).

Technological advances, including cDNA and TMAs, have allowed the characterization of gene expression in hundreds of breast tumors and the establishment of a new breast cancer classification (Abd El-Rehim et al. 2005; Perou 2002). Breast tumors are now divided into four main subtypes: luminal A (ER+ good prognosis), luminal B (ER+ poor prognosis), HER2+ (Her2+, ER−), and basal like (ER−, PR−, Her2−) (Cheang et al. 2009; Hu et al. 2006; Sorlie et al. 2001, 2003). Compared to luminal A subtype, luminal B tumors are characterized by an increased proliferation status (Cheang et al. 2009). Curiously, few immunohistochemical approaches including the characterization of Her2 and KI67 (an indicator of proliferation) expressions currently allow the distinction between good (luminal A) and poor prognosis ER+ (luminal B) patients (Cheang et al. 2009).

The Steroid receptor RNA activator gene, SRA1, has the peculiar property (Colley and Leedman 2011; Cooper et al. 2011) to encode both a non-coding functional RNA (SRA) and a protein (SRAP). If it now clear that SRA can act as a positive or negative transcriptional regulator (Colley and Leedman 2011; Cooper et al. 2011; Vicent et al. 2013), less is known regarding the function of SRAP. It has however been demonstrated that SRAP was able to modulate ER activity (Borth et al. 2010; Chooniedass-Kothari et al. 2010) and potentially the transcriptional activity of multiple other transcription factors (Chooniedass-Kothari et al. 2010). It has also been shown that abolishing SRA expression in MDA-MB-231 breast cancer cells decreased their invasive properties, indicating the involvement of either SRA RNA, SRA protein, or both, in breast cancer invasion (Foulds et al. 2010). Western blot analyses of breast tumors has originally shown that a high expression of the 30-kDa protein, detected by an antibody targeting the N-terminal extremity, was associated with good breast cancer survival (Chooniedass-Kothari et al. 2006). Interestingly, similar analyses performed using an antibody targeting SRAP C-terminus extremity revealed the presence, beside the expected 30-kDa protein, of multiple other peptides in different tumor samples (Yan et al. 2009). Using TMA, the global expression of these peptides was found associated with poor breast cancer survival in ER+ but not in ER− tumor cases (Yan et al. 2009). Interestingly, we have recently observed that SRAP expression could predict response to tamoxifen treatment in premenopausal patients with estrogen receptor-alpha negative early breast cancers (Yan et al. 2013). These apparent conflicting observations could result from the use of antibodies recognizing different epitopes and/or different tumor cohorts used. In order to establish the potential use of SRAP or its related peptides as a new prognostic marker, it is critical to compare SRAP expression patterns using different antibodies as well as to establish associations with clinical outcome in the same subset of breast tumors.

In the present study, we have validated two anti-SRAP antibodies targeting the N- and C-terminal extremity, respectively, for their use in Western blot and IHC. Western blot analyses of breast tumor tissues revealed the differential detection of SRAP-like peptides by these two
antibodies. To further assess their potential of these SRAP-like peptides as prognostic marker, we performed TMA analysis on a selected cohort of 170 ER+ breast cancer cases using both anti-SRAP antibodies.

**Methods**

**Cell culture**

MCF-7 breast cells (Cedarlane Laboratories Ltd., Burlington) were cultured as previously described (Yan et al. 2009).

**Western blot**

Total proteins were extracted from cells pellets or frozen breast tumor sections, and Western blot performed as previously outlined (Yan et al. 2009). Briefly, samples containing 75 μg of total protein were analyzed by Western blot using anti-SRAP antibodies (cat # A300-742A; cat # A300-743A; Bethyl Laboratories, Montgomery, TX, USA), respectively, raised against the N-terminal extremity (peptide 50–100 aa) or C-terminal (peptide 180–237 aa) of SRAP (Fig. 1a). For neutralization experiments, 2 μg of blocking peptide (cat # BP300-742 or cat # BP300-743; Bethyl Laboratories, Montgomery, TX, USA) was preincubated without (no BP) or with peptides (+BP742 or +BP743) as indicated.

**Fig. 1** Immunodetection of SRAP-related peptide in breast cancer cells. 

**A** Schematic representation of human SRAP sequence showing sites recognized by 742A and 743A antibodies, amino acids 50–100 and 180–237, respectively.

**B** Total proteins, extracted from MCF-7 breast cancer cells, were analyzed by Western blot using both antibodies as described in the “Methods” section. Antibodies have been preincubated without (no BP) or with peptides (+BP742 or +BP743) as indicated. 

**C** MCF-7 breast cancer cells were analyzed by IF using both antibodies with control or blocking peptide as described in the “Methods” section. Bar 20 μM
preincubated with 1 µg of antibody (A300-742A or cat # A300-743A) for 2 h at room temperature. For fractionation experiment, nuclear and cytoplasmic extracts from MCF7 cells were separated using PARIS™ Kit (cat # AM1921; Ambion) according to the manufacturer’s instruction. The samples were analyzed by Western blot using anti-SRAP antibodies (A300-742A or cat # A300-743A; Bethyl Laboratories), and SP3 antibody (cat # sc-644; Santa Cruz Biotechnology).

Immunofluorescence

Immunofluorescence experiments were performed on MCF7 cell as previously described (Yan et al. 2009). Briefly, fixed cells were incubated 1 h at 37 °C with 743A or 742A SRAP antibodies diluted at 1/200 or 1/100 in 3 % BSA/PBS. For the neutralization experiment, antibodies were preincubated with excess corresponding or control blocking peptide for 2 h at room temperature. Cell nuclei were stained with 1 µg/ml Hoechst (Invitrogen). Fluorescent images were captured and visualized with a Nikon Eclipse E1000 epifluorescent microscope equipped with filters at emission wavelengths of 552–620 nm (CY3) and 440–450 nm (Hoechst) using ACT-1 software (Nikon).

Breast samples and tissue micro-arrays (TMAs)

All invasive breast cancer and normal breast samples used in the current study were obtained from the Manitoba Breast Tumor Bank (MBTB), which operates with the approval of the Faculty of Medicine, University of Manitoba, Research Ethics Board (Yan et al. 2009). The research reported in this manuscript has been performed with the approval of the Bannatyne Campus, University of Manitoba, Research Ethics Boards. Tissue collection and selection of samples has been reported before (Skliris et al. 2006; Yan et al. 2009). Matched frozen and paraffin-embedded sections corresponding to 6 invasive breast tumors were selected and evaluated for protein expression by Western blot and immunohistochemistry by both antibodies.

Tissue micro-arrays corresponding to 450 ER+ cases were constructed from primary invasive breast carcinomas, as described before (Yan et al. 2009). For each tumor, two core tissue samples (0.6 mm diameter) were taken from areas selected as containing a maximum of cancer cells. TMAs were stained with two antibodies (cat # A300-743A and cat # A300-742A) using an automated tissue immunostainer (Discovery Staining Module, Ventana Medical Systems, AZ, USA) at a dilution of 1:250 or 1:100, as described previously (Yan et al. 2009). Slides were viewed using standard light microscopy by two different investigators including one pathologist (C.C.P). Cases with lost follow-ups, unknown grade or nodal status, or data on SRAP expression using either antibody (loss of a core during processing) were removed. All characteristics were ultimately available for 170 ER+ breast cases (Table 1).

Following surgery, all corresponding patients had been treated with tamoxifen. None of the patients in the cohort received chemotherapy; however, 37 % (63 out of 170) received adjuvant radiation therapy. The cohort had ER levels ranging from 3.2 to 331 fmol/mg protein (median

<table>
<thead>
<tr>
<th>Predictors Value</th>
<th>N</th>
<th>% of sample</th>
<th>HR</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgR</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≤10</td>
<td>143</td>
<td>84</td>
<td>0.80</td>
<td>0.37–1.71</td>
<td>0.56</td>
</tr>
<tr>
<td>≤10</td>
<td>27</td>
<td>16</td>
<td>2.44</td>
<td>1.36–4.38</td>
<td>0.0028</td>
</tr>
<tr>
<td>Node Negative</td>
<td>97</td>
<td>57</td>
<td>1.79</td>
<td>1.01–3.18</td>
<td>0.046</td>
</tr>
<tr>
<td>Age* &lt;66</td>
<td>79</td>
<td>46</td>
<td>1.41</td>
<td>0.79–2.53</td>
<td>0.25</td>
</tr>
<tr>
<td>Node Positive</td>
<td>73</td>
<td>43</td>
<td>1.41</td>
<td>0.79–2.53</td>
<td>0.25</td>
</tr>
<tr>
<td>Node Negative</td>
<td>91</td>
<td>54</td>
<td>1.41</td>
<td>0.79–2.53</td>
<td>0.25</td>
</tr>
<tr>
<td>Age* 66+</td>
<td>79</td>
<td>46</td>
<td>1.41</td>
<td>0.79–2.53</td>
<td>0.25</td>
</tr>
<tr>
<td>742A Lowest 25 %</td>
<td>45</td>
<td>26</td>
<td>1.82</td>
<td>1.21–2.74</td>
<td>0.0042</td>
</tr>
<tr>
<td>742A Middle 50 %</td>
<td>87</td>
<td>51</td>
<td>1.35</td>
<td>0.91–2.01</td>
<td>0.14</td>
</tr>
<tr>
<td>742A Highest 25 %</td>
<td>38</td>
<td>22</td>
<td>1.35</td>
<td>0.91–2.01</td>
<td>0.14</td>
</tr>
<tr>
<td>743A Lowest 25 %</td>
<td>42</td>
<td>25</td>
<td>1.35</td>
<td>0.91–2.01</td>
<td>0.14</td>
</tr>
<tr>
<td>743A Middle 50 %</td>
<td>85</td>
<td>50</td>
<td>1.35</td>
<td>0.91–2.01</td>
<td>0.14</td>
</tr>
<tr>
<td>743A Highest 25 %</td>
<td>43</td>
<td>25</td>
<td>1.35</td>
<td>0.91–2.01</td>
<td>0.14</td>
</tr>
<tr>
<td>Group Both low</td>
<td>22</td>
<td>13</td>
<td>2.24</td>
<td>1.28–3.92</td>
<td>0.0050</td>
</tr>
<tr>
<td>Group Mixed</td>
<td>129</td>
<td>76</td>
<td>2.24</td>
<td>1.28–3.92</td>
<td>0.0050</td>
</tr>
<tr>
<td>Group Both high</td>
<td>19</td>
<td>11</td>
<td>2.24</td>
<td>1.28–3.92</td>
<td>0.0050</td>
</tr>
</tbody>
</table>

* The median value of age in this cohort 66 years old has been used as cutoff.
44.5 fmol/mg protein) and spanned a wide range of PR levels (1.3–659 fmol/mg protein, median 29 fmol/mg protein). Nottingham grade was also known for all 170 tumors, which were assigned to low (n = 38, scores 3–5), moderate (n = 114, scores 6–7), or high (n = 18, scores 8–9) categories. Age of patients ranged from 37 to 92 years old (median 67). The clinical follow-up ranged from 22 to 179 months (median 99 months). Out of 170 cases, 71 had a recurrence of the disease and 47 died from the disease.

Quantification and SRAP staining cutoff selection

Steroid receptor RNA activator protein expression was assessed using a previously described semi-quantitative scoring consisting in an assessment of both staining intensity (scale 0–3) and the percentage of positive cells (0–100 %), which, when multiplied, generate a IHC score ranging from 0 to 300 (Skliris et al. 2006; Yan et al. 2009). Both cytoplasmic and nuclear signals have been taken into account. TMA slides were independently scored by two investigators (C.C.P and Y.Y). Average of values scored by the two investigators was calculated. IHC scores of 742A antibody ranged from 10 to 232.5 with median value of 135, and IHC scores of 743A antibody ranged from 7.5 to 175 with median value of 76.25. The expression of SRAP was defined as low expression (0–25 percentile), medium expression (26–75 percentile), and high expression (76–100 percentiles) for each antibody.

Statistical analysis

Correlation of SRAP expression between the two antibodies was tested using a two-sided Spearman’s test. Survival analyses were performed using the Mantel–Cox log-rank test to generate Kaplan–Meier curves. Breast-cancer-specific survival (BCSS) was defined as the time from initial surgery to the date of death attributable to breast cancer only. Deaths caused by other known diseases were censored.

Cox regression models with the proportional hazard assumption were used to test the statistical independence and significance of the different predictors on BCSS (events modeled: deaths attributable to breast cancer only) via the PHREG procedure in SASTM. The predictors considered in the model were the following: age at diagnosis (<66, ≥66); progesterone receptor status (PgR ≤10 considered negative, PgR >10 are positive); node status (positive, negative); SRAP-742A (low SRAP ≤87.5, medium 90–175, and high SRAP ≥180); and SRAP-743A (low SRAP ≤60, medium 62.5–100, and high SRAP ≥105).

Results

742A anti-SRAP antibody validation

Total protein extracts from MCF7 cells were used to first assess the ability of 742A anti-SRAP antibody to specifically recognize SRAP protein, as described in the “Methods” section. For a control, the previously validated 743A antibody that targets a C-terminal SRAP epitope was used in parallel (Yan et al. 2009). As expected, a doublet migrating at the previously observed size of ~30 kD is detected using both antibodies, and the signal from 742A is substantially diminished when the antibody is preincubated with an excess of corresponding blocking peptide (BP742) but not C-terminal blocking peptide (BP743) (Fig. 1b). Inversely and as previously observed, the band detected by 743A is neutralized by BP743 blocking peptide but not BP742 blocking peptide. To further validate the use of two anti-SRAP antibody for in situ analyses, similar experiments were performed using immunofluorescence detection of SRAP on fixed cells. As shown in Fig. 1c, similar SRAP staining patterns are detected in the nucleus and the cytoplasm of MCF7 cells when using both antibodies. As expected, the signals are decreased when the primary antibodies are preincubated with the corresponding blocking peptide but not with the peptide corresponding to the other region. The dual localization of SRAP ~30 kDa in the nucleus and cytoplasm has been confirmed by analyzing independent nuclear and cytoplasmic protein extracts (supplementary Fig. 1).

Differential detection of SRAP-like peptides using 742A and 743A antibodies by Western blot

We have previously reported the differential detection, using 743A antibody, of several SRAP-like peptides in breast tumor samples (Yan et al. 2009). Total protein extracted from six selected frozen breast tumor cases was analyzed in parallel by Western blot using both 742A and 743A antibodies (Fig. 2a). Using 742A, a signal corresponding to SRAP ~30 kDa, varying in intensity, was detected in most cases except in T2 tumor (Fig. 2a). Additional bands migrating at apparent sizes of 26 kDa are also detected in all tumor cases as well as control uterus cell line HeLa. A ~48 kDa is seen at weak level in T1 and strong level in HeLa cells. Using 743A antibody, a signal corresponding to SRAP ~30 kDa was detected in most tumors (except T2) at similar intensity level as that seen with the 742A antibody. Additional bands migrating at apparent sizes of 40 kDa (see Tumors 3, 4, 5); 48 kDa (see Tumor 1); and ~25 kDa (see Tumors 2, 3) are also
detected. All these bands are specifically recognized as shown by extinction of the signals in neutralization experiments.

