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ORIGINAL PAPER

Differential Expression of Ki-67 and Sex Steroid Hormone Receptors Between Genders in Peritoneal Mesothelioma

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Abstract Gender influence on survival in mesothelioma has been observed in several large clinical series. However, this gender effect has not been investigated. Female patients often have less aggressive tumors and survive longer. However, few studies in the literature have explained the molecular basis of this finding. Understanding this difference at a molecular level may offer the hope of improving survival via hormonal manipulation.We investigate the expression of Ki-67 and sex steroid receptors; estrogen receptors (ER), progesterone receptors (PR) and androgen receptors (AR) to elucidate any pathognomonic difference that characterize this gender difference. Positive expression of markers was observed in 95% (Ki-67), 80% (ER), 100% (PR) and 65% (AR) of patients. Expression of markers between gender showed a higher Ki-67 in males (M=1.3%, F=0.6%), higher estrogen receptor in females (M=0.6%, F=1.7%) and higher progesterone receptor in females (M=1.0%, F=1.4%). Twenty patients were treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy in our peritonectomy unit. Paraffin sections of the tumor specimens were retrieved for immunohistochemical analysis. The immunostaining was performed using monoclonal mouse anti-human antibodies on an autostainer (Autostainer Plus; Dako, Inc.). The intensity of the stains were quantified using the Image-Pro Plus (IPP) 4.5 (Media Cybernetics, Silver Spring, MD). For the first time, we demonstrate the presence of sex steroid receptors in peritoneal mesothelioma. Once the exact functional effects of these receptors are understood, the use of established therapeutic options that are clinically available to target the sex steroid pathway may become a reality.

Keywords Mesothelioma · Ki-67 antigen · Estrogen receptor · Progesterone receptor · Androgen receptor · Cytoreductive surgery

Synopsis Higher expression of Ki-67 protein, lower ER and PR in peritoneal mesothelioma tumor of males compared to females. We provide preliminary evidence of the possible involvement of sex steroid receptors in the cancer biology of peritoneal mesothelioma.

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Introduction

Mesothelioma is an aggressive and fatal malignancy. Owing to the immense mining of asbestos in the 1970s, we are witnessing an unprecedented increase in the incidence of mesothelioma worldwide [1]. There is a preponderance towards the male gender [2]. This is explained by the higher incidence of asbestos-related occupations held by men [3]. The peritoneum is the second most common site where mesothelioma arises comprising of one-third of all cases. This tumor arises from mesothelial cells within the serosal lining of the peritoneum. This disease often presents insidiously with non-specific gastrointestinal symptoms of increasing abdominal girth, pain and

weight loss. Due to the heterogeneity of these clinical symptoms, diagnosis is often delayed [4]. The prognosis of this disease remains poor with a median survival of about one year in patients treated with the combined chemotherapy regime of Cisplatin and Pemetrexed [5–7]. Loco-regional treatment using cytoreductive surgery (CRS) and hyperthermic intraperitoneal chemotherapy (HIPEC) have shown to result in a survival benefit, extending the median survival to between 34–92 months [8].

Results from several clinical series have shown that the prognosis of peritoneal mesothelioma is related to various patient and tumor factors. Non-epithelial tumors such as biphasic and sarcomatoid tumors are associated with a poorer prognosis compared to the epithelioid tumors [9]. Lymphatic invasion indicate a poorer prognosis [10–12]. Patient factors such as poor performance status and male gender have also been shown to result in a poorer prognosis [13]. It has been consistently reported that female patients survive longer and have a better prognosis than male patients [13–20]. The biological basis for this survival difference between male and female patients is unclear.

Sex steroid hormones, estrogen, progesterone and androgen are involved in the growth, differentiation and function of both male and female reproductive organs. In addition, these hormones have been shown to mediate tumorigenesis in hormone sensitive cancers through intracellular proteins; estrogen receptor (ER), progesterone receptor (PR) and androgen receptors (AR) [21]. Binding of the hormone to its receptor site activates the signal transduction cascade that leads intracellular signalling and production of transcription factors that regulate gene expression that promotes cellular differentiation [22]. Mutations, aberrant or abnormal expression of sex steroid receptors may result in the development and/or progression of cancers.

