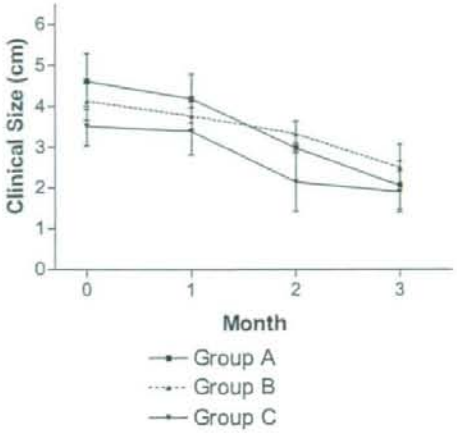

Fig. 1. Reduction in clinical tumor size after three different types of neoadjuvant treatment.

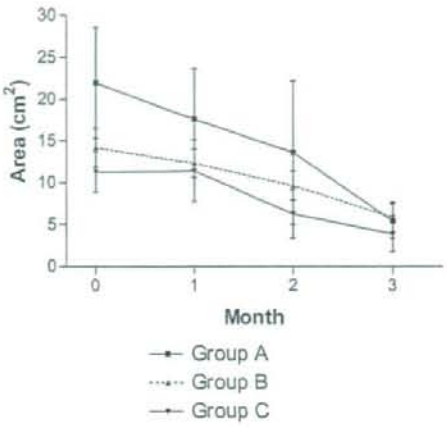

Fig. 2. Reduction in clinical tumor area after three different types of neoadjuvant treatment.

analysis was performed for the 12 patients who had completed 3 months of treatment, there was one complete response and two partial responses in group A, two partial responses in group B and three partial responses in group C. All the other patients had response but not up to 50%. The changes in tumor area for these 12 patients were shown in Fig. 3.

The changes in CEA and CA15.3 were shown in Tables 2 and 3. There was a slight reduction in markers levels. The differences between the three groups were not statistically significant.

Table 2
Changes of blood CEA levels during the period of neoadjuvant treatment

	Pre-treatment	1st month	2nd month	3rd month
Group A	3.33 (0.96)	3.09 (0.73)	3.20 (0.93)	2.0 (0.36)
Group B	2.34 (0.33)	2.40 (0.47)	3.13 (1.26)	2.00 (0.20)
Group C	2.14 (0.38)	1.77 (0.53)	1.57 (033)	0.70 (0.01)

The values in parenthesis represent standard error of mean.

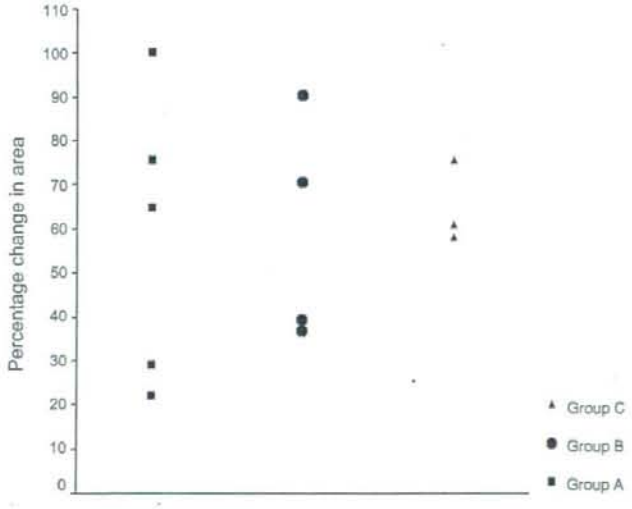

Fig. 3. Percentage reduction from baseline of tumor area for the 12 patients who completed three cycles of neoadjuvant treatment.

Table 3
Changes of blood CA15.3 levels during the period of neoadjuvant treatment

	Pre-treatment	1st month	2nd month	3rd month
Group A	27.96 (8.59)	28.34 (7.54)	18.52 (4.16)	19.17 (11.68)
Group B	39.43 (12.02)	36.41 (12.48)	37.93 (17.06)	23.50 (11.50)
Group C	46.10 (17.33)	18.80 (6.20)	28.00 (0.01)	9.40 (0.01)

The values in parenthesis represent standard error of mean.

4. Discussion

Cyclooxygenase inhibition has been implicated in the blockage of angiogenesis [7,8]. Results from epidemiological studies suggest that use of non-steroidal anti-inflammatory drugs, such as aspirin and indomethacin that inhibit COX-2 activity, reduces the incidence of breast cancer as well as colon cancer in the human [9–13]. Celecoxib is a selective COX-2 inhibitor. It has chemopreventive and chemotherapeutic properties in rodent models of breast cancer [5]. In patients with familial adenomatous polyposis, 6 months of twice-daily treatment with 400 mg of celecoxib leads to significant reduction in the number of colorectal polyps [14].