In tumor T1, signal from 30 and ~26 kDa bands detected by 742A as well as 30 and ~48 kDa bands detected by 743A appears to localize in both nucleus and...
cytoplasm (Fig. 2b). In tumor T3, signal from 30 and 
~26 kDa bands detected by 742A as well as 30 and 
~40 kDa bands detected by 743A is also localized in 
both nucleus and cytoplasm. These suggest that overall 
SRAP-like peptides can be observed in both cellular 
components.

TMA analysis of ER-positive cases using 742A 
and 743A antibodies

Steroid receptor RNA activator protein expression was 
subsequently assessed via IHC analysis on TMAs con-
taining solely ER+ breast cancer cases with different 
established clinical parameters. Staining and scoring were 
performed as described in the “Methods” section. Exam-
pies of staining and corresponding IHC scores are shown in 
Fig. 3. Overall, SRAP staining was slightly higher using 
742A antibody as compared to when using 743A antibody. 
As shown in Fig. 4, a significant correlation exists between 
scores obtained with the two antibodies (Spearman’s 
coefficient $r = 0.46$, $P < 0.001$). SRAP staining varies 
from one sample to another, with H-scores ranging from 10 
to 232.5 using 742A antibody ($n = 170$, median = 135) 
and from 7.5 to 175 using 743A antibody ($n = 170$, 
median = 76.25) (Fig. 4b, c).

Low SRAP expression is associated with better survival 
among ER-positive breast cancer patients

For each antibody, we have then arbitrarily divided the 
cohort into low, medium, and high SRAP expressor, by 
using 25 and 75 % percentile of normalized scores as cut-
off values. When using 742A antibody, 45 cases fell in the 
low SRAP category (742A-IHC score 10–87.5), 87 cases 
had a medium SRAP expression (742A-IHC score 
90–175), and 38 belonged to the high SRAP subgroup 
(742A-IHC score 180–232.5). When using 743A, low 
(743A-IHC score 7.5–60), medium (743A-IHC score 
62.5–100), and high SRAP (743A-IHC score 105–175) 
subgroups contained 42, 85, and 43 cases, respectively.

In the single predictor analysis, larger tumor size and 
positive node status were associated with a poor prognosis 
(Table 1). When considering 742A results (Fig. 5a), high 
SRAP was also associated with a poor BCSS ($P = 0.014$). Cox regression analysis clearly showed that high SRAP 
subgroups had an increased risk of dying of the disease.
compared to low SRAP (HR = 1.71, P = 0.014). This advantage continued in models including other risk factors (Table 2). In a regression model with backward selection, only S742A and tumor size were significant predictors.

When considering S743A results, similar observations were obtained (Fig. 5b). Patients with low SRAP had longer BCSS time than patients whose primary tumor expressed medium or high SRAP although this difference was not statistically significant (P = 0.14, HR = 1.35).

Since 742A and 743 recognize distinct SRAP peptides as showed previously, the predicative information might not be entire if results from only one antibody are taken into consideration. To establish whether combining 742A and 743A results could provide additional information, three combined groups—both low, both high, and rest mixed group—were arbitrarily grouped as illustrated in Fig. 5c. Within the resulting defined subgroups (Fig. 5c), the “green group” corresponding to “both low” appears to have a distinct survival profile (breast-cancer-specific deaths 1 out of 22, mean survival 113 months). As such, both high and mixed groups have a significantly much poorer BCSS (both high group: breast-cancer-specific deaths 9 out of 23, mean survival: 97.26 months; mixed group: breast-cancer-specific deaths 37 out of 125, mean survival: 97.67 months, Mantel–Cox: P = 0.005, HR = 2.24).

In multivariate regression analyses of this cohort, variable of group by 742A and 743A was omitted because same variable could not be measured twice in this model. Only 742A and tumor size remained significant with poor prognosis (Table 2). No analysis significantly violated the proportional hazard assumption.

Discussion

In the present study, we have analyzed SRAP expression in breast cancer tissues by Western blot and TMA using two antibodies (742A and 743A) recognizing the N- and C-terminal region of the protein, respectively. Even though several SRAP-like peptides were differentially detected, both antibodies established that low SRAP expression is associated with a better prognosis for patients whose primary tumor is estrogen receptor positive.

We have previously reported the detection of multiple SRAP-like peptides, migrating at apparent molecular weights of ~40, ~30, and ~25 kDa using 743A antibody (Yan et al. 2009). As expected, all these peptides were observed in the present study, with various levels of expression (Fig. 2a). Indeed, some tumors expressed only one form (~30 kDa tumor T6 and 25 kDa tumor T2), whereas multiple forms are detected in other tumors (~40, ~30, and ~25 kDa in tumor T3). Interestingly, an additional band is also observed with this antibody in Tumor 1, migrating at ~48 kDa. The exact identity of only one of these peptides is currently known. The doublet migrating at ~30 kDa has indeed consistently being recognized in other systems using other antibodies (Chooniedass-Kothari et al. 2004, 2006; Cooper et al. 2009; Kurisu et al. 2006). 742A antibody also detected this band (Fig. 2a), confirming the presence on the corresponding peptide of both N- and C-terminal domains. The ~30-kDa signal is therefore currently believed to represent the full-length 236–237 aa SRAP protein. The localization of this SRAP has been confirmed in both nucleus and cytoplasm by Western blot analysis in MCF7 cells (supplementary Fig. 1).

The 40 kDa as well as the 25 kDa is not seen when using 742A. This suggests that these peptides might be missing the N-terminal extremity of SRAP or that the
The corresponding epitope is masked. Inversely, 743A antibody fails to detect the 26 kDa (seen in all tumors) and the 48 kDa (seen in HeLa cells) peptides recognized by 742A. Similar reasons can be invoked to rationalize this observation: loss or masking of the corresponding epitope. The exact origin of such SRAP-like peptides is currently unknown. We have, however, recently proposed that alternative splicing events, known to occur in breast cancer cells (Cooper et al. 2009; Hube et al. 2006; Leygue 2007; Yan et al. 2009), might be responsible for the differential SRAP patterns observed. Further studies are obviously needed to establish the nature of the different splicing events potentially involved in the generation of the different SRA transcripts and their potential protein products.

In regard to the cellular localization of specific SRAP-like peptides in tumor sections, it is not known which, if any, is preferentially located in the nucleus, cytoplasm, or both (Fig. 2b). To address this issue would necessitate performing cellular fractionation of tumor samples followed by Western blot analysis. However, given the limited amount of tumor samples available from the Manitoba Tumor Bank as well as sub-proportion of material within the tumor that actually represents bona fide cellular material, it is unlikely that enough starting material could be

---

**Table 2** Cox multivariate analysis of BCSS

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR</th>
<th>95 % CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>PgR</td>
<td>1.06</td>
<td>0.48–2.35</td>
<td>0.88</td>
</tr>
<tr>
<td>Size</td>
<td>2.35</td>
<td>1.29–4.28</td>
<td>0.0051</td>
</tr>
<tr>
<td>Node</td>
<td>1.54</td>
<td>0.85–2.79</td>
<td>0.15</td>
</tr>
<tr>
<td>Age</td>
<td>1.10</td>
<td>0.59–2.03</td>
<td>0.77</td>
</tr>
<tr>
<td>S742</td>
<td>1.71</td>
<td>1.12–2.62</td>
<td>0.014</td>
</tr>
<tr>
<td>S743</td>
<td>1.13</td>
<td>0.75–1.71</td>
<td>0.55</td>
</tr>
</tbody>
</table>

Variables as described in Table 1 except for grouped variable of 742A and 743A were omitted from this multivariate analysis.
obtained to yield adequate amounts of nuclear/cytoplasmic protein necessary for subsequent Western blot analysis. Alternatively, antibodies recognizing specific SRAP peptides could theoretically be generated, perhaps through identification of these peptides via purification followed by mass spec analysis; however, this is technically beyond the scope of our present study.

Interestingly, even though two antibodies independently show that lower expression of SRAP peptides is associated with a better outcome, the effect by 743A was not statistically significant in the present analysis ($P = 0.14$). This is probably due to the fact that the group with medium SRAP expression by 743A has a similar trend of prognosis compared to the group with high SRAP expression by 743A (Fig. 5b). This could suggest that even a slight increase in expression detected by 743A is a factor of risk in these patients. The variation in prognostic value between two antibodies could also be explained by two antibodies differentially recognizing specific SRAP-like peptides. When both scores are combined, patients whose primary tumors expressed double high SRAP by 742A and 743A had a poor prognosis of BCSS (Mantel–Cox: $P = 0.005$); double high SRAP, therefore, appeared as a very powerful indicator of poor prognostic for BCSS ($HR = 2.24$). Interestingly, mixed group with either medium or highly expressed 742A and/or 743A recognized SRAP-like peptides also showed worse outcomes compared to double low SRAP patients (Fig. 5c). This probably suggests that the expression of any SRAP-like peptide is potentially associated with a bad outcome in ER+ breast cancer patients. Whether or not specific SRAP peptides are associated with tumor phenotypes and patient survival need to be further determined.

As outlined earlier, the poor outcome ER+ tumor (luminal B) is associated with an increasing Ki67 levels and higher expression of Her2 (Cheang et al. 2009). Considering the predictive value of SRAP in a particular ER+ sub-cohort, we propose that SRAP expression might correlate with Ki67 and Her2 expression. Hypothetically it could be potentially used to separate luminal A and luminal B subgroups. Further studies are required on large cohort-based trials to test this hypothesis before routine clinical practice.

**Conclusion**

Altogether, we have confirmed that several SRAP-like peptides are expressed in breast tumors and that their detection by two antibodies could be used as a new prognostic marker in ER+ patients who received tamoxifen treatment. Additional studies need to be performed to establish whether specific splicing SRA variants have additional prediction values.

---

**Acknowledgments** This work was supported by grants from the Canadian Institute of Health Research (CIHR), the CancerCare Manitoba Foundation (CCMF), the Manitoba Health Research Council (MHRC), and the Canadian Breast Cancer Foundation (CBCF). Y.Y. received a CIHR Graduate Studentship Award.

**Conflict of interest** No competing interests to be declared.

**References**


Conclusion of chapter IV

1. Two commercial anti-SRAP antibodies 742A and 743A, respectively (recognizing the N- and C-terminal extremity of the protein), specifically recognizes SRAP in breast cancer cell line and breast tumor samples. Both antibodies led to similar nuclear and cytoplasmic staining in breast tissue section by immunochemistry (IHC). A solid correlation was found between 742A and 743A IHC scores.

2. Anti-SRAP antibody at its C-terminus (743A) could detect in breast tumours at least four peptides migrating at ~25 kDa and ~30kDa ~40kDa and ~48kDa whereas the one targeting SRAP at its N-terminus (742A) recognized three bands migrating at ~26 kDa, ~30kDa and ~48kDa when assessing multiple tumours by western blot analysis.

3. In a cohort of 270 ER positive breast tumors from Manitoba Breast Tumor Bank (MBTB), tissue microarray (TMAs) results from both antibodies independently showed that the higher expression of SRAP was associated with a significantly worse breast cancer specific survival (BCSS) than those with lower SRAP expression. This is consistent with the results presented in Chapter III, but not with those of a previous study [145]. Potential reasons behind this apparent discrepancy will be further discussed in Section VII-2-1.

4. When both scores of IHC are combined, patients whose primary tumors expressed “double high” SRAP by 742A and 743A had a worst prognosis of breast cancer specific survival (BCSS). This suggests that “double high” SRAP appeared as a very powerful indicator of poor prognostic for BCSS.
CHAPTER V Expression of both Estrogen Receptor-beta 1 (ER-β1) and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with Estrogen Receptor-alpha (ER-α)-Negative Early Breast Cancer (EBC)

I first noticed that the expression of ER-β1 and SRAP (a potential co-regulator of ER-β1) were positively correlated in TMAs previously constructed by the Manitoba Breast Cancer Bank (MBCB). This observation prompted us in collaboration with Dr. L. Murphy to assessed the expressions of ER-β1 and SRAP in TMAs generated from tumour samples representative of a large breast cancer cohort in a randomized placebo controlled clinical trial (NCIC CTG MA12). This trial was originally designed to determine the benefit of tamoxifen following chemotherapy for early Breast Cancer in pre-menopausal women. A TMA representing 492 of recruited cases (including 68% ER-α positive) was constructed. Adjacent TMA sections were assessed by immunohistochemistry (IHC) for ER-β1 and SRAP expression, using semi-quantitative IHC-scoring. Survival analyses were performed using Cox modeling to investigate associations between SRAP/ER-β1 and clinical outcome. Similar to previous observation, we confirmed that the expression of ER-β1 positively correlated with that of SRAP and that high expression of both molecules significantly predicted a favorable tamoxifen response in the ER-α negative cohort. The experimental approaches and results were published on March 4, 2013 in *Ann Oncol.* (8):1986-93 entitled “Expression of both ER-β1 and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with Estrogen Receptor-alpha (ER-α)-Negative Early Breast Cancer (EBC).”

The fact that expression of these proteins characterize a subset of ER-α negative cancers more likely to favourably respond to tamoxifen treatment is of potential clinically importance since ER-α negative patients are currently not treated by endocrine therapy.
This is the first time that the predictive value of SRAP has been suggested.

**My contribution to this article**

I performed SRAP antibody validation as well as scored all the TMA slides.
Expression of both Estrogen Receptor-beta 1 (ER-β1) and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) are predictive for benefit from tamoxifen therapy in patients with Estrogen Receptor-alpha (ER-α)-Negative Early Breast Cancer (EBC)

Y. Yan1, X. Li2, A. Blanchard3,4, V. H. C. Bramwell5, K. I. Pritchard6, D. Tu2, L. Shepherd2, Y. Myal3,4, C. Penner3, P. H. Watson7, E. Leygue1 & L. C. Murphy1*

1Department of Biochemistry and Medical Genetics, Manitoba Institute of Cell Biology, CancerCare Manitoba, University of Manitoba, Winnipeg; 2NCIC Clinical Trials Group (NCIC CTG), Queens University, Kingston; 3Department of Pathology, University of Manitoba, Winnipeg; 4Department of Physiology, University of Manitoba, Winnipeg; 5Cross Cancer Institute, University of Alberta, Edmonton; 6Sunnybrook Odette Cancer Centre, University of Toronto, Toronto; 7Deely Cancer Research Centre, BCCA, Victoria, Canada

Received 28 August 2012; revised 17 December 2012 and 27 February 2013; accepted 4 March 2013

Background: Roles of Estrogen Receptor-beta 1 (Erb-B1) and its co-regulator Steroid Receptor RNA Activator Protein (SRAP) in breast cancer remain unclear. Previously, ER-β1 and SRAP expression were found positively correlated in breast cancer and, therefore, expression of these two molecules could characterize cancers with a distinct clinical outcome.