We hypothesized that the Ki-67 protein, a marker of cellular proliferation, and sex steroid receptor expression would be differentially expressed in tumor specimens between sexes. Demonstrating this difference will serve as a prelude towards understanding the underlying biology of the sex difference and its implication on tumorigenesis that may potentially allow the use of clinically available drugs that target the sex steroid pathway.

Patients and Methods

Patients

Twenty patients with a clinical and histopathological diagnosis of peritoneal mesothelioma underwent CRS and HIPEC by a single surgeon team (D.L.M) according to Sugarbaker's protocol [23]. HIPEC was performed for 90 min using an open abdomen technique with cisplatin (50 mg/m^2) and doxorubicin (15 mg/m^2) in 3 l of 1.5% dextrose peritoneal dialysis solution at 42°C. A signed informed consent was obtained from all patients for their clinical information to be used in research and to donate the left-over tissue after the completion of histological diagnosis.

Clinical Data

All clinical data were obtained prospectively and stored. The clinical variables recorded include; age at time of surgery, sex, previous treatment (neoadjuvant chemotherapy), peritoneal cancer index (PCI), completeness of cytoreduction, disease free survival and overall survival.

Immunohistochemistry

Tumor specimens from patients were harvested and preserved as paraffin-embedded tissue. Paraffin blocks were sectioned as 4 to 5 μm sections and mounted on positively charged Superfrost slides (Fisher Scientific Co., Houston, TX) by S.E.A.L.S. Pathology, St George Hospital. These sections were used for immunohistochemistry staining for Ki-67 protein and sex steroid receptors; estrogen receptor (ER), progesterone receptor (PR) and androgen receptor (AR). One section from each sample was also stained with hematoxylin and eosin to facilitate histological assessment. The staining process was performed according to a standard protocol. Briefly, sections were baked at 60°C, cooled and deparaffinized in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and de-ionized water. The slides were treated with EDTA retrieval solution (pH9.0) or citrate-based retrieval solution (pH6.0) for 20 min at 95°C, and blocked with 0.3% hydrogen peroxide before the application of primary antibodies (Dako Aust. Pty Ltd, Botany, Australia). Immunostaining was performed on an autostainer (Autostainer Plus; Dako, Inc.). This involved incubating with monoclonal mouse anti-human antibodies (Ki-67, ER, PR, and AR antibodies) for 1 h at room temperature. The sections were further incubated with goat anti-mouse HRP-Streptavidin immunoglobulin (Abcam) for 30 min at room temperature. The slides were developed with diaminobenzidine (DAB; Dako) for 10 min0 and counterstained with hematoxylin. Two types of negative controls, substituting the matched mouse IgG isotype and goat nonimmune IgG in the staining protocol, were used.

Following immunostaining, the slides were first analyzed manually. Any nuclear staining for ER, PR, AR, or Ki-67 was considered positive. The immunostaining results were evaluated by defining a threshold of positive staining for all sections before automated processing. Briefly, the

threshold of positive signal was defined for each antibody for all the sections following different treatments. Color signal above the threshold for each antibody defined was deemed to be positive, whereas any signal below the threshold was regarded as negative. The intensity was averaged from ten fields of view. This was performed using Image-Pro Plus (IPP) 4.5 (Media Cybernetics, Silver Spring, MD). All images analyzed with IPP 4.5 were counter-checked by a histopathologist (U.S.).

Statistical Analysis

The data were analyzed using SPSS® for Windows version 16.0 (SPSS, Munich, Germany). The patient characteristics between gender were compared using unpaired t-test and Fischer's exact test. The Kaplan-Meier method was used to analyze survival. The log-rank test was used to compare differences in survival. Survival was measured from the time of surgery. No patient was lost from follow-up. The immunostaining, which demonstrates the expression of the markers, was compared using an unpaired t-test.