It is known that the aromatase gene expression is regulated by prostaglandin E_2, which is a product of COX-2 [15]. In fact, there is a linear relationship between aromatase activity and COX-1 and COX-2 expression within the human breast tissue [16]. This significant relationship between the aromatase and cyclooxygenase enzyme systems suggests that autocrine and paracrine mechanisms may be involved in hormone-dependent breast cancer development via growth stimulation from local estrogen biosynthesis. Indeed, recent research on the signaling pathway in the regulation of aromatase and COX-2 expression showed that both the breast epithelial cells and the stromal cell compartment play important roles in the progression of tumor growth [8]. The interconnecting pathway may involve epidermal growth factor (EGF), transforming growth factor-β (TGF-β) and tetradecanoyl phorbol acetate (TPA).

The therapeutic potential of combining celecoxib and exemestane was tested in the DMBA rat model [17]. An objective response rate of 48% was achieved when the rats were treated with both exemestane and celecoxib. This contrasted with OR rates of only 5% when treated with exemestane alone and 0% when treated with celecoxib alone. The development of new tumors follows a similar pattern. The study demonstrated that the addition of celecoxib could enhance the inactivation of aromatase activity.

The CAAN trial is conducted in postmenopausal hormonal-sensitive breast cancer patients to investigate the efficacy of neoadjuvant therapy combining aromatase inhibitors with COX-2 inhibitor. Neoadjuvant treatment of breast cancer offers several advantages. Firstly, the successful therapy would down-stage large tumors to sizes suitable for conservative surgery. Secondly, the sensitivity of the tumor to the therapy administered could be assessed and agents that are effective could be used as adjuvant therapy after the operation. Thirdly, as the breast cancers could be easily accessible, the biological as well as the genetic changes of the tumor could be followed and studied. Recent studies using aromatase inhibitors as neoadjuvant therapy in postmenopausal women have demonstrated that these agents are effective [18–20]. Eleven of the 12 patients given 1 mg and seven of 11 patients given 10 mg of anastrozole had shrinkage of tumors by over 50% [18]. The median reduction from baseline for the whole group was 75.5%. Another non-randomized study showed that letrozole has an apparent superior pathologic response than anastrozole, although the clinical response is similar [19]. Both have a better clinical and pathologic response than tamoxifen. Exemestane treatment was associated with a marked reduction of aromatization peripherally and in non-malignant breast tissue [20]. Eight of the 10 patients that would have required mastectomy were able to undergo breast-conserving surgery after exemestane treatment. There was a median reduction of tumor volume by about 85%.

Based on these results, the CAAN trial is designed to study the neoadjuvant use of exemestane with and without celecoxib. Exemestane is chosen because it is a type I agent and it has marked reduction of aromatization in malignant and non-malignant tissues [20]. In this study, exemestane is given at 25 mg daily with and without celecoxib. Celecoxib is given at 400 mg twice a day. This is the dosage used in the chemopreventive study on familial adenomatous polyposis [14]. A third arm using letrozole 2.5 mg daily is also added as control. The objectives of the study are to confirm the superior laboratory results from treatment combining exemestane with celecoxib, to determine whether the addition of celecoxib would cause different changes in angiogenesis and apoptosis markers, and to evaluate the safety and side effect profiles of the three treatment arms. This preliminary report shows that all of the three anti-aromatase therapies are effective. However, the results presented here are only in the initial phase of the study. It is hoped that at the conclusion of the trial, we would be able to determine the contribution of cyclooxygenase-2 inhibition in the management of hormonal-dependent breast cancers.

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Evaluation of neoadjuvant inhibition of aromatase activity and signal transduction in breast cancer

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Abstract

Purpose: To evaluate the efficacy and safety of combing aromatase inhibitor (AI) and signal transduction inhibitor neoadjuvantly in postmenopausal patients with invasive hormone-sensitive breast cancer.

Patients and methods: Postmenopausal women with hormone-sensitive breast cancer were given three months of letrozole 2.5 mg daily and imatinib 400 mg twice daily preoperatively. End-points of this study included clinical and pathologic responses, toxicities, and change in [^{18}F]fluorodeoxyglucose (FDG) uptake in tumor. Expression of c-Kit was also evaluated in breast cancer tissue by immunostaining.

Results: Thirteen patients, aged 52–78, were accrued. Five patients (38.5%) experienced grade 3 toxicity including neutropenia, skin rash, dermatitis, hypokalemia, shortness of breath, acute coronary syndrome, and acute chronic gastritis. Three patients were withdrawn after two months of treatment due to hematoma in tumor and toxicity. Of the ten evaluable patients, nine patients (90%) achieved clinical partial response and one patient (10%) had stable disease. One patient (10%) achieved pathologic complete response. Average relative changes of FDG uptake was −69.5% among responders. Eight out of 13 tissue samples were tested for c-Kit expression and the expression was detected in all.