Patients and methods: ER-β1 and SRAP expression was determined by immunohistochemistry (IHC) in tissue microarrays from a randomized, placebo-controlled trial (NCIC-CTG-MA12), designed to determine the benefit of tamoxifen following chemotherapy in premenopausal early breast cancer (EBC). Expression was dichotomized into low and high using median IHC scores. Relationships with survival used Cox modeling.

Results: In the whole cohort, ER-β1 and SRAP were not prognostic. However, high ER-β1 and SRAP significantly predicted tamoxifen responsiveness [overall survival, interaction test, \( P = 0.03 \); relapse-free survival (RFS), interaction test, \( P = 0.01 \)]. Stratification by ER-α-status found predictive benefit only in ER-α-negative cases. The difference in RFS between tamoxifen and placebo was greater in patients whose tumors expressed both high SRAP and ER-β1 [hazard ratio = 0.07; 95% confidence interval (CI) 0.01–0.41; \( P = 0.003 \)] versus those with low SRAP or ER-β1 [interaction test, \( P = 0.02 \)]. The interaction test was not significant in ER-α-positive cohorts.

Conclusions: This study provides evidence that both ER-β1 and SRAP could be predictive biomarkers of tamoxifen benefit in ER-α-negative premenopausal EBC.

Key words: early breast cancer, estrogen receptor alpha, estrogen receptor beta, NCIC-CTG-MA12, steroid receptor RNA activator protein, tamoxifen sensitivity

introduction

Estrogen receptor beta’s (ER-β) role in breast cancer remains unclear [1, 2]. Often higher levels of ER-β are associated with a better prognosis in tamoxifen-treated patients. Since most of the patients treated with tamoxifen are ER-positive (here referred to as ER-α-positive) tumors, little opportunity exists to determine the value of ER-β expression alone for its prognostic and/or predictive value with hormonal therapies, despite consistent observations, that approximately 5%–10% of ER-α-negative tumors show responses to tamoxifen [3, 4]. Analyses from the Early Breast Cancer Trials Collaborative Group (EBCTCG 1998) suggested that there might be a small (6%) reduction in mortality for patients with ‘ER poor’ tumors receiving tamoxifen [5], but this was no longer evident in later meta-analyses including more trials [6, 7]. With the discovery of ER-β expression alone in breast tumors representing ~17% of primary breast cancers [8, 9], the possibility now exists that, in this ER-negative cohort, tamoxifen could mediate activity via ER-β1, the full-length ligand-binding receptor isomorph. Other isoforms, e.g. ER-β2/cx, are C-terminally truncated and...
## Table 1. Predictive analysis for ER-β1 and SRAP

<table>
<thead>
<tr>
<th>Patient subset</th>
<th>Marker status</th>
<th>Treatment</th>
<th># of patients</th>
<th>5-year OS (%)</th>
<th>Adjusted hazard ratio (95% CI)*</th>
<th>P-value for interactiona</th>
</tr>
</thead>
<tbody>
<tr>
<td>OS</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>115</td>
<td>85</td>
<td>0.86 (0.50–1.47)</td>
<td>0.20</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>136</td>
<td>86</td>
<td>P = 0.58</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>107</td>
<td>86</td>
<td>0.45 (0.22–0.90)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>99</td>
<td>78</td>
<td>P = 0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>114</td>
<td>81</td>
<td>0.92 (0.54–1.59)</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>128</td>
<td>84</td>
<td>P = 0.78</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>113</td>
<td>90</td>
<td>0.59 (0.32–1.11)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>107</td>
<td>83</td>
<td>P = 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>153</td>
<td>84</td>
<td>1.03 (0.66–1.60)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>176</td>
<td>86</td>
<td>P = 0.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>65</td>
<td>92</td>
<td>0.40 (0.18–0.89)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>55</td>
<td>78</td>
<td>P = 0.03</td>
<td></td>
</tr>
<tr>
<td>ER+</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>82</td>
<td>89</td>
<td>1.04 (0.56–1.92)</td>
<td>0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>100</td>
<td>89</td>
<td>P = 0.90</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>60</td>
<td>92</td>
<td>0.64 (0.27–1.52)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>57</td>
<td>88</td>
<td>P = 0.29</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>70</td>
<td>87</td>
<td>1.23 (0.64–2.33)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>84</td>
<td>89</td>
<td>P = 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>74</td>
<td>93</td>
<td>0.70 (0.34–1.45)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>73</td>
<td>89</td>
<td>P = 0.33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>101</td>
<td>88</td>
<td>1.16 (0.66–2.03)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>121</td>
<td>90</td>
<td>P = 0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>39</td>
<td>95</td>
<td>0.38 (0.13–1.16)</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>33</td>
<td>85</td>
<td>P = 0.09</td>
<td></td>
</tr>
<tr>
<td>ER−</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>33</td>
<td>79</td>
<td>0.85 (0.30–2.43)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>36</td>
<td>80</td>
<td>P = 0.76</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>47</td>
<td>81</td>
<td>0.27 (0.11–0.70)</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>42</td>
<td>67</td>
<td>P = 0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>44</td>
<td>73</td>
<td>0.99 (0.44–2.25)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>44</td>
<td>75</td>
<td>P = 0.98</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>39</td>
<td>85</td>
<td>0.33 (0.12–0.88)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>34</td>
<td>73</td>
<td>P = 0.03</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>52</td>
<td>75</td>
<td>0.76 (0.36–1.60)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>55</td>
<td>76</td>
<td>P = 0.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>26</td>
<td>88</td>
<td>0.16 (0.03–0.82)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>22</td>
<td>68</td>
<td>P = 0.03</td>
<td></td>
</tr>
<tr>
<td>Patient subset</td>
<td>Marker status</td>
<td>Treatment</td>
<td># of patients</td>
<td>5-year RFS (%)</td>
<td>Adjusted hazard ratio (95% CI)*</td>
<td>P-value for interactiona</td>
</tr>
<tr>
<td>----------------</td>
<td>---------------</td>
<td>-----------</td>
<td>---------------</td>
<td>---------------</td>
<td>---------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>RFS</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>115</td>
<td>78</td>
<td>0.82 (0.52–1.29)</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>136</td>
<td>75</td>
<td>P = 0.39</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>107</td>
<td>79</td>
<td>0.36 (0.20–0.65)</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>99</td>
<td>64</td>
<td>P = 0.0007</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>114</td>
<td>75</td>
<td>0.81 (0.50–1.29)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>128</td>
<td>72</td>
<td>P = 0.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>113</td>
<td>81</td>
<td>0.49 (0.29–0.84)</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>107</td>
<td>69</td>
<td>P = 0.009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>153</td>
<td>77</td>
<td>0.88 (0.60–1.30)</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>176</td>
<td>75</td>
<td>P = 0.53</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>64</td>
<td>84</td>
<td>0.28 (0.14–0.56)</td>
<td>0.0003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>55</td>
<td>62</td>
<td>P = 0.0003</td>
<td></td>
</tr>
</tbody>
</table>

*Continued*
expression in tamoxifen-treated ER-negative patients [13, 14] and concluded that high ER-α expression in tamoxifen-treated ER-negative cancers, a consistent finding is a positive association of ER-β1 expression with Ki67, a marker of proliferation [10–12]. Recently two studies published analyses on ER-β expression in tamoxifen-treated ER-α-negative patients [13, 14] and concluded that high ER-β expression in tamoxifen-treated ER-α-negative patients associated with a better clinical outcome. Both studies had relatively small numbers of cases and all patients were treated with tamoxifen; so only prognostic value was concluded.

We recently observed that steroid receptor RNA activator protein (SRAP), a regulator of ER activity [15–17], could be a prognostic marker in ER-α-positive breast tumors [18]. In this cohort [18], we also noted that expression of SRAP correlated with ER-β1 expression, leading us to hypothesize that cases expressing both biomarkers might represent a specific tumor subset.

Here, we investigated ER-β1 and SRAP expression in tissues from a randomized, placebo-controlled trial to determine the benefit of tamoxifen added to chemotherapy of early breast cancer (EBC) in premenopausal women, NCIC-CTG-MA12 [19].

**methods**

**patients**

The patient population, treatment design and research focus for NCIC-CTG-MA12 were previously published [19]. A unique aspect of NCIC-CTG-MA12 was the eligibility of patients with ER-negative tumors for a study evaluating the benefit of tamoxifen therapy. At the time of initiation of NCIC-CTG-MA12 (1992), a potential benefit in patients with ER-negative tumors had not been excluded [5]. ER/progesterone receptor (PR) status was previously defined [19]. Tumors from this cohort were recently assessed for the frequency of intrinsic molecular subtypes as determined by the PAM50 assay and were ∼22% basal-like [20].

Table 1. Continued

<table>
<thead>
<tr>
<th>Patient subset</th>
<th>Marker status</th>
<th>Treatment</th>
<th># of patients</th>
<th>5-year OS (%)</th>
<th>Adjusted hazard ratio (95% CI)</th>
<th>P-value for interaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER+</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>82</td>
<td>79</td>
<td>0.88 (0.53–1.46)</td>
<td>0.34</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>100</td>
<td>76</td>
<td>P = 0.61</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>59</td>
<td>83</td>
<td>0.50 (0.25–1.01)</td>
<td>0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>57</td>
<td>74</td>
<td>P = 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>70</td>
<td>78</td>
<td>0.90 (0.53–1.55)</td>
<td>0.44</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>84</td>
<td>72</td>
<td>P = 0.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>73</td>
<td>82</td>
<td>0.61 (0.33–1.01)</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>73</td>
<td>78</td>
<td>P = 0.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>101</td>
<td>79</td>
<td>0.91 (0.57–1.45)</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>121</td>
<td>77</td>
<td>P = 0.68</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>38</td>
<td>84</td>
<td>0.27 (0.10–0.71)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>33</td>
<td>73</td>
<td>P = 0.008</td>
<td></td>
</tr>
<tr>
<td>ER–</td>
<td>ERβ1 low</td>
<td>Tamoxifen</td>
<td>33</td>
<td>76</td>
<td>0.97 (0.37–2.53)</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>36</td>
<td>75</td>
<td>P = 0.95</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 high</td>
<td>Tamoxifen</td>
<td>47</td>
<td>77</td>
<td>0.22 (0.09–0.54)</td>
<td>0.0009</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>42</td>
<td>52</td>
<td>P = 0.0009</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP low</td>
<td>Tamoxifen</td>
<td>44</td>
<td>70</td>
<td>0.99 (0.45–2.20)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>44</td>
<td>73</td>
<td>P = 0.99</td>
<td></td>
</tr>
<tr>
<td></td>
<td>SRAP high</td>
<td>Tamoxifen</td>
<td>39</td>
<td>82</td>
<td>0.23 (0.09–0.61)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>34</td>
<td>52</td>
<td>P = 0.003</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ERβ1 or SRAP low</td>
<td>Tamoxifen</td>
<td>52</td>
<td>73</td>
<td>0.73 (0.37–1.45)</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>55</td>
<td>71</td>
<td>P = 0.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Both ERβ1 and SRAP high</td>
<td>Tamoxifen</td>
<td>26</td>
<td>85</td>
<td>0.07 (0.01–0.41)</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Placebo</td>
<td>22</td>
<td>45</td>
<td>P = 0.003</td>
<td></td>
</tr>
</tbody>
</table>

*Adjusted for age, performance status, time from diagnosis to randomization, nodal status, t-stage, receptor status and type of chemotherapy treatment.

**statistical analysis**

Clinical end points in this analysis are as defined in NCIC-CTG-MA12 [19]. Relapse-free survival (RFS) was defined as time from randomization to earliest date of recurrence or death or censored on the last date the patient was known to be alive, and overall survival (OS) as the time from randomization to date of death or censored on the last date the patient was known to be alive. Both RFS and OS by expression and treatment status were described by Kaplan–Meier curves and compared by multivariate Cox models adjusting for treatment (when appropriate), age, performance status, time from diagnosis to randomization, nodal status, t-stage, receptor status and type of chemotherapy. Tumor grade was not assessed in the original data collection and retrospective assessment resulted in a large portion of missing values. It was not found to be an independent prognostic factor [20]. Therefore, it was not included in multivariate Cox models. Multivariate Cox models with the same covariates and an interaction term between treatment and expression status were used to assess predictive significance of markers. All statistical tests were two sided.
NCIC-CTG-MA12 recruited 672 breast cancer patients. A TMA representing 492 (73.2%) cases was constructed at a later time. Expression of ER-β1 and SRAP were determined as previously described [10, 18, 24]. Due to heterogeneity of cellular content of tumor biopsies, as TMAs are sectioned through the cores, there was loss of the invasive tumor compartment in some sections or for technical failures, e.g. sectioned core flipped over and therefore, not every case provided interpretable IHC data. Therefore, tumor numbers (n) analyzed for different markers were <492: 457/492 (92.9%) for ER-β1 and 462/492 (93.9%) for SRAP. As shown in supplementary Table S1, available at *Annals of Oncology* online, baseline characteristics for those samples providing assessable IHC staining for ER-β1 and SRAP did not differ from the whole cohort of patients randomized in the trial.

A total of 457 patients with ER-β1 and 462 with SRAP results are available. The median IHC score for ER-β1 was 5.0 (range 0–205), resulting in 251 (54.9%) patients classified into the low and 206 (45.1%) into the high ER-β1 group. Based on the median IHC score for SRAP, 77.5 (range 0–225), 242 (52.4%) patients were classified into the low and 220 (47.6%) into the high SRAP group.

As observed previously [10, 18, 24], ER-β1 and SRAP expression were significantly associated (Spearman coefficient \( r = 0.314, P < 0.0001, n = 449 \) in this trial cohort. When tumors were dichotomized according to low or high ER-β1, there were significant differences in SRAP expression (median SRAP IHC score 70 versus 83, Mann–Whitney \( P < 0.0001 \)).

There were no associations of ER-β1 or SRAP with either OS or RFS in the whole patient cohort regardless of treatment (supplementary Table S2, available at *Annals of Oncology* online).
online). When patients were separated into those treated with tamoxifen and those receiving placebo, there were no statistically significant associations with expression of any marker (supplementary Table S2, available at *Annals of Oncology* online). Therefore, neither marker had a prognostic value in the whole patient cohort or within each treatment group.