Results

Comparison of Patient Characteristics Between Gender

There were 14 males and 6 females. The mean age (standard deviation) at the time of surgery was 56 [9] years. Sixteen patients had epithelioid tumor, three had non-epithelioid tumor (one had biphasic tumor, two had sarcomatoid tumor) and one patient had cystic mesothelioma. Ten patients received neoadjuvant chemotherapy prior to surgery. Between gender, males tend to present with higher volume of disease, as indicated by the PCI (mean PCI=males 19, females 13). The detailed comparison of the patient characteristics between gender is presented in Table 1. Amongst the 6 females, the age range from 43 years to 62 years with a median of 53 years. By assuming that patients who had neoadjuvant chemotherapy treatment and those above the age 50 are considered menopausal, there is one patient, aged 47, who did not receive neoadjuvant chemotherapy and is pre-menopausal.

Table 1 Gender comparison of patient characteristics

	Male	Female	p value
Total	14	6	
Age (years)			**0.34**
Mean (s.d.)	57 (10)	53 (7)	
Asbestos exposure			**0.04**
No	2	4	
Yes	12	2	
Neoadjuvant chemotherapy			**1.00**
No	7	3	
Yes	7	3	
Tumor type			**1.00**
Epithelioid	12	5	
Non-epithelioid	2	1	
Peritoneal cancer index			**0.05**
Mean (s.d.)	19 (7)	13 (5)	
Completeness of cytoreduction			**0.35**
CC0	8	5	
CC1/2	6	1	
HIPEC			**1.00**
No	0	0	
Yes	14	6	

Expression of Markers

Tumor cells demonstrated staining for Ki-67, ER, PR and AR. The expression of the molecular markers was positive in 95% (Ki-67) 80% (ER) 100% (PR) 65% (AR) of the patients' tumor specimens. The intensity of the immunostain was homogenous within a given case, and the percentage of stained tumor cells varied depending on the evaluated marker. The patient with cystic peritoneal mesothelioma was excluded from comparison of the molecular markers between sexes. Female tumors had lower expression of Ki-67 (females 0.6%, males 1.3%), higher expression of ER (females 1.7%, males 0.6%), higher expression of PR (females 1.4%, males 1.0%) and similar expression of AR (females 0.1%, males 0.1%) (Fig. 1). In the pre-menopausal female patient, the ER was 0.002% and the PR 0.05%. In the remaining menopausal patients, the mean (s.d.) ER was 1.7 (1.9)% and PR was 1.4 (2)%. Comparing expression of markers with tumor histology, epithelioid tumors had a higher ER expression than in non-epithelioid tumors (1.0% v. 0.3%, p=0.005). PR expression was similarly significantly higher in epithelioid tumors than in non-epithelioid tumors (1.3% v. 0.05%, p=0.01). Representative sections showing the expression of the various markers between sexes are shown in Fig. 2.

Survival Analysis

The median follow up period from time of surgery was 18 months (range 1–89 months). The overall median survival of patients with peritoneal mesothelioma analyzed using the Kaplan-Meier method was 30 months [Fig. 3a]. The 1–and 3-year survival rates were 79% and 48% respectively. Five-year survival rate was not reached. The median disease free survival was 8 months (range 1–58 months). There