Conclusions: In this pilot study, the dramatic response to this neoadjuvant combination treatment warrants further clinical trials. Further investigation on the involvement of c-Kit pathway in the treatment response is also suggested. However, dosage reduction of imatinib may be required to avoid its potential toxicity.

Keywords: Aromatase; Signal transduction; Neoadjuvant; Breast cancer

1. Introduction

Breast cancer is the most prevalent cancer in the word and, among females, it is still the most frequent cancer [1,2]. It is commonly associated with female

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hormones exposures. Our previous study showed that about 55% of patients possessed hormonal receptors, with the frequency of hormonal receptor positively increased with advancing age [3]. Endocrine therapy was, therefore, used for treatment of advanced breast cancer and nearly 30% of breast cancer patients were responsive to endocrine therapy [4]. Currently, researchers are further exploring the use of aromatase inhibitors for treatment of breast cancer.

Aromatase, an enzyme complex consisting of a cytochrome P-450 hemoprotein and a flavoprotein, converts C-19 androgen such as testosterone and androstenedione to C-18 estrogen such as estradiol and estrone. The aromatization of adrenal androgens to estrogen is taking place mostly in the peripheral tissue like fat and muscle. In post-menopausal women, major source of estrogen is derived from peripheral aromatization via aromatase enzyme [5,6].

Letrozole is a nonsteroidal competitive inhibitor of the aromatase enzyme system. Its superiority to tamoxifen was presented in adjuvant aromatase inhibitor study that the incidence of breast cancer and total time to disease recurrence were significantly reduced [7]. The clinical efficacy and tolerability of the aromatase inhibitor was well demonstrated. However, much evidence suggested that enhanced signal transduction pathways may be one of the key adaptive changes accounting for endocrine-resistant growth in breast cancer [8–11]. Inhibition of these pathways may treat or even prevent endocrine-resistant tumor growth. *In vitro* data suggested that combined treatment with tamoxifen and the EGFR TKI may provide greater anti-proliferative effects and delay hormone-resistant outgrowth in hormone-sensitive cells [12]. Such a strategy of combination therapy could prove more effective than either therapy alone in hormone-sensitive breast cancer and, in particular, could delay the emergence of acquired resistance.

Imatinib was developed as a receptor-targeted agent for chronic myelogenous leukemia (CML) [13]. This phenylaminopyrimidine derivative was selected from a screen of molecules for its ability to competitively target the ATP-binding site of the platelet-derived growth factor receptor (PDGFR). *In vitro* analysis revealed that imatinib also selectively inhibits the ABL and KIT (CD117) tyrosine kinase receptors. Imatinib has demonstrated activity against conditions in which either KIT or PDGFR is activated. Autocrine stimulation of KIT and PDGFR by stem cell factor and PDGF, respectively, is observed in breast tumors and may enhance mitogenic signaling.

2. Patients and methods

2.1. Study design

This open label pilot study assessed the efficacy of letrozole and Imatinib in postmenopausal women with hormone sensitive and invasive breast cancer and was conducted in Surgery Department of University of Hong Kong Medical Centre. As this is a proof-of-principle study, it was initially planned to recruit 15 patients. Combination treatment of letrozole 2.5 mg daily and imatinib 400 mg twice daily was given orally in the form of 100-mg capsules for three months before surgery. The study was conducted in accordance with the International Conference on Harmonization Good Clinical Practice. The study protocol and informed consent were reviewed and approved by the appropriate local scientific and ethics committee. All patients gave written informed consent to participate into this study.

2.2. Inclusion criteria

Postmenopausal women with histologically confirmed hormone sensitive and invasive breast cancer were accrued for this study. Eligible patients were untreated for primary invasive breast cancer, confirmed by core needle biopsy, with positive estrogen receptor (ER) and/or progesterone receptor (PR) determined by immunohistochemistry. Other eligibility criteria were as follow: tumor size of 3 cm or more; Eastern Cooperative Oncology Group (ECOG) performance status $\leqslant 3$; acceptable cardiac function with left ventricular ejection fraction (LVEF) $\geqslant 50\%$; acceptable liver function with bilirubin, aspartate aminotransferase (AST) and alanine aminotransferase (ALT) within institution normal range; acceptable renal function with serum creatinine <150 μmol/L and glomerular filtration rate (GFR) >40 ml/min. All patients were able to give a written informed consent following the recommendation of the Helsinki Declaration and to follow prescription instructions reasonably well.