**predictive analysis in the whole patient cohort**

As summarized in Table 1, patients with high ER-β1 expression when treated with tamoxifen had better OS [adjusted hazard ratio (HR) of tamoxifen to placebo 0.45 with 95% confidence interval (CI) 0.22–0.90, P = 0.02]. As well, patients who received tamoxifen and whose tumors had either high ER-β1 or high SRAP had better RFS (adjusted HR of tamoxifen to placebo 0.36 with 95% CI 0.20–0.65, P = 0.0007 for women with high ER-β1 and 0.49 with 95% CI 0.29–0.84, P = 0.009 for women with high SRAP). In patients with low ER-β1 or SRAP expression, no significant difference was found between tamoxifen and placebo in either RFS or OS. Therefore, while neither ER-β1 nor SRAP were prognostic markers, these data suggest that they might be predictive markers for tamoxifen treatment benefit. The test of interaction between tamoxifen treatment and the marker, however, was not significant for either ER-β1 or SRAP (Table 1).

Due to the positive relationship between ER-β1 and SRAP, an analysis was undertaken to determine whether the detection of high levels of both these biomarkers together would be associated with the benefit of tamoxifen treatment. Data in Table 1 and Figure 1 suggest that, when considering all patients, women with high levels of both ER-β1 and SRAP benefited from tamoxifen treatment, while women with low levels of ER-β1 or SRAP did not. Specifically, the HR in OS between tamoxifen and placebo groups was 0.40 (95% CI 0.18–0.89; P = 0.03) for those with both high ER-β1 and SRAP (n = 120) and 1.03 (95% CI 0.66–1.60; P = 0.90) for those with either low ER-β1 or SRAP (n = 329), and the HR in RFS between tamoxifen and placebo groups was 0.28 (95% CI 0.14–0.56; P = 0.0003) for those with both high ER-β1 and SRAP (n = 120) and 0.88 (95% CI 0.60–1.30; P = 0.53) for those with either low ER-β1 or SRAP (n = 329). The interaction tests for OS and RFS were both significant (P = 0.03 and 0.01, respectively). These data lend further support to the possibility that high ER-β1 and SRAP are predictive markers for the benefit of tamoxifen treatment in this cohort of breast cancer patients [19].

**predictive analysis stratified by ER status**

Since patients with both ER-α-positive or -negative tumors were recruited into NCIC-CTG-MA12, a subgroup analysis was undertaken stratifying cases by ER-α status.

In ER-α-positive tumors, levels of ER-β1 and SRAP expression, alone or together, were not predictive of treatment benefit from tamoxifen (Table 1).

In ER-α-negative tumors with high ER-β1 expression, a longer RFS was observed for tamoxifen-treated patients than those receiving placebo (adjusted HR = 0.22, 95% CI 0.09–0.54, P = 0.0009, Table 1). The interaction value between expression of ER-β1 and treatment in RFS was also significant, P = 0.03 (Table 1). Similarly, a longer OS was observed for those patients with high levels of ER-β1 and treated with tamoxifen (adjusted HR = 0.27, 95% CI 0.11–0.70, P = 0.007, Table 1). The interaction was not significant (P = 0.10). There was evidence that, in ER-α-negative placebo patients, high ER-β1 levels in tumors were associated with worse outcome since the HR for low versus high ER-β1-expressors was 0.23 (95% CI 0.09–0.61, P = 0.003) for OS and for RFS was 0.23 (95% CI 0.09–0.57, P = 0.002), implying that ER-β1 was also prognostic for ER-α-negative patients.

Similarly, in the ER-α-negative cohort, high SRAP expression was associated with a longer RFS in patients treated with tamoxifen (RFS, adjusted HR = 0.23, 95% CI 0.09–0.61, P = 0.003) compared with those receiving placebo. The interaction test for predictive analysis was significant, P = 0.02 (Table 1).

Importantly, high expression of both SRAP and ER-β1 was predictive for the benefit of tamoxifen treatment (interaction test P = 0.02, Table 1 and Figure 2).

These data suggest that high expression of ER-β1 especially when combined with high SRAP expression is a predictive marker for the benefit of tamoxifen treatment primarily in ER-α-negative EBC.

**discussion**

Results of this study suggest that high expression of both ER-β1 and SRAP is predictive of better clinical outcome to tamoxifen treatment in premenopausal women previously treated with chemotherapy. The surprising finding was that most benefit was found in ER-α-negative breast cancer. This study is the first to provide evidence that ER-β1 expression particularly when co-expressed with high SRAP levels is predictive of the efficacy of adjuvant tamoxifen treatment in ER-α-negative breast cancer in premenopausal women previously treated with chemotherapy.

With regard to ER-β1 expression, the current study is consistent with and adds valuable new information to the conclusions of two recently published studies (non-placebo control, non-randomized) where the relationship of ER-β expression and clinical outcome was determined in ER-α-negative tamoxifen-treated breast cancer [13, 14]. Our study, like that of Honma *et al.* [13], specifically measured ER-β1, the ligand-binding ER-β isoform. The data support the hypothesis

---

**Figure 2.** Expression of high levels of both ER-β1 and SRAP is predictive of response to tamoxifen only in ER-α-negative breast cancer. (A) Kaplan–Meier curves for patients with high ER-β1 and SRAP for OS (left-hand panels) and RFS (right-hand panels) in tamoxifen treated (filled lines) versus placebo controls (dotted line), stratified for ER-α status, ER-α-negative (top panel), ER-α-positive (bottom panel). (B) Kaplan–Meier curves for patients with low levels of ER-β1 or SRAP, OS (left-hand panels) and RFS (right-hand panels) in tamoxifen treated (filled lines) versus placebo controls (dotted line).
that tamoxifen effects might be mediated in ER-α-negative breast cancers, at least in part, through ER-β1 within the tumor cells and not through alternative, non-ER-mediated activities [25]. Effects through ER-positive immune and endothelial cells present within the heterogeneous cellular composition of any breast tumor in vivo cannot be excluded [26–28].

Previously small subsets of patients with apparently ER-α-negative breast cancer were reported to respond to tamoxifen treatment [3, 4]. The reasons behind such apparently paradoxical observations remain unknown [29, 30], although false negative assays for technical reasons or ER-independent tamoxifen action [30] have been suggested. However, a role of ER-β1 in this group of so-called ER-negative cancers is now a possibility. In ER-α-negative tumors, correlations of ER-β1 with Ki67, a proliferation marker, were found [10–12], suggesting that ER-β1 may have a role in driving proliferation of these tumors, and that tamoxifen treatment, through inhibiting ER-β1 activity [31], could slow the progression of such tumors. This has important implications since an already approved treatment of breast cancer which is mostly well tolerated, has a low cost, is orally administered and has over 30 years of clinical use, may be an attractive alternative for a group of patients with generally few options other than cytotoxic therapies.

A confounding issue associated with the MA12-trial is suboptimal treatment compliance in both tamoxifen treated and placebo arms [19]. This may underlie results showing a marginal benefit from tamoxifen in the whole population with no evidence of greater benefit for ER-α-positive compared with the ER-α-negative subgroup. Furthermore, it may in part be the reason that we only saw a trend towards high ER-β1 levels being associated with better RFS in all patients treated with tamoxifen irrespective of ER-α status.

The present study population was not representative of the normal breast cancer population since the MA12-trial focused on premenopausal women. However, at the molecular level, the cases did represent the usual population, i.e. cases represented on the TMA were 65% ER-α-positive and 35% ER-α-negative, but when receptor positivity was expanded to include those which were ER-α-negative but PR+, the receptor-positive population became 74%. Furthermore, analysis of frequency of intrinsic molecular subtypes using the 50 gene subtype predictor, PAM50, suggested that the MA-12 breast cancer cohort is similar to the usual population [20].

The positive relationship found between SRAP and ER-β1 is interesting and may have a mechanistic basis. SRAP physically interacts with both ER-α and ER-β1 and affects activity [15–17]. As well, altering SRAP expression in breast cancer cells resulted in altered expression of several genes, including ER-β [32]. Furthermore, our unpublished data suggest that over-expressed ER-β1 in MCF7 breast cancer cells may bind to sequences in the SRA-gene promoter under some conditions, suggesting potential regulatory interactions of the two genes in breast cancer. This requires further investigation since our results show that combined assessment of ER-β1 and SRAP expression in a tumor provided better predictive information than measurement of either alone. If a regulatory relationship exists between ER-β1 and SRAP, this may be analogous to measurement of PR a downstream marker of ER-α-signaling in traditionally ER+ breast cancer [33, 34]. It is also possible that ER-β1 and SRAP expression are associated with the luminal molecular subtype of breast cancer, which was shown to predict better clinical outcome to tamoxifen [20, 35]. Interestingly, ER-β1 seems to localize evenly across the main intrinsic subtypes of breast cancer [36], but using multiple correspondence analysis, ER-β1 expression in node-positive cases was associated with classically ER-negative cancers with more aggressive clinical outcomes [36].

In conclusion, our data together with those reported in two other studies support the exciting possibility that ER-β1 may be a treatment target in some ER-negative breast cancers. This deserves further study and if true, means that a group of patients previously considered only for aggressive chemotherapies would now be candidates for better tolerated hormonal-like therapies.

**funding**

This work was supported by the Canadian Institutes of Health Research (CIHR, #70199), the CancerCare Manitoba Foundation (CCMF) and the Canadian Breast Cancer Foundation (CBCF- Prairies/NWT Chapter). This study was also supported by the Manitoba Breast Tumour Bank, a member of the Canadian Tumour Repository Network and is funded in part by CCMF and CIHR (#80155).

**disclosure**

The authors have declared no conflicts of interest.

**references**


Conclusion of Chapter V

1. The expression of ER-β1 and SRAP were positively correlated in the cohort represented on the TMAs previously constructed by the randomized placebo controlled clinical trial NCIC CTG MA12.

2. Both high ER-β1 and high SRAP levels significantly predict favorable tamoxifen responsiveness in the ER-α negative cohort. This suggests that a group of ER-α negative patients previously considered only for aggressive chemotherapies would be potential candidates for better-tolerated hormonal therapy such as tamoxifen.
CHAPTER VI The Steroid receptor RNA Activator protein (SRAP) controls cancer cell migration/motility

We have previously observed that breast cancer patients whose primary tumour expressed the estrogen receptor α had a decreased chance of survival when their tumours contain high levels of SRAP (chapter III and IV). This suggests that in this specific group of patients, SRAP acts as a poor prognostic marker and might even contribute to increasing tumour aggressiveness.

However, as described in chapter V, contradictory data showed that high expression of SRAP (or SRAP like peptide) could also predict a benefit from tamoxifen treatment primarily in ER-α negative patients. This could suggest a protective value of this marker in specific subgroups of breast cancer patients upon certain treatment. Therefore, it is urgent to understand the potential function of SRAP and how it may contribute to different oncologic phenotypes. Several groups have used expression microarrays in different cell line models to identify endogenous genes and cellular processes regulated by SRA in order to gain insight into SRA function [112,123,136]. However, inconsistent results have been obtained. Vicent et al. (2009) first stated that gene expression potentially changed upon SRA silencing overlapped mostly with PR- regulated gene candidates [124]. Foulds et al. (2009) suggested only a small portion of hormone target genes was affected by SRA depletion. Instead they validated eight *bone fide* SRA downstream targets, none of which were hormone-regulated [136]. In contrast to both reports, McKay et al. (2014) recently suggested that neither SRA RNA nor SRAP is a major transcription regulator [123]. To investigate SRAP potential roles, we have in this study established the gene expression profile of cervical cancer Hela cells and MDA-MB-231 breast cancer cells following treatment with siRNAs targeting SRA and/or overexpression of SRAP. Overall, the
expression of very few genes was altered in our experiments. Interestingly however, ontology analysis indicated that the expressions of the few genes identified were positively associated with cellular movement. This prompted us to further investigate the effect of SRAP depletion and overexpression on cell motility. We further confirmed that cells transfected with SRAP showed an increased motility compared to control cells and inversely SRAP silenced cells showed a significant decrease in cell motility. The corresponding results have been published on Dec 21, 2015 in *FEBS letters; 589 (24PtB): 4010-8* entitled “The Steroid receptor RNA Activator protein (SRAP) controls cancer cell migration/motility.” Our results highlight for the first time a link existing between SRAP expression and cell motility.

**My contribution to this article**

I performed SRA/SRAP “overexpressing” and “silencing” experiment and cell sorting preparations. I conducted all the “live-cell imaging” experiments and statistical analysis. I helped the RNA sequencing statistical analysis.
The steroid receptor RNA activator protein (SRAP) controls cancer cell migration/motility

Yi Yan\textsuperscript{a,b,1}, Charlton Cooper\textsuperscript{a,1}, Mohammad K. Hamedani\textsuperscript{a,b}, Brent Guppy\textsuperscript{a,b}, Wayne Xu\textsuperscript{a}, Deborah Tsuyuki\textsuperscript{a}, Christine Zhang\textsuperscript{d}, Zoann Nugent\textsuperscript{a}, Anne Blanchard\textsuperscript{a,c}, James R. Davie\textsuperscript{a,b}, Kirk McManus\textsuperscript{a,b}, Leigh C. Murphy\textsuperscript{a,b}, Yvonne Myala\textsuperscript{a,c}, Etienne Leygue\textsuperscript{a,b,*}

\textsuperscript{a}Manitoba Institute of Cell Biology, 675 McDermot Ave., R3E0V9 Winnipeg, Manitoba, Canada
\textsuperscript{b}Department of Biochemistry and Medical Genetics, University of Manitoba, 770 Bannatyne Avenue, R3E0W3 Winnipeg, Manitoba, Canada
\textsuperscript{c}Department of Physiology, University of Manitoba, 770 Bannatyne Avenue, R3E0W3 Winnipeg, Manitoba, Canada
\textsuperscript{d}Department of Immunology, University of Manitoba, 413 Apotex Center, 750 McDermot Ave., R3E0T5 Winnipeg, Manitoba, Canada

A R T I C L E   I N F O

Article history:
Received 19 September 2015
Revised 3 November 2015
Accepted 5 November 2015
Available online 17 November 2015

Edited by Ned Mantei

Keywords:
Steroid receptor RNA activator
Non-coding RNA
Functional RNA
Cell motility
Cancer
Steroid receptor RNA activator
Steroid receptor RNA protein

A B S T R A C T

The steroid receptor RNA activator gene (SRA1) produces both a functional RNA (SRA) and a protein (SRAP), whose exact physiological roles remain unknown. To identify cellular processes regulated by SRAP we compared the transcriptome of Hela and MDA-MB-231 cancer cells upon depletion of the SRA/SRAP transcripts or overexpression of the SRAP protein. RNA-seq and Ontology analyses pinpointed cellular movement as potentially regulated by SRAP.

Using live cell imaging, we found that SRA/SRAP depletion and SRAP overexpression lead respectively to a decrease and increase in cancer cell motility.

Our results highlight for the first time a link existing between SRA1 gene expression and cell motility.