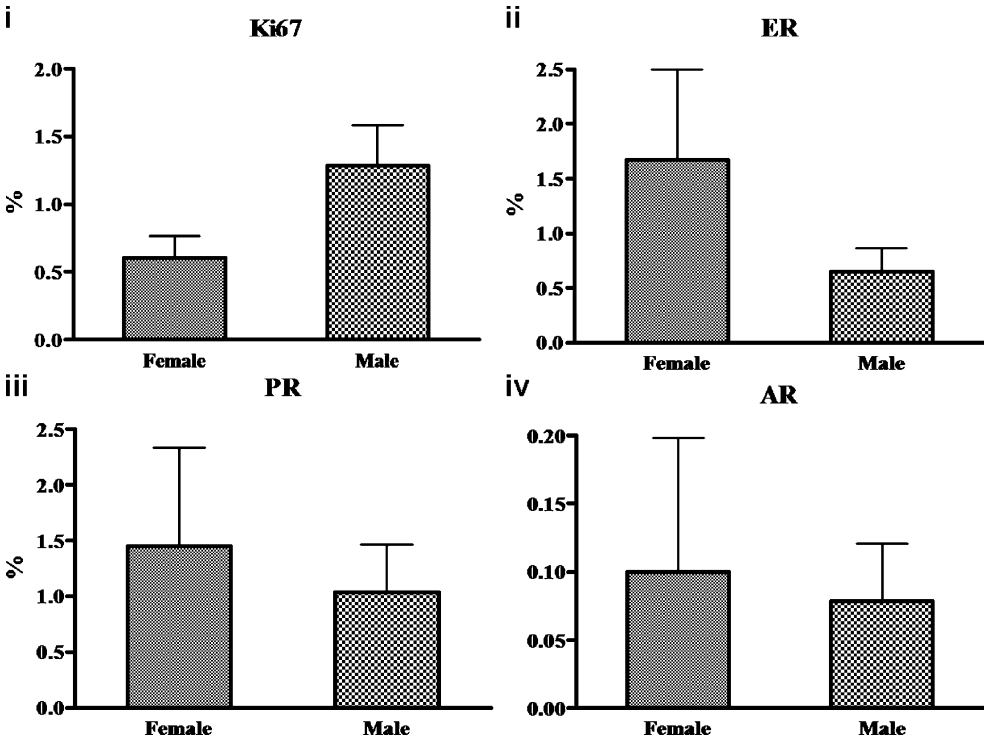

Fig. 1 Bar graphs showing the differential expression of the various immunostains between sexes; **a** Ki-67, **b** estrogen receptor, **c** progesterone receptor and **d** androgen receptor

was a trend towards a better survival in female patients [Fig. 3b].

Discussion

Peritoneal mesothelioma is a fatal malignancy that results in severe morbidity and mortality. Treating this malignancy aggressively with CRS and HIPEC has provided patients with a prolonged median survival. However, due to the aggressive nature of this tumor, recurrence following treatment is inevitable. Patients in our series had a disease free survival of 8 months. At the time of recurrence, patients may be treated surgically with either a repeat CRS and HIPEC, palliative debulking or systemic chemotherapy using Cisplatin and Pemetrexed, depending on their overall performance status and disease burden which determines the appropriate selection of treatment. As aforementioned, the efficacy of systemic chemotherapy is poor and the prognosis is dismal [5, 24]. Therefore, should newer adjuvant therapy following curative CRS and HIPEC become available, it would potentially offer the hope of a longer term survival in patients. In addition, combining the treatment regimes may also reduce the number of patients requiring redo CRS and HIPEC or palliative debulk following primary treatment.

In our study, firstly, we investigated the expression of Ki-67 and quantified its intensity to elucidate any difference between the proliferative activity of the tumor cells between sexes that could explain the volume of disease indicated by the PCI at the time of presentation where males presented with a higher volume of disease compared to females. Secondly, we investigated the expression of sex steroid hormone receptors; ER, PR and AR to elucidate any gender difference in the expression of these markers. The basis of our investigation follows the findings of several large mesothelioma clinical series, including a national registry study, that have shown that gender is a prognostic factor. Females tend to survive longer [13–20]. Biologically, this reason is not well understood. However, one study did show that there are differences in the histological appearance of the tumor. Tumors of females had a smaller nuclear size and a granular chromatin pattern [16].