2.3. Exclusion criteria

Patients with known sensitivity to anti-aromatase drugs or imatinib were excluded. Major cardiac disease or LVEF <50%, renal impairment, and prior history of other malignancy within 5 years of study entry, aside from basal cell carcinoma or the skin or carcinoma-in-situ of the uterine cervix were other criteria for exclusion.

2.4. Clinical assessments

Patients were evaluated at baseline and every 4 weeks by physical examination, ECOG performance status

[14], vtial signs, adverse event assessments, and blood tests including hematology, blood chemistry, lipid profile, carcinoembryonic antigen (CEA) level and CA15.3 level. Relevant medical history and echocardiogram (ECHO) were performed at baseline for eligibility assessment.

2.5. Efficacy assessments

The primary endpoints of this study were tumor response determined by clinical measurement and ultrasound. Clinical and radiological (ultrasound) assessments for tumor sizes were performed at baseline and every 4 weeks. Positron Emission Tomography (PET) with [^{18}F]fluorodeoxyglucose (FDG), and Mammography (MMG) were also performed at baseline and before surgery as additional tumor assessments. Clinical response assessments was determined according to standard Union International Centre Cancer criteria [15] defining complete remission, partial remission, no change, progressive disease, and not assessable. The surgical tissues including primary tumor and dissected axillary or sentinel lymph nodes were collected and investigated at time of surgery for pathologic response. Pathologic complete response (pCR) was defined as complete disappearance of invasive tumor cells in breast and dissected lymph nodes.

2.6. Safety assessments

Safety was monitored by physical examination, vital signs, hematology, blood chemistry, and adverse event assessments every 4 weeks since commencement of therapy. Severity of adverse events were graded according to National Cancer Institute common toxicity criteria (NCI-CTC) version 3.0 [16].

2.7. Immunohistochemistry for C-kit

The formalin-fixed, paraffin wax–embedded pre-operative breast tissues were immunostained for c-KIT using standard methods. Primary monoclonal antibodies of c-KIT (CD117) (Thermo Fisher Scientific, CA, USA; dilution 1:200) was used and the staining was visualized by DAB chromogen staining using UltraVision LP Detection System. The c-KIT expression level was scored as follow: 1+: the cytoplasm was discretely and weakly to moderately stained in 10% or more of cells; score 2+: the cytoplasm was strongly stained with or without membrane staining in 10% or more of cells; 0 or negative: no staining was observed or staining was observed in less than 10% of cells. Cases with a score of 1+ and 2+ were considered positive.

2.8. Statistical analysis

Statistical analyses were performed using SPSS for Windows 11.0 computer software (SPSS Inc., Chicago, IL). One-way ANOVA tests were used to compare parameters. All values were expressed as mean and standard deviation (SD) unless otherwise stated. $P < 0.05$ was considered as statistically significant.

3. Results

3.1. Patient characteristics

A total of 13 patients with operable breast cancer were recruited from September 2004 to September 2005. The median age was 68 years, ranged from 52 to 78 years. All of them had ECOG performance status of grade 1. Histopathology showed that 100% of primary tumor samples were invasive ductal carcinoma, and 100% and 92% of them had positive ER and positive PR status, respectively. Additional immunohistochemistry for c-kit expression in eight pre-operative breast tissue samples were done and all of them gave moderate expression with scores between 1+ and 2+. 3 out of 13 patients stopped neoad-

Table 1
Patients' characteristics ($n = 13$)

Characteristics	No. of patient	%
Age, years		
Median	68	
Range	52–78	
Side		
Left	5	38.5
Right	8	61.5
Primary Histology		
Ductal carcinoma	13	100
Initial tumor size		
Mean LD in cm (SD)	4.7	2.81
Mean Area in cm^2 (SD)	27.44	39.25
Hormonal receptor status/oncogene expression[a]		
Estrogen receptor		
Weak positive (1+)	–	
Moderate positive (2+)	2	15.4
Strong positive (3+)	11	84.6
Progesterone receptor		
Weak positive (1+)	2	15.4
Moderate positive (2+)	3	23.1
Strong positive (3+)	8	61.5
CerbB2 oncogene		
Non-overexpressed (2 + or below)	11	84.6
Overexpressed (3+)	2	15.4
C-kit expression[a]		
Negative	–	
Positive[b]	8	61.5
ND	5	38.5

Abbreviations: LD, longest diameter; SD, standard deviation; ER, estrogen receptor; PR, progesterone receptor; ND, not determined.

[a] Determined by immunohistochemistry.

[b] All are moderately stained with scores between 1+ and 2+.

juvant treatment prematurely and, therefore, only 10 patients were evaluated for response. Table 1 shows their baseline characteristics.