\textcopyright 2015 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

1. Introduction

The steroid receptor RNA activator (SRA) was first identified in 1999 as a non-coding RNA able to increase the activity of steroid receptors [1]. Since then, many studies have investigated the mechanisms of action of this functional RNA [2–8]. Through its core sequence (corresponding to exons 2–3–4 and part of exon 5), SRA behaves as a scaffold molecule that physically interacts with a number of RNA binding proteins and transcription factors via specific and critical secondary RNA structures [1–4,9]. These SRA-containing complexes involve a wide range of molecules including, but not limited to, multiple nuclear receptors, nuclear receptor co-regulators, and proteins involved in gene silencing and gene insulation [2,3,8,10–13]. SRA therefore is believed to be a major transcriptional regulator potentially involved in numerous mechanisms including myogenesis, glucose uptake, tumorigenesis and tumor progression [4,6,14–17].

Interestingly, the steroid receptor RNA gene (SRA1), in addition to the pre-cited functional non-coding SRA RNA, also produces a coding mRNA, fully spanning the above-mentioned functional core but also encoding a 236/237 amino acid protein referred to as SRAP [18,19]. Both coding and non-coding SRA transcripts co-exist in human cells and alternative splicing events involving intron 1 control their respective levels [16,20]. As its RNA counterpart, SRAP physically interacts in vitro with several transcription factors [5,21] and might co-activate or repress their action depending on the promoter and the cellular context considered [5,21,22]. We have previously observed that in specific breast cancer subgroups, high levels of SRAP were associated with poor patients survival.
This suggests that SRAP could be a potential prognostic marker and/or contributor to increasing tumor aggressiveness. Even though several groups have now tried to identify SRAP cellular functions, the exact roles played by this protein remain to be identified [23,24].

To identify pathways and processes potentially controlled by SRAP in cancer cells we have herein compared transcriptome modifications occurring upon SRA1 gene silencing and SRAP overexpression.

2. Material and methods

2.1. Cell culture

Hela (ATCC, cat# ATCC-CCL2, passage number 55-65) and MDA-MB-231 (ATCC, cat# HTB-26, passage number 21-31) breast cancer cells were routinely maintained in high-glucose (4.5 g/l) DMEM (Gibco, cat# 11965-092) supplemented with 10% FBS (Hyclone, cat# SH30396.03) and 2 mM l-glutamine (Gibco, cat# 25030).

2.2. RNA interference

Hela cervical or MDA-MB-231 breast cancer cells were plated at 2.5 × 10^5 cells per well in 6-well plates 24 h prior to siRNA treatments. Each well was then transfected with either 100 nM siRNA Non-targeting Control Pool (Dharmacon, cat# D-001810-10-05) or individual ON-TARGETplus SRA1 siRNA: Si-1, Si-2, Si-3, Si-4 (Dharmacon, cat#-J-027192-09-0002, cat#-J-027192-10-0002 cat#-J-027192-11-0002 cat#-J-027192-12-0002) for 72 h using Dharmafect1 (Dharmacon, cat# T-2001) according to the manufacturer’s instructions.

2.3. Cell sorting of cells expressing GFP-only-SRAP and GFP

The details of the artificial “only-SRAP” vector sequence were previously described [22]. This construct was sub-cloned into the commercially available vector pmaxFP-GFP-N (Amaza, cat#VDF-1012) to generate pmaxFP-GFP-SRAP. For SRAP over-expressing experiments, Hela cells were transfected with a pmaxFP-GFP-SRAP (Green fluorescent SRAP representing “only-SRAP”) or an empty GFP vector (Amaza, cat#VDF-1012) in 10 cm dishes for 24 h using Lipofectamine 2000. Transfected Hela cells were washed twice with DMEM supplemented with 2 mM EDTA and harvested in 10 ml sterile capped conical tubes. Approximately 10 million transfected Hela cells were re-suspended in 2 ml sorting buffer containing 1XPBS, 1% FBS, 2 mM EDTA and 10 units DNase per ml. All procedures before sorting were performed at room temperature. The GFP expressing fluorescent Hela cells were sorted using a BD FACSAriaIII cell sorter (BD Biosciences, San Jose, CA) and in 24-well Trans-well insert (BD Biosciences, cat#354578), respectively. After 72 h Si-cont or Si-pool treatment, a total of 100000 cells, resuspended in serum-free medium alone (invasion assay) or supplemented with 2.5 ng/mlTGF-b1 (migration assay), was added to the insert and incubated in the bottom chamber filled with 10% fetal bovine serum-containing medium. After 24 h (invasion) or 6 h (migration assay), cells on the upper surface of the trans-well were removed using cotton swabs, and the cells that migrated to the bottom of the membrane were fixed with 4% paraformaldehyde in PBS (USB Corporation, Cleveland, OH) and stained with 0.25% crystal violet. Cell micrographs were taken at 100x on standard bright field microscope equipped with a digital camera. Cell counts were performed using the ImageJ software (http://rsb.info.nih.gov/ij/) and the Cell Counter plug-in. Cells were counted from a minimum of four random fields per experimental conditions. Two independent experiments were performed in triplicate. For each experiment, results were expressed as the percentage of the average number of cells counted in Si-cont treatment.

2.4. SOLiD sequencing

cDNA libraries were prepared from RNA-depleted total RNA (200–500 ng) of MDA-MB-231 or Hela cells transfected with the various siRNAs or overexpressing GFP or GFP-only-SRAP, using the SOLID™ Total RNA-Seq Kit protocol according to the manufacturer’s instructions (Life Technologies).

The SOLID paired read sequences were mapped onto human reference genome hg19 using LifeScope software package (Life Technologies) with 2-mismatch setting and the best score mapping when multiple mapping occurs. The sequence quality was inspected by Bamstats module. For a given gene the mapped sequence reads were counted against the current gene annotation General Transfer Format (hg19.gtf). Reads that map to overlapping exons or features of a given gene are counted only once. For each gene, the raw read data were normalized and expressed as reads per kilobase per million reads (rpkm). Genes with 0 rpkm in any one sample were not considered for analysis.

The log of the ratio “experimental point (Si-pool, Si-1, Si-2, Si-3, Si-4, or only-SRAP) rpkm”/“Corresponding Control rpkm” was calculated for each gene. Gene Ontology (GO) analysis was performed using the Ingenuity Pathway Analysis (IPA, Ingenuity Systems, Inc) [27].

2.5. RT-qPCR

Total RNA was extracted from cells with RNeasy Plus kit (Qiagen) and first strand cDNA synthesis was carried out using M-MLV reverse transcriptase and random primers (Invitrogen) following the manufacturer’s instruction. SYBR Green quantitative PCR amplifications were performed on the Biorad iCycler Real-Time PCR system with the following thermal profile: 95 °C for 4 min followed by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. The following human primers were used to amplify SRA1 (forw: TTGGAACAGCCATTGGAAC, in exon 3, rev: ACAACTTCTTCCAGCCCAC, in exon 4), and GAPDH (forw: ACCCCTCTCCTCACCTTTG, rev: CTCTTGTCCTTGTGCGG). Data is expressed as a percentage of control treated cells using the 2^(-ΔΔCt) method with GAPDH gene expression as RNA input control for three independent samples measured in duplicate per condition.

2.6. Cell invasion and trans-well migration assays

MDA-MB-231 cell invasion and motility were assayed using a two-chamber trans-well system containing an 8-μm cell culture insert pre-coated with growth factor-reduced Matrigel (BD Biosciences, San Jose, CA) and in 24-well Trans-well insert (BD Biosciences, cat#354578), respectively. After 72 h Si-cont or Si-pool treatment, a total of 100000 cells, resuspended in serum-free medium alone (invasion assay) or supplemented with 2.5 ng/mlTGF-b1 (migration assay), was added to the insert and incubated in the bottom chamber filled with 10% fetal bovine serum-containing medium. After 24 h (invasion) or 6 h (migration assay), cells on the upper surface of the trans-well were removed using cotton swabs, and the cells that migrated to the bottom of the membrane were fixed with 4% paraformaldehyde in PBS (USB Corporation, Cleveland, OH) and stained with 0.25% crystal violet. Cell micrographs were taken at 100x on standard bright field microscope equipped with a digital camera. Cell counts were performed using the ImageJ software (http://rsb.info.nih.gov/ij/) and the Cell Counter plug-in. Cells were counted from a minimum of four random fields per experimental conditions. Two independent experiments were performed in triplicate. For each experiment, results were expressed as the percentage of the average number of cells counted in Si-cont treatment.

2.7. Wound healing assay

Twenty-four hours post Si-cont or Si-pool treatment, cell suspensions were prepared in 10% FBS-DMEM (Gibco cat# 11966-025) supplemented with 0.9 g/l glucose and 2 mM l-glutamine.
MDA-MB-231 cells ($1.5 \times 10^5$) were seeded into 24-well plates and grown overnight. The next day, confluent cell monolayers were scratched with a p200 tip then washed 3 times in PBS before replacing growth media. To image the same area pre- and post-wound healing, marker lines were made perpendicular to the scratch on the bottom of the plate to orient images. Images were taken at time of scratch (T0) and 24 h post scratch. Cell micrographs were taken at 40× magnification on a standard phase contrast microscope equipped with a digital camera. Wound areas were delineated using the ImageJ software and the freehand selection tool. Average area of wounds before and after 24 h healing were obtained from four independent experiments, with each experiment having a
minimum of three independent scratch areas measured per condition. For each scratch, results were expressed as percentage of open wound (T0) remaining after 24 h. Differences between Si-cont and Si-pool tested with the two-tailed Student’s t-test.

2.8. Time-lapse live cell migration assay

MDA-MB-231 cells were transfected in triplicate with 25 nM Non-targeting Control, Pooled or individual SRA siRNA using Dharmafect1 for 48 h. Cells transfected with SRA siRNA and control siRNA were incubated with 10 mM CMTMR (red fluorescence, Invitrogen Cat: #C2927) and CMAC (blue fluorescence, Invitrogen cat: #C2110), respectively, for 10 min. Transfected and fluorescence-labeled cells were subsequently re-seeded together with a mixture of unlabeled cells at a ratio of 1:5 in an optically clear bottom 96-well cell culture plate (Nunc). The re-seeded cells were cultured in DMEM medium (Gibco, cat# 11965-092) supplemented with 10% FBS. To allow for acclimation prior to imaging, plates were incubated at 37 °C, 5% CO2, for 2 hrs, in a Cytation 3 imager (Biotek) equipped with a live cell module. Live cell images were then acquired at 30-min intervals, using a 10× dry objective, for a total of 10 hrs. Image files (.tiff) were exported to Imaris (Bitplane), and cell mobility tracks were generated using a semi-automated cell-tracking feature. Wind-rose plots were generated from at least 3 independent experiments. Single cell speed was derived from dividing total cell path length by the total time of migration. Cell displacement length (μm) is defined as the linear distance between the first point to the last point of migrating cells. Total distance (μm) depicts total length of a migratory path.

For “only-SRAP” over-expression experiments, Hela cells were first co-transfected with a pmaxFP-GFP-SRAP-only vector (green) representing “SRAP-only” [22] and empty PM-cherry vectors (red, cat: #C2927). Transfected cells were tracked for 10 h. Cell migration data were generated using a Cytation 3 imager (Biotek) equipped with 10× phase-contrast objectives. Cell tracks were generated using semi-automated cell tracking and represent single cell tracks over 10 h with 30 min intervals.

2.9. Western blotting

Total cell lysates were separated on 10% SDS–PAGE and analyzed by Western blotting as described previously [23].

2.10. Statistical analysis

Differences of total cell track length, displacement length and average speed between SRA siRNA or control siRNA treated Hela cell were tested using a two-tailed Wilcoxon test. Box and whiskers representation was performed with boxes at 25–75% and whiskers at 5–95%. Regression model was used to test the statistical significance of the different SRA siRNA treatments on dependent variables such as cell track length, and displacement length.

3. Results and discussion

3.1. Gene Ontology analysis of genes inversely regulated by silencing the SRA1 gene and overexpressing SRAP identifies cell movement as potentially regulated by the protein

To identify cellular processes potentially regulated by SRAP in cancer cells, we chose to monitor the changes in gene expression induced by silencing the SRA1 gene and by overexpressing the protein. Indeed, we reasoned that genes inversely regulated following these two manipulations of SRAP expression would provide a reliable indicator of the cellular events involving this protein. Three sets of complementary experiments were performed. First, SRA1 gene expression was silenced using a pool of siRNAs (Si-pool) in both MDA-MB-231 breast cancer and Hela cervical cancer cell lines as described in the Section 2. Two different cell lines were used to try to identify “global” rather than cell type specific processes. In order to minimize potential erroneous results linked to documented off-target effects of pooled Si-RNAs [25], a second set of experiments was performed on Hela cells using pooled as well as individual Si-RNAs (Si-1, Si-2, Si-3 and Si-4). All the SRA siRNAs used in these experiments led to a robust decrease of SRA RNA and SRAP levels both in MDA-MB-231 (data not shown) and Hela Cells (sFig. 1). Interestingly, if Si-1, 2 and 3 have efficiencies similar to that of Si-pool, Si-4 has always a smaller impact on both SRA RNA and SRAP expressions (sFig. 1, last column). All SRA isoforms, those functional as RNA (i.e., containing a full core exon-2 to exon-5) as well as those encoding SRAP (i.e., containing exon-1 in frame with this core, are similarly down-regulated with the different combinations used (sFig. 2). It should be noted that this simultaneous down-regulation of coding and non-coding isoforms by these same interference RNAs has already been reported by McKay et al. [25].

Because interference approaches do not allow to discriminate between processes regulated by SRA RNA and SRAP, we performed a third complementary set of experiments in Hela cell overexpressing only SRAP and not its functional RNA. We used a previously described construct, “only-SRAP”, in which SRA sequence has been extensively mutated by silent mutations throughout the whole functional core SRA sequence. These mutations alter the critical...
secondary structure of SRA, necessary for its RNA action, but do not interfere with its coding properties [9,22,28]. In order to easily identify, sort and isolate cells overexpressing SRAP, this “only-SRAP” construct was cloned in frame with GFP. Hela cells were transfected with GFP-SRAP (control) or with GFP alone for 24 h. Transfected cells were subsequently sorted using a BD FACSAriaII cell sorter, and GFP-positive cells were re-suspended and re-seeded before analysis for 24 h as described in the Section 2. The transfection efficiency as well as the final enrichment in GFP-tagged molecules have been checked by immunofluorescence and Western blot (data not shown).

Deep RNA sequencing (RNA-seq) of transcriptomes corresponding to the differently treated cells has been performed as described in the Section 2. Results (18 gene sets) have been deposited in the Gene expression Omnibus (GEO) database (accession number #GSE74461).

For control and Si-pool treated MDA-MB-231 cells, two independent experiments were performed (A and B), producing 39.2, 30.3, 25.1 and 24.5 million reads for Cont-Si-231-A, Si-pool-231-A, Cont-Si-231-B and Si-pool-231-B, respectively (Table S1).