Detection of the Ki-67 antigen within the cell nucleus occurs during all phases of cellular proliferation in the cell cycle; Interphase (G1, S and G2) and M phase. During the G0 phase of Interphase, commonly known as the post mitosis phase, Ki-67 antigen is undetectable as there is no cellular differentiation. Therefore, the Ki-67 protein is regarded as an excellent marker of cellular proliferation and provides an inference of the aggressiveness of the tumor [25]. Our finding of a higher expression of Ki-67 protein in the tumors of male as compared to female validates the current understanding in the literature of tumors in men being more aggressive compared to tumors in women. In addition, the prognostic significance of nuclear size can also be explained by the expression of Ki-67 protein which we have demonstrated. The correlation between nuclear size and proliferative activity as indicated by the Ki-67 protein expression has been previously

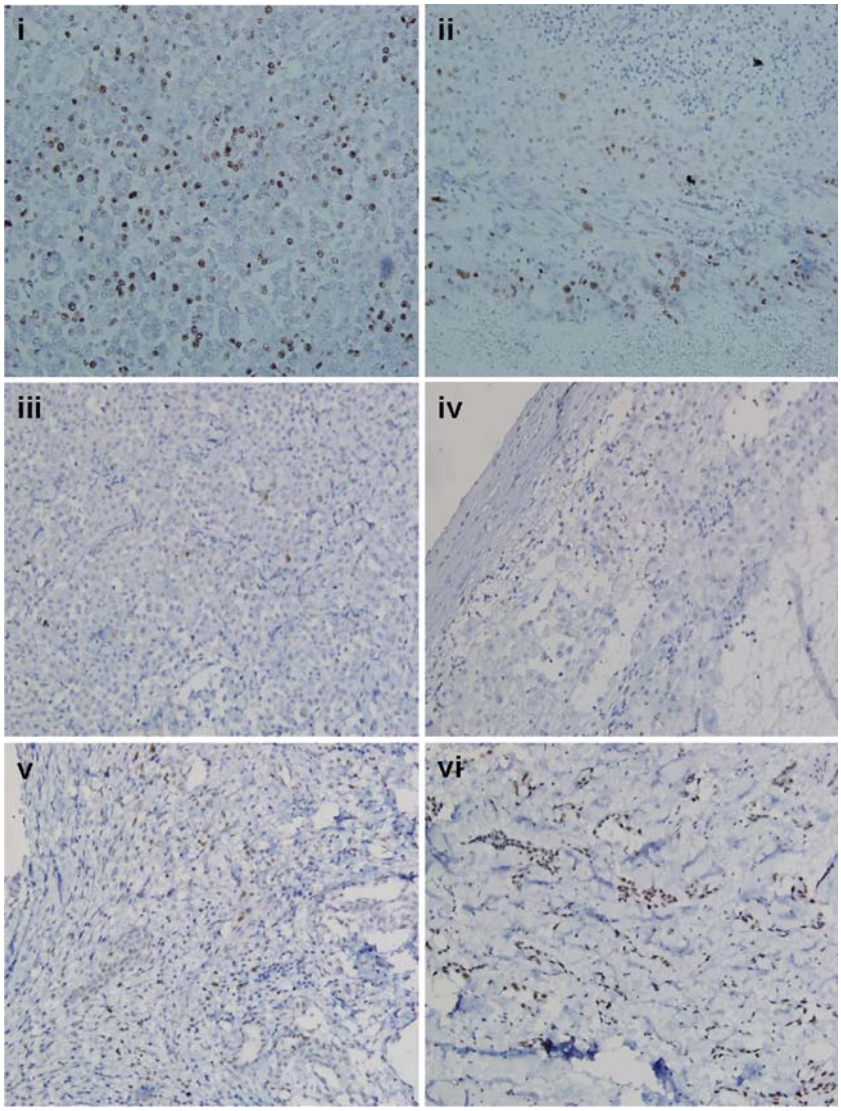

Fig. 2 Representative sections of immunostains **a** Ki-67 in male, **b** Ki-67 in female, **c** estrogen receptor in male, **d** estrogen receptor in female, **e** progesterone receptor in male and **f** progesterone receptor in female (*magnification 200x*)

demonstrated in other cancers [26]. Therefore, the association of proliferative activity and the interpretation of histomorphometry of the tumor cell nuclei may provide useful information about the degree of proliferative activity, which may become a valuable prognostic marker.