3.2. Efficacy

The 10 patients completed three months of letrozole and imatinib treatment whereas 3 patients stopped after 8 weeks of the neoadjuvant treatment. One patient developed hematoma in breast tumor, and therefore, surgery was performed prematurely. Primary tumor was measured 16 cm in longest diameter (LD) which increased by 28% from baseline whereas pathology showed grade I invasive ductal carcinoma and two metaststic lymph nodes. Neither extensive intraductal component nor lymphovascular permeation was present in resected specimen. Deep resection margin were also clear. Another two patients were dropped out due to drug-related toxicities. Of the ten evaluable patients, nine patients (90%) achieved partial response and one patient (10%) had stable disease from clinical assessments. Though no clinical complete response was observed, one patient (10%) achieved pathologic complete response (pCR). Of the clinically responders, the metabolic activity in the tumor evaluated as standardized uptake value maximum (SUV_{max}) by PET scan showed relative changes of FDG uptake after neoadjuvant treatment of -69.5% ($SD = 0.09$). The patient with pCR showed approximate 68% improvement with FDG (Fig. 1). Analysis performed according to PR status among responders showed that tumors with strong positive PR expression showed greater relative FDG uptake change than that with moderate positive PR expression ($P = 0.016$).

3.3. Safety

The safety population include all recruited subjects. More than 90% patients experienced grade 1 or 2 treatment-related toxicity and 38.5% patients experienced grade 3 toxicity including neutropenia, skin rash, dermatitis, hypokalemia, shortness of breath, acute coronary syndrome, and acute chronic gastritis.. Two patients (15%) were withdrawn due to reported grade 3 acute coronary syndrome and intolerable grade 2 generalized edema. No grade 4 toxicity was observed. The incidences of major toxicities are reported in Figs. 2 and 3.

4. Discussion

In recent years, aromatase inhibitors (AI) are the mainstay of endocrine therapy for hormone sensitive breast cancer in postmenopausal women [17–19]. Their superiority to traditional hormonal treatment such as tamoxifen, a selective estrogen receptor modulator, was well demonstrated in most clinical trials [7,20–22]. However, nearly half of patients with ER positive tumors develop resistance to hormonal therapy [23] and the underlying mechanism remains unclear. But, crosstalk between ER and other cellular signalling pathways are generally deemed as the cause of endocrine resistance [24]. Different combinations of AIs with signal transduction inhibitors (STI) are of interest for many researchers to maximize the use of existing endocrine therapy.

This pilot study addresses the efficacy and safety of combination of letrozole and imatinib as neoadjuvant treatment for postmenopausal women with hormone sensitive breast cancer. Being the commonly prescribed aromatase inhibitor, letrozole was used together with imatinib, a selective tyrosine kinase inhibitor (TKI), in an attempt to improve the response rate of single therapy and to overcome endocrine resistant tumors. In our previous neoadjuvant trial of groups of exemestane versus letrozole [25], approximate 60% of patients were clinically

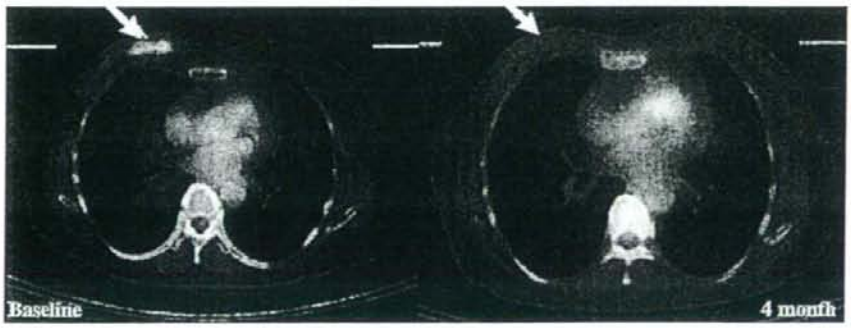

Fig. 1. [^{18}F]-FDG-PET scan obtained in the patient with pathologic complete response at baseline and 4 month after letrozole and imatinib treatment. Significant reduced FDG uptakes at primary tumor was noted in the PET scan.