For control and Si-pool treated Hela cells, two independent experiments were performed (A and B), producing 18.2, 28.1, 31.8 and 18.0 million reads for Cont-Si-Hela-A, Si-pool-Hela-A, Cont-Si-Hela-B and Si-pool-Hela-B, respectively (Table S2).

For Hela cells treated with pooled or individual Si-RNAs, one experiment (Hela-C) was performed, leading to 33.6, 43.0, 31.9, 48.5, and 60.4 million reads for Si-Cont-Hela-C, Si-pool-Hela-C, Si-1-Hela-C, Si-2-Hela-C, Si-3-Hela-C and Si-4-Hela-C, respectively (Table S3).

For Cont-GFP and “GFP-only-SRAP”-expressing Hela cells, two independent experiments were performed (A and B), producing 65.3, 19.0, 42.6 and 51.7 million reads for Cont-GFP-Hela-A, SRAP-Hela-A, Cont-GFP-Hela-B and SRAP-Hela-B, respectively (Table S4).

For each transcript and each experiment, corresponding reads were normalized and expressed as reads per kilobase per million (rpkm). Genes without read in any of the 18 sets obtained were discarded. As such, information was gathered on 16557 genes. The log of the ratio “experimental point (Si-pool-231-A, Si-pool-231-B, Si-pool-Hela-A, Si-pool-Hela-B, Si-pool-Hela-C, Si-1-Hela-C, Si-2-Hela-C, Si-3-Hela-C, Si-4-Hela-C, SRAP-Hela-A or SRAP-Hela-B) rpkm”/”Corresponding Control rpkm” was calculated for each gene (Table S5).

Out of 16557 genes monitored, 50 genes had a negative and positive log value in the nine “silencing” and two “SRAP overexpression” gene sets, respectively. Inversely, 56 had a positive and negative log value in the nine “silencing” and two “SRAP overexpression” gene sets, respectively. As such, 106 genes had their levels of expression modified in opposite direction when silencing the SRA1 gene or overexpressing SRAP (Table S6, Fig. 1). It should be noted that, in this analysis, we have not applied any cut-off value and only focused on log values strictly negative or positive in the “silencing” and “overexpression” datasets. As such, the aforementioned changes in gene expression are mostly very small. Interestingly, McKay et al. concluded in their recent study, using a cut-off of two fold changes, that not a single gene appeared to be significantly regulated upon silencing SRA1 expression with individual siRNAs [25]. Our results corroborate their conclusion and suggest that SRAP does not act as a major transcriptional regulator.

Gene Ontology (GO) analysis of the 106 genes inversely regulated in “silencing” and “overexpressing” experiments identified...
cell movement/migration as the largest functional group (with 20 genes) potentially associated with the cellular role of SRAP (Fig. 1 genes in red, Table S7).

3.2. Silencing of SRA/SRAP expression decreases the motility of MDA-MB-231 cells

To further investigate the potential involvement of SRA/SRAP in the movement of cancer cells, we first assessed the effect of silencing the SRA1 gene in the highly invasive MDA-MB-231 cell lines. Cells were treated with Si-cont or Si-pool and their ability to move across 8-µm pore culture inserts was monitored as described in the Section 2. MDA-MB-231 cells expressing decreased levels of both SRA and SRAP migrated significantly less through a matrigel pre-coated insert than did control cells (sFig. 3A). This corroborates the previous observation by Foulds, et al., who concluded that silencing SRA1 decreased invasion properties of MDA-MB-231 cells, as assessed using the same “invasion assay” [15]. It should however be noted that this assay does not allow to truly differentiate between change in motility and modification of the ability to move through matrigel. As such, in the absence of matrigel cells expressing decreased level of SRA/SRAP also seemed

Fig. 4. Decrease in motility of individual MDA-MB-231 cells upon SRA knock-down. Cells were treated and tracked as in Fig 3. (A) For each condition (Si-cont Ci, Si-pool, Si-1, Si-2, Si-3, Si-4), Wind-rose plots were generated from at least 3 independent experiments. (B) For each condition (Si-cont Ci, Si-pool, Si-1, Si-2, Si-3, Si-4), Total cell track length (µm), cell displacement track (µm) and average speed (µm/h) were determined using cell tracking module and analyzed by the Imaris software, as described in the Section 2. Track length depicts the total length of a migratory path (µm). Track displacement (disp) length (µm) is defined as the linear distance between the first point to the last point of migrating cells. Single cell speed (µm/h) was derived from dividing the total cell path length by the total time of migration. $n > 45$ red cells; $n > 45$ blue were included in the analyses. The box indicates 25th and 75th percentile and the whiskers 5th and 95th percentile. The line in the box indicates the median of the distribution. Wilcoxon test was used to calculate the statistical significance between control and SRA siRNA (** $P < 0.001$, * $P < 0.05$).
Fig. 5. Increase in motility of individual Hela cells upon overexpression of SRAP. Hela cells were co-transfected with pmaxFP-GFP-SRAP-only (green) and empty Pmcherry vectors (red) as detailed in the Section 2. Orange cells express both vectors after co-transfection. Live cell images were then acquired at 30-min intervals, for 10 h and cell mobility tracks generated and analyzed as described earlier. (A) The left panels show the same still picture of one of the field analyzed to track SRAP-only overexpressing cells (green cells, top panel), SRAP-only and control overexpressing cells (orange cells, middle panel) and control only overexpressing cells (red cells, bottom panel). Cell tracks represent the displacement of a single cell over the 10 h period. Corresponding videos are provided as Supplementary videos S3–S5. (B) Wind-rose plots (left panels) were generated from at least 3 independent experiments. Total number of cells assessed are indicated. (C) Total cell track length (\(l_m\)), cell displacement track (\(l_m\)) and average speed (\(\mu m/h\)) were determined using cell tracking module and analyzed as described in the Section 2. The box indicates 25th and 75th percentile and the whiskers 5th and 95th percentile. The line in the box indicates the median of the distribution. Wilcoxon test was used to calculate the statistical significance between “SRAP-only” overexpressing cells (green and orange cells) and control cells (red cells). Wilcoxon test was used to calculate the statistical significance of differences observed (***\(P < 0.001\)).
to migrate less toward the lower chamber than did control cells (sFig. 3B). This suggests that the results from the invasion assays (sFig. 3A) were at least partially linked to an overall decreased migration of cells under-expressing SRA/SRAP. This negative effect of silencing SRA1 gene on cell migration was further confirmed using a classical wound-healing assay (Fig. 2). In this latter assay, cell treated with Si-pool migrated less in the wound compared to Si-cont treated cells.

It should be noted that even though invasion-, transwell- and wound-healing assays clearly hint that depletion of SRA/SRAP in MDA-MB-231 cells decreases their ability to migrate as a whole, they do not provide information as to whether SRA1 silencing in individual cells impacts directly on their motility. Actually, observed phenotypic changes could result in change in the ability to detect chemo-attractant, a hallmark of all trans-well insert assays. Similarly, in wound-healing assays, a decrease in “wound-closing” may also result from a defect in a secreted factor affecting other cells.

To address these as well as other described potential limitations of conventional motility assays such as depending on proliferation rate or transfection efficiency [29], we used live-cell imaging to monitor the motility properties (track length, mean track speed and directional displacement track length) of individual MDA-MB-231 cells depleted in SRA/SRAP. Motility properties were assessed as described in the Section 2. Briefly, cells were treated 48 h with Si-SRA-RNA (Si-1, Si-2, Si-3 and Si-4 individually or pooled) or with Si-cont (Ci) before being incubated with 10 nM CMTMR (red fluorescence) or CMAC (blue fluorescence), respectively. Cells were subsequently re-seeded together with a mixture of unlabeled cells, and live cell images acquired at 30-min intervals for a total of 10 hrs. Cell motility tracks were generated using a semi-automated cell-tracking feature. Fig. 3A illustrates an example of a still picture of a field used to monitor motility of cells treated by Si-pool (red cells, top left panel) and Si-cont Ci (blue cells, bottom left panel). The corresponding videos are attached as Supplementary videos 1 and 2. The respective wind-rose plots, visually displaying cell tracks emanating from a centralized point to allow cell motility to be compared across various treatment, are shown for 31 and 30 individual cells treated by Si-pool (red, top right panel) and Si-cont Ci (blue, bottom right panel), respectively (Fig. 3B).

Overall, cells treated with Si-pool, Si-1, Si-2 and Si-3 migrated constantly less and with a lower velocity compared to control siRNA treated cells (P < 0.0001, Wilcoxon test) (Fig. 4A and B). In contrast, cells treated with Si-4 moved with apparent characteristics similar to those of the control cells. Average speed and track length were highly correlated during the statistical analyses (data not shown). By using a regression model where track length or speed are the dependent variables, no significant differences are found between Si-pool and individual Si-1, Si-2, Si-3 groups. Treatment with Si-4 however differs from all other SRA siRNA treatments (data not shown).

As outlined earlier, Si-4 siRNA was always less efficient than others in decreasing SRA and SRAP expression (sFig. 1). Interestingly, this specific Si-4 was also less efficient when used by others [25]. The apparent lack of action of silencing SRA/SRAP with Si-4 on cell motility as assessed here could potentially result from the fact that low remaining amounts of SRA/SRAP (i.e. 7% and 11% of initial levels for SRA and SRAP, respectively (sFig. 1)) are still sufficient for the cell to move with no apparent modifications. To investigate the function of the protein, we have favored this live cell assay for several reasons. This assay allows the simultaneous visualization in the same field of “only-SRAP” expressing cells (green), negative control transfected cells (red), as well as “positive-control” cells (co-transfected with both SRA and control plasmid, orange). As opposed to transwell- or wound-assays, results here do not depend upon transfection efficiency or intercellular differences in expression levels, key parameters to consider in overexpression experiments. More importantly, this live imaging assay allows to clearly establish whether individual cells affected by the change monitored (in our case visible detection of only-SRAP overexpression) are the ones with a modified motility. Overall, it provides a powerful way to observe the exact cellular role played by the protein SRA1 independently of its RNA counterpart.

Cell motility was assessed as described earlier. Fig. 5A left panels show the still images of one of the fields analyzed to track only-SRAP transfected cells (top panel, green), only-SRAP and control transfected cells (orange, middle panel) and control only transfected cells (red, bottom panel). The corresponding videos are presented in Supplementary videos S3–S5, respectively. The wind-rose plots showing the specific tracks analyzed are presented Fig. 5B right panels. Compared to red cells, both orange and green cells, which over-express only-SRAP, migrated faster and farther away from the original point of recording compared to control cells (P < 0.0001, Wilcoxon test) (Fig. 5C). Average speed and track length were highly correlated during the statistical analyses (data not shown).

Altogether our results show that depleting cells of the products of the SRA1 gene leads to a decrease in their ability to move. Inversely, overexpressing the only-SRAP construct that encodes the protein without its functional RNA counterpart allows cells to move faster and further. This is, to our knowledge, the first time that a clear link is established between the very unusual bi-faceted SRA/SRAP system and cell motility. The mechanisms involved in this apparent action of SRA1 remain to be identified.

**Competing interests**

No competing interests to be declared.

**Acknowledgement**

This work was supported by a grant from the Canadian Breast Cancer Foundation (CBCF).

**Appendix A. Supplementary data**

Supplementary data associated with this article can be found, in the online version, at [http://dx.doi.org/10.1016/j.febslet.2015.11.007](http://dx.doi.org/10.1016/j.febslet.2015.11.007).

**References**


Supplementary Figures legends

sFig. 1: SRA RNA and SRAP expression upon different SRA siRNAs silencing
SRA RNA and SRAP expression level were assessed by RT-qPCR (A) and Western blotting (B), respectively, after knocking down SRA1 gene expression using SRA Si-pool and individual SRA siRNAs (Si-1, 2, 3 4) in Hela cells, as described in the Materials and Methods section. Because primers used in the RT-qPCR anneal with exon 3 and exon 4 sequences, they can amplify both coding and functional isoform sequences. Mock transfections by scrambled siRNAs (Ci) was used as control. Bars represent standard deviation from 3 independent experiments.

sFig. 2: Both functional and coding isoforms are similarly decreased by Si-RNA treatment
Average ~45 million pairs (50bp×35bp) of SOLiD sequences per sample were generated for 6 Hela cell samples, treated with Si-Control (orange), Si-pool (red), Si-1 (dark blue), Si-2 (light blue), Si-3 (green), or Si-4 (yellow). The quality check and quality trimming were applied to the sequence files. The sequence reads were mapped on human reference genome (hg19) using Lifescope package2.5.1 (LifeTech) with 2-mismatch settings. The mapped bam files were used for genomic feature visualization by using Partek Genomics Suite v6.6 (Partek Inc. St Louis, USA). The X-axis represents the gene location and genomic features. The Y-axis represents the mapped read coverage of each sample track. E1-E5 represents SRA1 exons. The functional RNA core is defined by an integral exon-2, exon-3, exon-4 and part of exon-5 [1, 19].

sFig. 3: Silencing SRA1 expression reduces MDA-MB-231 cell migration as assessed by Invasion- and trans-well assays
MDA-MB-231 cells were treated with Si-cont or Si-pool and cell invasion and motility were assayed using trans-well systems containing an 8-µm cell culture insert pre-coated with Matrigel (A) or not (B) as described in the Materials and Methods section. Left: Representative micrographs of cells stained with crystal violet stained from the lower surface of the Transwell insert. Right: Quantification of number of crystal violet stained cells per field was performed as described in Materials and Methods. Columns: normalized average cell counts, bars: standard deviation. Two experiments were performed
in triplicate. Star: significant differences between Si-cont and Si-pool (Student's $t$-test, two-sided ($P<0.05$)).
A

relative % SRA

<table>
<thead>
<tr>
<th>ci</th>
<th>Si-pool</th>
<th>Si-1</th>
<th>Si-2</th>
<th>Si-3</th>
<th>Si-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

B

SRAP

GAPDH

relative % SRAP

<table>
<thead>
<tr>
<th>ci</th>
<th>Si-pool</th>
<th>Si-1</th>
<th>Si-2</th>
<th>Si-3</th>
<th>Si-4</th>
</tr>
</thead>
<tbody>
<tr>
<td>117</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Fig. 2

transcript -

E5  E4  E3  E2  E1
A

Si-cont

Si-pool

B

Si-cont

Si-pool

sFig. 3
Conclusion of Chapter VI

1. The characterizations of transcriptome of Hela cervical cells and MDA-MB-231 breast cells upon depletion of \textit{SRA1} gene or overexpression of SRAP identified 106 genes, which had their levels of expression modified in opposite direction upon silencing or overexpression of SRAP.

2. Gene ontology analysis of genes inversely regulated by silencing and overexpressing SRAP identifies cell movement as a potentially function regulated by changes of SRAP expression.