From a molecular standpoint, it is well known that sex steroid hormones are involved in tumorigenesis [27]. Binding of oestrogen to the ER result in a series of molecular events such as receptor phosphorylation, dimerisation, and facilitation of the binding of the receptor complex to the promoter region of target genes which ultimately leads to the activation of transcription. These transcriptional effects are modulated by interactions with co-regulatory proteins that function as either co-activators or co-repressors. The conformational change induced by the binding of estrogen to the ER favours the recruitment of co-activators that augment transcription and hence cellular proliferation [22, 28]. Likewise, activation of PR occurs as a result of the ligand binding action of co-activators. When this occurs, activation of the PR through the various intracellular signaling mechanisms results in proliferative effects at various target tissues [29]. Binding of androgen to the AR result in a conformational change in the receptor which causes dissociation of heat shock proteins and their transportation from the cytoplasm into the nucleus. This interaction between AR and proteins in the nucleus results in gene transcription [30]. AR is also known to activate the production of Insulin Growth Factor I (IGF-I) which function as a stimulator of cell growth and an inhibitor of apoptosis [31]. Our results demonstrate a higher expression of ER and PR in female tumors and an equivocal expression of AR in tumors of both sexes. From these findings, we speculate that ER and PR expression may confer a protective effect to female patients, thus explaining the longer survival seen in females and in male patients. The poorer prognosis in males may be explained by the