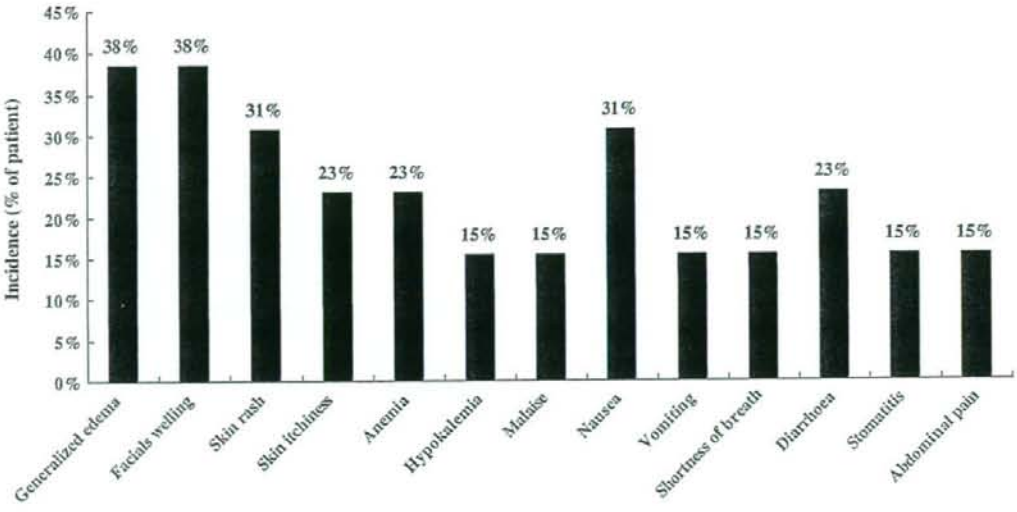

Fig. 2. Grade 1/2 reported adverse events occurring in ⩾10% of patients.

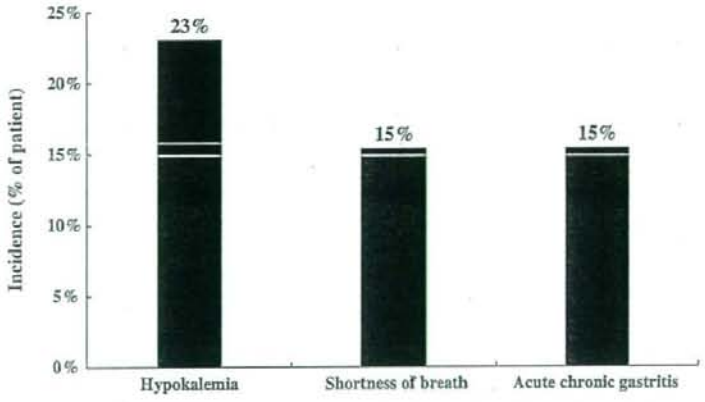

Fig. 3. Grade 3/4 reported adverse events occurring in ⩾10% of patients. No grade 4 toxicity was observed.

responsive to 3-month neoadjuvant use of letrozole. Notwithstanding the high overall response rate (ORR), only 2 out of 28 patients achieved pCR. After addition of imatinib, an improvement of nearly 30% of ORR was observed and 1 out of 10 patients achieved pCR which was comparable to previous trial. In our study, c-Kit expression was also investigated and almost all of our patients' pre-operative tumors possessed c-Kit expression although only eight tumor samples were performed. Repression of tumor growth through inhibition of c-Kit pathway by imatinib might be possible. Nevertheless, further exploration of a detailed molecular profile is highly recommended to investigate the relationship between high ORR and c-Kit expression. Though the sample size is too small to draw a solid conclusion in this stage, the result clearly demonstrated the potential additional clinical benefit of imatinib.

The dose of 400 mg twice daily for imatinib was chosen in this study according to the maximum tolerated dose from a Phase I study on other cancers [26]. No dose reduction was observed in this study, but 2 out of 13 patients were unable to tolerate the

combination resulting in treatment discontinuation whereas others were well-tolerated. The toxicity profile including edema, rash, nausea and vomiting, gastrointestinal disturbance as well as anemia is similar to other clinical trials [27]. However, unexpected high incidence of severe hypokalemia was observed possibly due to the concomitant use of diuretics for edema which results in electrolyte imbalance. More importantly, it is well known that imatinib may potentially develop cardiotoxicity [28]. In our study, one patient experienced acute coronary syndrome which clearly indicated the potential cardiac risk of using imatinib at dosage of 400 mg twice daily. Therefore, reduction of imatinib dose should be considered to improve the toxicity profile and reduce the chance of cardiotoxicity.

This is indeed the first study to report on the combination of letrozole and imatinib for treatment of hormone sensitive breast cancer in neoadjuvant setting. Yet, the results presented here is an initial phase of study which needs further research. We believe that, apart from combination of letrozole and imatinib, there would be more immense research on different kinds of combination of AI and TKI in the near future in order to promote treatment efficacy and fight against drug resistance in cancers.

5. Conclusions

The pilot study gives promising result of the combination therapy which warrants further investigation. The regimen is however quite toxic. Therefore, reduction of imatinib dose might be required to avoid its potential toxicity. Also, in the future, biological studies should be conducted to unveil the underlying mechanism contributing to response and resistance to therapy.