3. Silencing of \textit{SRA1} gene expression decreases the migration and invasion of MDA-MB-231 cells as assessed by classical transwell migration, matrigel pre-coated invasion assays and wound-healing assays. This negative effect was further confirmed by live cell imaging.

4. Using live cell imaging, I found that MDA-MB-231 breast cells treated with individual or pool of SRA siRNA migrated constantly less with a lower velocity than control siRNA treated cells.

5. Inversely, overexpressing SRAP protein allowed to cells move faster and further compared to control cells. Overall this result suggests the existence of an association between \textit{SRA1} gene expression and cell motility.
CHAPTER VII  Discussion

VII-1  Overall summary

I first reported that different SRAP peptides could be detected by western blotting and have variable expression patterns in breast tumours. I have subsequently tried and defined the clinical relevance of SRAP expression in breast tumours using tissue microarrays (TMAs). Overall, a higher expression of SRAP peptides was associated with poor prognosis in ER-α-positive patients, suggesting it might be considered as a poor prognostic marker in this tumour subgroup. Using the same TMA approach, I have further demonstrated that high expression of SRAP-like peptides not only correlated with high ER-β1 levels but also significantly predicted better tamoxifen responsiveness in ER-negative patients. This later finding highlights the potential for SRAP level of expression to be considered as a new predictive factor of response to endocrine therapy in these patients. Lastly, I have explored the cellular processes potentially regulated by this protein. Upon depletion and overexpression of this protein, I have identified cellular movement as potentially affected by changes in SRAP expression. I have further corroborated this hypothesis by using classical trans-wells assays as well as a novel live-cell imaging assay.

My study has raised critical questions that remain to be addressed.

VII-2  What is the real significance if any of assessing SRAP expression in breast tissue?

I have earlier emphasized in the introduction section of this thesis the need for identifying new prognostic and predictive markers. Can SRAP be one of them?
Is SRAP a prognostic marker?

At first, when evaluating the prognostic value of SRAP, the opposite prognostic values of SRAP expression were observed. In a previous study emanating from our laboratory, it was found using western blot analysis by “in house” antibody (recognizing N-terminal of SRAP) [Fig VII-1], that the detection of a signal corresponding to the expected 30kDa SRAP-peptide was associated with a better overall survival from breast cancer in ER-α positive patient [148]. However, in Chapter II and III, I present evidence suggesting that a global higher expression of peptides recognized by antibodies specifically targeting SRAP was associated with poor prognosis in a similar ER-α positive cohort [148]. A possible explanation for the apparent discrepancy of these findings could lie in the traditional pitfalls of the different techniques used in these studies. First, multiple frozen tissue sections for western blot analyses were homogenized and therefore the extract will come from a mixture of cell types present in any tumour biopsy sample including normal and neoplastic epithelial, fibroblast, vascular and inflammatory cells, all of which express SRAP. Therefore the final signal results from the sum of the signals corresponding to multiple cell types. In other words, assessed SRAP expression consists of the expression of cancer as well as other normal cells. In contrast to what was highlighted

Figure VII-1. Schematic profile of SRAP antibodies used.
The two evolutionary conserved domains, CD1 and CD2, in are highlighted in green and pink color. Proline-rich area is also labelled in yellow. Different SRAP epitopes are recognized by the “in house” anti SRAP rabbit polyclonal antibody (RP), the commercially available anti-SRAP rabbit polyclonal antibodies from Bethyl Laboratories A300- 742A (742) targeting at N-terminus and A300-743A (743) targeting at C-terminus.
for Western blots, the studies involved tissue microarray (TMA) where only neoplastic epithelial cancer cells were scored within each tissue section. Because any tumour sample is heterogeneous with respect to cellularity in addition to that already inherent in the tumour cells themselves, each assay will assess different part of any tumor samples. This will also introduce difficulties in comparing one to the other.

Also, Western blotting analysis limits the number of samples that is feasible to analyze. The first study by Chooniedass-Kothari et al has therefore an obviously limited statistical value. The two studies reported in this thesis have used tissue micro-arrays. Unlike western blotting this approach allows to investigate SRAP expression in more than 120 paraffin core tissue sections at a time on a single slide. Tissue micro-arrays is currently one of the most commonly used methods of biomarker studies for breast cancer [153]. We have tried to minimize analytical errors often associated with this approach by carefully characterizing the antibodies used and processing samples with an automated Ventana machine. To overcome potential inconsistency of interpretation of staining, tissue microarray staining was evaluated by at least two investigators independently; in cases where discordance was found, slides were re-evaluated together and an agreement was reached.

Further differences between the two techniques might also result from different sample processing conditions (including transport of samples), times to fixation, and/or length of fixation; all of which could indeed be also responsible for the apparent inconsistency observed between previous and present studies. Overall, we cannot completely exclude the possibility that the differences observed between “bad” and “good” prognostics in ER-α positive patients might be technical. Therefore, it is imperative that these results are further validated.

Besides the inherent pitfalls of the approaches used, other factors such as the diverse origins and natures of SRAP peptides detected by the different techniques could also
explain opposite tendency of prognostic indications of SRAP. In the previous publication, two SRAP peptides ~30kDa and ~25kDa were detected by Western blotting [148], using an antibody raised against the very well conserved N-terminal domain [Fig VII-1]. The detection of these bands was considered to be specific as the corresponding signals disappeared when the antibody was pre-incubated with an excess of the peptide it was raised against. It is important here to note that even if the expression of the ~30 kDa SRA peptide positively correlated with a better overall survival [148], the expression of the ~25kDa SRAP peptide did not. In the Chapter IV, I have re-assessed SRAP expression in multiple breast tumour tissues using commercially available SRAP antibodies recognizing N- and C-terminal epitopes (742A and 743A, respectively) [Fig VII-1]. During the antibody characterization phase of my study, I found that the antibody targeting SRAP at its C-terminus (743A) could detect in breast tumours at least four peptides migrating at ~25 kDa and ~30kDa ~40kDa and ~48kDa whereas the one targeting SRAP at its N-terminus (742A) recognized three bands migrating at ~26 kDa, ~30kDa and ~48kDa when assessing multiple tumours by western blot analysis. Different pattern of SRAP peptides expression in some tumours were noted as some tumours expressed only one SRAP peptide at ~30kDa whereas others expressed other bands. All signals disappeared when the antibody was pre-incubated with the corresponding blocking peptide, suggesting that the corresponding recognition is specific. We are confident that the ~30kDa peptide, recognized by both N- and C- terminal SRAP antibodies, represents the full-length 236-237 amino acid long SRAP protein. Unfortunately, identities of the others bands remain unknown. Among them, a “short” form at ~25kDa band detected by N-terminal SRAP recognizing antibodies in both previous and attached studies but absent with C-terminal SRAP antibody (743A) may represent a C-terminally truncated form of SRAP peptides possibly resulting from alternative splicing. The other relative “longer” forms of SRAP peptides migrating at ~ 40kDa detected by 743A but not 742A might suggest that
they contain additional unknown sequences (amino-acids) or alternative post-translational modifications [150]. In contrast, 743A antibody fails to detect the ~26kDa peptides recognized by 742A, potentially for similar reasons. Additionally, only the ~30kDa SRAP peptide was detected in the parallel control protein extracts from normal breast tissue obtained from reduction mammoplasty samples [154]. This leads to the intriguing hypothesis that tumor specific mechanism may be responsible for the generation of the alternate SRAP-like peptides. If differential levels of those SRAP peptides indeed possess distinct prognostic values in tumour specific manner, this could potentially explain the discordance noticed between two studies. Therefore, it is crucial to clarify the distinct origin of individual SRAP peptides. This would be further illustrated in the future direction part of this discussion.

The apparent bad prognostic value of high SRAP expression found in the ER-α positive cohort from the Manitoba Breast Tumour Bank (MBTB) was not seen in a similar sub-cohort of National Cancer Institute of Canada (NCIC-CTG-MA12) patients (Chapter VI) [Table VII-1]. One should note that these two independent ER-α positive cohorts are slightly different. Indeed, all ER-α positive patients recruited in the Manitoba Tumour Bank (MBTB) subsequently received tamoxifen treatment, whereas in the National Cancer Institute of Canada (NCIC) cohort, patients on the trial had all previously received chemotherapy and then subsequently only a portion of ER-α positive patients underwent endocrine therapy; the rest of patients being recruited as placebo-controlled group. The age of the patients also differed. Age of patients included in the Manitoba Breast Tumour Bank (MBTB) cohort ranges from 37 to 92 years old with median of 67 years old whereas the age of National Cancer Institute of Canada (NCIC-CTG-MA12) patients are much younger ranging from 26 to 58 years old with medium of 45.7 years old. Alternatively, previously mentioned technical variations such as processing conditions in multiple different locations could be also associated with the inconsistency observed.
Is SRAP a predictive marker?

With regard to the predictive value aspect of SRAP clinical significance, high SRAP levels significantly predicted a better responsive to tamoxifen in the patients with ER-α-negative tumours from the cohort recruited by the National Cancer Institute of Canada (NCIC-CTG-MA12) (Chapter V). This indicates the potential for SRAP to be considered as a new predictive marker of tamoxifen sensitivity in patients affected by ER-α-negative tumours. Normally, these patients are primarily treated by chemotherapy instead of tamoxifen due to the absence of expression of ER-α in their primary tumours. It is thus a bit counter-intuitive that this specific subgroup still benefit from tamoxifen treatment. However, as outlined in chapter V, tumours expressing high level of SRAP also expressed high levels of ER-β1. Thus, it is tempting to speculate that the positive tamoxifen effect seen in this cohort might be mediated alternatively through ER-β1 rather than ER-α. Interestingly, ER-β1 level declines during breast tumourigenesis [155], and a tumour suppressor role for this receptor has even been suggested [156]. Most, but not all,
studies assessing ER-β1 expression in relation to clinical outcome in breast cancer have indeed showed a significant association of higher expression with better clinical outcome [156].

Interestingly, several groups have already mentioned the existence of potential crosstalks between SRA/SRAP and ER-β1 signaling pathways. We have indeed previously suggested the possibility of a physical interaction between SRAP and ER-β1 in vitro [147]. We have also found that SRAP was recruited to gene promoter regions where ERE binding sites were significantly enriched [147]. These studies suggest that SRAP could form complexes with ER-β1 and modulate its activity in a promoter specific manner. Further, Klinge et al. (2004) showed that SRA RNA could also modulate ER-β1 activity in a ERE dependent manner [157]. Thus, overall it can be speculated that the apparent cumulative predictive value of a high expression of ER-β1 and SRAP might result from a synergistic effect of these two molecules. Such hypothesis requires further investigation. Future studies investigating genes and pathways potentially co-regulated by SRAP and ER-β1 in ER-α-negative cells would indeed help understand how patients bearing primary tumours devoid of ER-α receptor could benefit from tamoxifen treatment.

Another possible explanation why such predictive value of SRAP is only seen in ER-α negative rather than ER-α positive cohort may perhaps lie in a differential expression of the different SRAP peptides I have described. If so, may be specific SRAP peptides could possess distinct even opposite predictive/prognostic values. This again needs to be further investigated with understanding, and characterizing the identity and functions of the different peptides specifically recognized with our antibodies.

VII-3 What are the exact roles played by SRA/SRAP in breast cancer cells?

Trying to decipher the exact roles of the system SRA/SRAP is inherently difficult as there are two players, which can have different and/or complementary/opposite functions.
As mentioned previously, alternative splicing of SRA is one of the mechanisms allowing cells to control the level of two SRA species in the cells. For example, cells can produce a functional non-coding SRA RNA through retention of partial or full intron-1. These transcripts, which have a function, will however not produce any SRAP protein. Alternatively, it appears challenging for cells to produce only SRAP proteins without functional SRA RNA. Indeed, the coding SRA transcripts still contain an intact functional core expected to also function at the RNA level.

One could hypothesize that specific post-transcriptional modifications of the transcripts, such as pseudo-uridinylation by pseudo-uridine synthases Pus1p and Pus3p (see Chapter 2.2), might contribute to the production of coding transcripts with altered protein function. Indeed, Zhao et al. (2007) found that SRA could be pseudo-uridinylated by these two enzymes at similar sites and that these events regulated the steroid receptor activation by SRA transcripts [110,111]. Interestingly these authors also showed that the mutation of a single residue U206A in STR-5 [Fig VII-2], which is a common site of pseudo-uridinylation by Pus1p and Pus3p led to an overall hyper-pseudo-uridylation of SRA RNA [110]. Further, the introduction of this silent mutation, which did not modify the reading frame of SRAP, however led the resulting RNA construct to act as negative regulator of steroid receptor activity [110]. One could therefore speculate that alternative pseudo-uridinylation of SRA RNA by the two pseudo-uridine synthases might regulate the SRA RNA activity without changing the ability of those transcripts to be translated.

In order to try and understand the biological roles potentially played by SRA/SRAP in breast cancer, it is important here to summarize and reassess the various functional data that have been gathered since the discovery of SRA in 1999.
VII-3-1 SRA RNA side of the SRA/SRAP bi-faceted system

The first series of functional assays have been performed mostly by overexpressing non-coding SRA constructs generated by Lanz et al [158] in various cell lines and using reporter luciferase assays to assess the effects on multiple transcription factors [109,113-116,159]. These studies overall suggested that SRA RNA forms ribonucleo-protein complexes with steroid receptors or other transcription factors as well as accessory proteins acting either as positive (such as the RNA helicase p68) or negative
modulators (such as the SRA stem-loop interacting RNA binding protein, SLIRP)[159]. SRA RNA has thus since been considered as a transcriptional co-activator. It is however important to consider the limitations of the techniques used to arrive to this conclusion. Luciferase reporter assay is commonly used to study regulation of gene expression as it is relatively inexpensive and allows quantitative measurements. However, this method does have known pitfalls. One of these is that the final luciferase activity readings depend upon the co-expression in the same cells of three constructs (SRA, transcription factor studied and reporter luciferase), an event difficult to monitor and which ultimately depends upon the efficiency of transfection of the cells investigated. Moreover, results also rely on the activity of a promoter in the absence of close chromatin, a well-known structure involved in gene expression regulation and interfering or promoting transcription. Another potential pitfall of luciferase assay is that excessive amounts of exogenous SRA RNA molecules are produced which may artificially drive interactions that do not occur normally when the components are present at endogenous physiological concentrations. Interestingly the conclusion that SRA RNA acts as a transcriptional co-regulator of estrogen receptor activity is not fully supported by cDNA microarray analysis of hormone inducibility of endogenous gene expression following silencing of the SRA gene in different cell line models [112,124,136]. Indeed, Foulds et al. (2010) only found a small portion of direct ER-α target genes impacted by SRA silencing in MCF7 breast cancer cells 72 hours post siRNA treatment [136]. Although Vicent et al. (2013) concluded that the majority of SRA putative downstream targets overlapped with progesterone receptor downstream targets in T47D breast cancer cells after 48 hours siRNA treatment. It should be stressed that, in these forementioned studies, the authors used a pool of SRA siRNAs. McKay et al. (2014) have however clearly demonstrated the likelihood of off-target effects of this approach. They showed that even if all individual siRNAs were effectively decreasing SRA expression, not all of them were modifying the expression of the same single gene. They
concluded that overall, SRA was unlikely to be a critical regulator of gene expression as no *bona-fide* downstream targets could be identified after *SRA1* gene silencing [123].