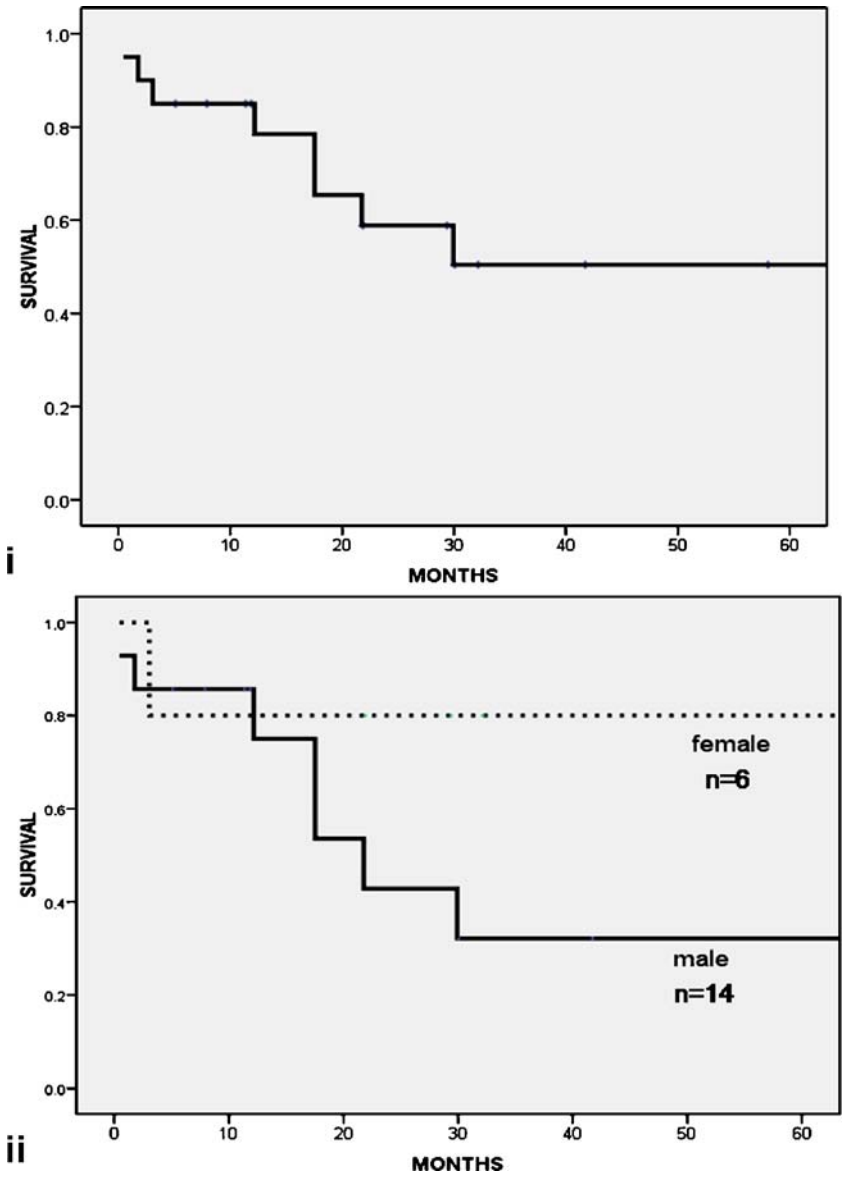

Fig. 3 **a** Overall survival of 20 patients with peritoneal mesothelioma treated with cytoreductive surgery and hyperthermic intraperitoneal chemotherapy, **b** Longer median survival seen in female patients with peritoneal mesothelioma

presence of AR that may be activated by circulating androgens.

Presently, three other studies have evaluated sex steroid hormones/receptors in mesothelioma [32–34]. Barnetson *et al* compared ER and PR status in peritoneal mesothelioma and secondary serous carcinoma of the peritoneum, and reported negative staining for ER and PR in peritoneal mesothelioma [32]. Horita *et al* demonstrated the suppression of cell proliferation and induction of apoptosis of mesothelioma cell line 211H by progesterone [33]. Swah *et al* reported focal presence of ER and/or PR in some lesions in cystic mesothelioma [34]. The negative finding of ER and PR staining in peritoneal mesothelioma in the study by Barnetson *et al* might be related to the lack of representative sections or their classification of weak staining as equivocal. In our study, evaluation of immunostaining was performed quantitatively using a standardized uniform computerized process.

The presence of sex steroid receptors in peritoneal mesothelioma provides preliminary evidence for the future use of hormonal therapy to augment the development and progression of this fatal malignancy, once the biological actions of hormone receptor expression are fully understood. Selective estrogen receptor modulator (SERM), which has an antagonistic effect on human breast tissue is useful as adjuvant hormonal treatment of ER positive breast cancers. Aromatase inhibitor (AI) blocks the enzyme aromatase, thereby preventing the conversion of androgens to estrogens in the adrenal gland. Hormonal treatment using SERMs and AIs have resulted in an improved survival, and hence become the standard of care in ER positive breast cancers [35, 36]. In addition, there is well established

evidence of a better response to treatment in both ER and PR positive tumors as compared to ER positive and PR negative tumors, thus reflecting some interdependence of PR expression on ER activity [37]. The action of selective progesterone receptor modulators (SPRM) on PR is more variable. Their effect is largely dependent on the concentration of existing coactivators or corepressors that are present which determines the overall agonist or antagonist effect [38]. At present, the use of SPRMs in clinical practice is not well established. Androgen ablative strategies such as surgical castration or medical treatment using luteinizing hormone-releasing hormone (LHRH) analogs or anti-androgens to inhibit AR activity and thus modulate the signal transduction pathways are treatment option in prostate cancer [39]. Therefore, with the numerous hormonal antineoplastic agents which are established and available for use in the treatment of various malignancies, further laboratory investigations to clarify the effect of hormonal therapy on the tumor cells in peritoneal mesothelioma is warranted.

In summary, we present evidence of a higher expression of Ki-67 protein in tumors of males compared to female patients, thus bearing prognostic significance in explaining the longer survival seen in females. The differential expression of steroid hormone receptors between sexes and the clinical correlation of differential survival outcomes between males and females provides an impetus for further investigation of steroid receptors as initiators of signal transduction pathways that regulate cellular proliferation, which may be suspected to play a role in the cancer biology of peritoneal mesothelioma. This study is a novel and provocative preliminary analysis but is limited by the small sample size. A replication of this study may be considered to provide a more conclusive finding.

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