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The aspartic protease napsin A suppresses tumor growth independent of its catalytic activity

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Members of the aspartic protease family have been implicated in cancer progression. The aspartic protease napsin A is expressed in type II cells of the lung, where it is involved in the processing of surfactant protein B (SP-B). Napsin A is also expressed in kidney, where its function is unknown. Here, we examined napsin A mRNA expression in human kidney tissues using *in situ* hybridization. Whereas strong napsin A mRNA expression was observed in kidney proximal tubules, expression was detected in only one of 29 renal cell carcinomas. This result is consistent with previous observations of loss of napsin A expression in high-grade lung adenocarcinomas. We re-expressed napsin A in the tumorigenic HEK293 kidney cell line and examined the phenotype of stably transfected cells. Napsin A-expressing HEK293 cells showed an altered phenotype characterized by formation of cyst-like structures in three-dimensional collagen cultures. Napsin A-expressing cells also showed reduced capacity for anchorage-independent growth and formed tumors in SCID mice with a lower efficiency and slower onset compared to vector-transfected control cells. Mutation of one of the aspartic acid residues in the napsin A catalytic site inactivated enzymatic activity, but did not influence the ability to suppress colony formation in soft agar and tumor formation. The mutation of the catalytic site did not affect processing, glycosylation or intracellular localization of napsin A. These data show that napsin A inhibits tumor growth of HEK293 cells by a mechanism independent of its catalytic activity.
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KEYWORDS: napsin A; renal cell carcinoma; aspartic protease; tumor suppression

The aspartic protease family includes several physiologically important enzymes such as pepsin, chymosin, renin, gastricsin, cathepsin D and cathepsin E. Some members of this protease family, in particular cathepsin D and cathepsin E, have been implicated in cancer progression. High cathepsin D expression is associated with shorter disease-free and overall survival in patients with breast cancer.[1,2] In patients with ovarian or endometrial cancer, cathepsin D expression has been reported to be associated with tumor aggressiveness.[3,4] Transfection of low-metastatic tumor cells with wild-type human cathepsin D results in stimulation of tumor growth and increased propensity for experimental metastasis.[5] Interestingly, the catalytic activity of cathepsin D is not required for stimulation of tumor growth.[6] Cathepsin E expression has been reported to be a prognostic marker in bladder cancer.[7] Cathepsin E has also been suggested to promote tumor growth independent of its catalytic activity.[8]

Napsin A is an aspartic protease expressed in the lung and the kidney.[9–12] Napsin A is expressed in type II cells in lung alveoli and is capable of cleaving the proform of surfactant protein B (SP-B) expressed in this cell type.[13,14] Studies using siRNA showed that downregulation of napsin-A in type II cells results in inhibition of SP-B processing.[13,14] Among the different types of lung cancers, only adenocarcinomas express napsin A, making napsin A a promising diagnostic marker for primary lung adenocarcinomas.[15–17] Lung adenocarcinomas with a low differentiation grade express napsin A less frequently than more differentiated tumors, suggesting an inverse association between napsin A and tumor progression.[15,16,18]

Napsin was first described in mouse kidney as a new member of the aspartic protease family (KAP; kidney aspartic protease).[9] A previous study reported napsin A localization to lysosomes in proximal tubules.[19] Napsin expression in kidney is first observed at embryonic day 13, preceding kidney tubulogenesis.[19] The function of napsin in the kidney remains unknown, but the pattern of embryonal expression raises the possibility that napsin may play a role in the

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differentiation of proximal tubules.[19] Considering the importance of lysosomal aspartyl proteases during carcinogenesis and the possible role of napsin during kidney differentiation, here we examined the expression of napsin A in renal carcinomas. Furthermore, we studied the effect of re-expressing napsin A in a tumorigenic kidney cell line. We report that napsin A suppresses anchorage-independent growth and tumor formation by a mechanism independent of its catalytic activity.