Additionally, classical knockdown approaches lead to both SRA RNA and SRAP decrease. The functional consequences on gene expression and/or other changes therefore result from the combined down-regulation of both molecules. More importantly, if SRA and SRAP possess opposite functions on gene expression for example, the neutralization of antagonist effects might not allow the visualization of the mechanisms involved. This could explain the lack of an obvious phenotype in SRA knockout mice model [160].

In summary, SRA RNA was initially thought to be a co-regulator modulating gene expression by associating with multiple transcription factors. However, this hypothesis is not completely supported by recent RNA deep sequencing analyses upon SRA silencing. Knockdown approaches cannot separate the roles played by SRA RNA and SRAP. Further approaches allowing the specific investigation of SRA and SRAP functions are required to understand the roles played by this bi-faceted system.

### VII-3-2 SRAP side of the SRA/SRAP bi-faceted system

Trying to focus on the “protein only” side of the system seems the best way to understand its function. For this purpose, I have used a modified construct “SRAP-only”, which has been designed to encode for SRAP but to drastically differ from the original SRA RNA sequence by introduction of multiple silent mutations [145]. In particular, specific secondary structures of the core sequence, shown to be necessary for the RNA to be functional have been extensively modified [128]. Using this modified “SRAP-only” construct, Chooniedass-Kothari *et al.* in our laboratory have found that SRAP physically interacts with multiple transcriptional regulators (such as Histone deacetylase 2) and transcription factors (including both estrogen receptors and ETS1)[147]. Using Chromatin Immunoprecipitation (ChIP) assays, Chooniedass-Kothari *et al.* also established that
SRAP was recruited to chromatin at the level of gene promoters [147]. Others have now confirmed that SRAP, independently of its RNA, modulates the activity of transcription factors including estrogen receptor, androgen receptor and ETS1 [115,144,145]. Altogether accumulated data have suggested that SRAP could act either as a positive or negative transcriptional regulator able to form complexes with transcription factors at specific promoter regions [149].

In order to identify genes whose expression might be controlled by SRAP, I performed a complementary set of experiments in Hela cell overexpressing “only SRAP” or silenced for SRA1 gene expression. In my study, I only focused on modifications of gene expressions which were in opposite direction when silencing the SRA1 gene or overexpressing SRAP. Few genes were identified which had trends of expression pattern modified in opposite direction when silencing the SRA1 gene or overexpressing SRAP. Moreover the amplitude of these changes was very subtle and no specific cut-off value could be applied in this analysis. These results corroborate the conclusion of McKay et al, which suggests that neither SRA RNA nor SRAP act as major transcriptional regulators [123].

Interestingly however, this modest response to SRA knockdown/SRAP overexpression suggested that modulation of this system could alter cell migration. To investigate the impact of modulating SRAP expression on cell motility, I have developed a novel real-time assay which overcomes most of the described potential limitations of conventional motility assays such as dependence on presence or response to a given chemo-attractant, proliferation rate or transfection efficiency. I have tracked, in the same field, individual cells overexpressing “GFP-green SRAP” and/or control RFP-red fusion protein. The main advantage of this method lies in its ability to explore the behavior of individual cells rather than that of the whole cell population. It is highly appropriate as it can be used even when transfection efficiency is low. Moreover, this approach allows the
simultaneous observation of test cells, negative control as well as positive control cells. Indeed green-test cells are tracked together with negative red cells and double transfected yellow cells. Using this novel real-time motility assay, I have shown that cells overexpressing “SRAP” migrated significantly faster and farther than control cells. This established for the first time a link between SRAP and cell motility. The specific mechanisms behind SRAP action remain to be further determined.

One should stress that the exact relevance of this observation to breast cancer and the extent of SRAP involvement in the mechanisms underlying invasion in vivo might be complex to investigate. Indeed, one might have to consider the previously discussed existence in breast tumours of multiple SRAP peptides with potential different mechanisms of action.

Overall, there is no consensus on the exact mechanisms of action of SRA/SRAP in breast cancer cells. Different research groups including ourselves have attempted to investigate this unique system using approaches focusing on either SRA RNA or SRAP. Although modified constructs representing “only SRA RNA” or “only SRAP” have been tried in different studies [112,117,145,146]. Both of the approaches display limitations as discussed above. The complexity of the problem at hand is increased when considering the potential existence of multiple functional SRA RNAs transcripts as well as SRAP peptides, which co-exist and may also possess complementary/opposite functions. Thus, perhaps trying to separate the functions of SRA RNA and SRAP is the wrong approach. In fact, alternative approaches to study both SRA RNA and SRAP such as specific point mutation could be used to study biological changes affecting both RNA and protein players. Mutations lying within both RNA and protein conserved regions are likely to have a profound effect on both SRA and SRAP action. Interestingly, such mutation of the SRA1 gene (Y35N) has recently been described in families affected by pubertal failure [161].
Conclusion and future Directions

The existence of a fascinating bi-faceted SRA1 gene system, involving functional RNA and protein has now been established. Characterizing SRAP expression in breast tissue is the first step toward understanding how this system affects the behavior of breast cancer cells. Herein, I have focused on the protein aspect of this system, and shown how its study might be clinically relevant to breast cancer. Overall, SRAP herein appears to be a clinically significant marker and a potential new therapeutic target in the fight against breast cancer. I have also extended our understanding of the cellular processes potentially regulated by this protein. I have thus taken a significant step in deciphering the role of SRAP. However additional studies are needed to further understand the roles played by this system.

Identification and characterization of SRAP peptides

The detection of several SRAP peptides recognized by different antibodies raised the hypothesis that different SRAP molecules exist in breast cancer tissue. What could be the origin of those peptides? First, different SRAP peptides may arise from various post-transcriptional events. Known alternative spliced events such as intron-1 retention conjugated with exon-3 deletion, predicts a 220 amino acid residue protein with molecular weight of 25kDa identical to SRAP in C-terminal extremity but differing in its N-terminal and central sequence [115]. This potential SRAP peptide is only recognized by 743A anti-SRAP antibody (targeting at C-terminal of SRAP, epitope 180-237aa), but not 742A anti-SRAP antibody (targeting at N-terminal of SRAP) as its epitope 50-100aa is missing if exon3 is truncated. Other events not yet identified may also occur. To establish the nature of the different splicing events potentially involved in the generation of potential truncated protein products, RT-PCR analyses could be performed on RNA extracted from the multiple tumours previously analyzed using different set of SRA primers. Products
corresponding to unknown splicing events potentially encoding protein can be confirmed by sequencing. In addition, alternative transcriptional initiation sites might also produce “short” forms of SRAP-like peptides. For example the transcript initiated at Methionine 3 could lead to a 162 amino-acids long peptides and migrating with a predicted molecular mass of 18 kDa. This peptide, recognized by 743A antibody would not be detected with 742A. Those SRA transcripts can be further confirmed by Rapid Amplification of DNA End (RACE) using SMARTer RACE cDNA Amplification Kit (Clontech). This strategy has previously been used to identify the transcription start sites of the SRA1 gene [162]. Furthermore, introducing a single mutation can also lead to the different SRAP peptides. Mutations modified to introduce a stop codon could lead to a truncated protein. If mutations are located within splicing factor binding site (alternative donor or acceptor consensus sequences), these mutations would likely also change alternative splicing events.

Other post-transcriptional modifications such as RNA editing could possibly contribute to the different SRAP peptides. RNA editing is mediated by the adenosine deaminase acting on RNA (ADAR) family of enzymes [163]. Adenosine to inosine conversion (A-to-I) is considered as the most common RNA modification, and inosines (I) are mostly interpreted by the ribosome as guanosines (G). Thus, A-to-I (G) editing of coding mRNAs by ADARs may lead to recoding peptides [163]. RNA editing events are fairly common in breast cancer and associated with poor prognosis[164]. If A-to-I (G) editing occurs at the stop codon of SRA leading to UGA-to-UGG or UAG-to-UGG modification[165], the SRAP translation would not be terminated. The continued translation of SRA transcripts would allow production of “longer” forms of SRAP peptides.

Interestingly it has been previously discussed in the introduction section I-2-2 that uridine residues at U206 of SRA RNA can be post-transcriptionally modified by
pseudo-uridylations. This modification is believed to consolidate the secondary structures of SRA RNA and promote co-activation function. Interestingly, pseudo-uridylation modifications of stop codons (ΨGA, ΨAA and ΨAG) have been shown to change protein length by suppressing translation termination [166]. Therefore, it would be interesting to see if SRA stop codons are also modified by pseudo-uridylations. If it is indeed happening, further tests would be required to confirm if the non-termination of translation of SRA transcripts leads to a longer form of its protein product. The conversional assay of CMCT modification (1-cyclohexyl-(2-morpholinoethyl) carbodiimide metho-p-toluene sulfonate) that inhibits U-to-Ψ conversion at the stop codons of SRA transcripts [103] could be performed with probe extension. If traditional methods are not sensitive enough to allow clear detection, more accurate quantitative techniques such as assay layer chromatography (TLC) analysis of [γ^32-P] ATP radiolabeled uridine/psudo-uridylated residue at the stop codon [166] would be used. Genome-wide detection method such as deep sequencing of pseudo-uridylation called pseudo-seq [167] has been recently developed with single-nucleotide resolution. This technique could be potentially used to confirm any pseudo-uridylation previously detected as well as detect novel SRA RNA modifications.

Lastly, alternative post-translational modifications might also be responsible for the relative “longer” forms of SRAP peptides. For example, S/T proline-rich region of SRAP has multiple serine and threonine kinases phosphorylation sites (S47, T50, S60, T66, T68, S69, K83 S87 and S92 S96) [123]. Mass spectrometric analyses have identified phosphorylated SRAP in different cell line models [150,168-170]. Phosphorylation of SRAP during mitosis could potentially participate in the shift in migration observed by western blotting (data not shown). Phosphor-SRAP antibody targeting specific phosphorylation sites could further test this. In addition, methylation at K218 [171] and ubiquitination at K83 of SRAP [172] identified by mass spectrometry could potentially
contribute to “longer” and “shorter” forms of SRAP, respectively. Interestingly, a lysine residue at position 21 of SRAP human sequence is conserved in all vertebrates and is predicted to be sumoylated (data not shown). Sumoylation of this residue could potentially contribute to the shift in migration from 30 kDa to 40 kDa [173]. Further studies are warranted to address all the existence of such post-translational modifications as well as their putative involvement in the genesis of the different SRAP peptides.

VII-4-2 Associations between the SRAP peptides and tumour characteristics

Once the origin and identity of those SRAP peptides are known, further associations between the expression of specific SRAP peptides and tumour/patient characteristics should be further assessed. A series of mouse monoclonal SRAP antibodies arising from hybridomas and specific for the unique sequences identified could be tested and optimized on selected tumours showing multiple SRAP bands using commercial SRAP antibodies. Using selected monoclonal antibodies corresponding to each SRAP peptide and tissue-microarrays, I could further clarify whether the detection of each specific SRAP peptide is associated with any particular histo-pathological characteristics during breast tumourigenesis and tumour progression. Ultimately, we could determine whether the expression of specific SRAP peptides is associated with breast cancer survival.

VII-4-3 Potential mechanism involved in SRAP-mediated regulation of cell mobility/invasion

Although the impact of SRAP expression on cell migration/mobility has been confirmed using both knock down and over-expression experiments, the mechanisms involved remain to be deciphered. The apparent discrete changes on genes expression upon silencing SRA1 or overexpressing SRAP suggest that regulation of gene expression is not the main driver behind SRAP action, at least in the model cell line used. Different
mechanisms other than gene transcriptional regulation might therefore be involved. Further studies are required to identify the mechanisms associated with SRAP-mediated cell mobility regulation.

Investigating SRAP protein partners/complexes will provide valuable information to understand its mechanism of action. Using commercially available antibodies against SRAP N- and C-terminal extremities, Jung et al. (2005) have established a list of SRAP interacting proteins by mass spectrometry analyses [139]. However, none of the SRAP protein partners has been validated by other protein interaction assays. Also, no consistent candidates overlapped when using SRAP antibodies recognizing N- and C-terminus [139]. This may result from the fact that different interacting domains of SRAP might overlap with targeted epitope, and thus be covered by the precipitating antibody. This is likely as SRAP is short and contains two conserved domains, covering most of the sequence and that are suspected to be actively involved in its action. In addition, an obvious limitation to the use of mass spectrometric analyses to identify and characterize protein complexes is that all antibodies have some degree of cross-reactivity, and they frequently co-immunoprecipitate a large number of cross-reactive polypeptides, referred to as non-specific false positive discoveries [174,175]. Indeed, some of the proteins candidates identified by Jung et al. (2005) such as Y box binding protein 1 (YBX1), DEAD box polypeptide 5 (DDX5) and heat shock 70kDa protein 1-like (HSPA1L) are actually among a list of non-specific binding partners often occurring during mass spectrometric analysis [175]. Furthermore, immunoprecipitation followed by mass spectrometry approaches suffer loss of candidates due to various protein insolubility and transient or weak interactions. Considering the described pitfalls of this otherwise traditional protein-protein interaction assay, we are now considering alternative techniques to identify SRAP interacting proteins.
BioID (biotin identification) is a technique based on proximity-dependent labeling of targeting protein by fusion of an E. coli biotin protein ligase to the protein of interest. The proximity-dependent biotinylation of proteins that are near-neighbors of the fusion protein may be isolated and identified by mass spectrometry following isolation of biotinylated proteins using streptavidin [Fig VII-3] [176]. BioID is a useful and generally applicable method to screen for both interacting and neighboring proteins in their native cellular environment [177]. Our laboratory is currently setting up this approach to investigate SRAP interacting proteins related to motility function.
Breast cancer is the second most frequent cause of death amongst women in North America. It therefore remains of crucial importance to develop additional approaches to overcome or circumvent the intrinsic mechanisms of resistance to existing standard therapies. The higher expression of SRAP in tumours of patients with poorer outcome upon tamoxifen treatment outlines the relevance of the proposed research and its potential future benefits. Overall SRAP should now be considered as a biomarker and a potential target for therapy. Understanding how this unique bi-faceted system works might open a new era of novel strategies to fight breast cancer.
Appendix: list of co-authored publications


References


helicases p68/p72 and the noncoding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. Dev Cell 11: 547-560.


S52-59.