MATERIALS AND METHODS

In Situ Hybridization

Twenty-nine renal cell carcinoma tissues, from patients treated in the Karolinska Hospital from 1995 to 1998, were randomly selected. The tissues consisted of 10 grade I, 12 grade II and 7 grade III cancers. The study was approved by the local ethical committee. Formalin-fixed, paraffin-embedded tumor sections were deparaffinized with xylene, treated with proteinase K (1 μg/ml, 37°C, 30 min), transferred to 0.1 M triethanolamine buffer (5 min) and treated with triethanolamine containing 0.25% acetic anhydride for 10 min. Sections were washed in 2 × SSC, dehydrated and allowed to air-dry. After overnight hybridization (2 × SSC, 50% formamide, 10% dextran sulfate, 55°C) with an ^{35}S-labeled RNA probe (1.16×10^5 c.p.m./μl), sections were washed (the most stringent step being 0.1 × SSC, 15 min at 60°C) and treated with RNase A (20 μg/ml, 37°C, 30 min). Finally, the slides were dehydrated, air-dried, dipped in Kodak NTB emulsion, exposed for 7–14 days at 4°C, developed and counter-stained with hematoxylin–eosin. The probes were made using T3 (antisense) and T7 (sense) RNA polymerase (Promega, Madison, WI, USA) from a pCMS-EGFP vector (Clontech, Palo Alto, CA, USA) containing an *Nhe*I/*Eco*RI napsin A full-length cDNA. Both sense and antisense probes were hybridized to all sections.

DNA Constructions

HA-tagged human napsin A cDNA was generated by polymerase chain reaction (PCR) using specific primers to human napsin A: 5′ primer, agcgctagcatgtctccaccaccgctgct (primer N1); 3′ primer, cgcgaattctcaagcgtagtctgggacgtcg tatgggtacccggggaactgcgcctgcg (primer N2). A PCR fragment was subcloned into pEGFP-N3 vector (Clontech) at *Nhe*I and *Eco*RI site. To generate a mutated napsin cDNA tagged with HA, PCR was performed using specific primers. To generate mutation of the catalytic site, the PCR-based overlap extension method was applied.[20] Primers were designed as follows: N-terminal fragment, 5′ primer, primer N1; 3′ primer, ccgtattcaggatggcagcacagccct; C-terminal fragment, 5′ primer, catcctgaatacgggcacgtccctcatc; 3′ primer, primer N2. To generate a control vector, the EGFP sequence was removed from pEGFP-N3 using restriction enzymes *Sal*I and *Not*I and both ends were filled in using the Klenow fragment, followed by self-ligation. None of the constructs were fused with EGFP. All constructs were subjected to bi-directional sequencing.

Stable Transfectants

Cells were maintained at 37°C in a 7% CO_2 atmosphere in DMEM (Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Invitrogen). Plasmids were transfected into HEK293 cells using LIPOFECTAMINE (Invitrogen). For stable expression, transfected cells were selected with G418 (Invitrogen) and G418-resistant colonies were analyzed for the expression of wild-type and mutant napsin A by immunoblotting with anti-HA antibody (clone 3F10; Roche Molecular Biochemicals, Mannheim, Germany).

Western Blotting

Samples were separated by electrophoresis in 12% polyacrylamide gel and transferred to nitrocellulose membranes. The membranes were incubated in blocking solution (5% nonfat dry milk in PBS containing 0.05% Tween 20) for 1 h at room temperature and then incubated overnight with anti-HA antibody (clone 3F10; 100 ng/ml; Roche Molecular Biochemicals) or antibodies directed against mature SP-B (number 28031) or SP-B proprotein (number 55522).[21] The membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated anti-rat IgG (Pierce, Rockford, IL, USA) or HRP-conjugated anti-rabbit Ig (Amersham Biosciences, Little Chalfont, UK) for 1 h. The membranes were washed six times and peroxidase activity was developed by SuperSignal West Pico (Pierce) according to the manufacturer's instructions.

Cell Proliferation Assay

For each cell line, 1×10^4 cells were seeded in 5 wells × 5 rows of 96-well plates in 100 μl DMEM containing 10% FBS. After 24, 48, 72 and 96 h, cell proliferation was assayed using CellTiter 96® Non-Radioactive Cell Proliferation Assay (Promega) according to the manufacturer's instructions. After growth curves were drawn, cell doubling time was calculated using the log-phase growth rate: cell doubling time = (2/the log phase growth rate for 24 h) × 24 h.

Colony Formation in Soft Agar

Five hundred cells from each of the stably transfected cell lines were suspended in 2.5 ml of 0.35% (W/V) agar in DMEM/20% FBS and overlaid onto 0.5 ml of 0.5% (W/V) agar in DMEM/20% FBS in three wells of a 12-well plate. After 10 days, colonies with more than 20 cells were scored as positive using an inverted microscope.

Cell Culture in Collagen Gel

Five hundred cells were suspended in 1.5 ml of type I collagen gel solution containing 66% vitrogen 100 (Cohesion Technologies, Palo Alto, CA, USA), 1 × DMEM, 0.004 g/l folic acid and 3.7 g/l sodium bicarbonate and overlaid onto 0.6 ml of collagen gel solution in a 12-well plate.

Tumor Growth in SCID Mice

Cells from each clone were suspended in PBS (2×10^7 cells/ml) and injected subcutaneously at the right and left

dorsal flanks of C.B-17/IcrCrl SCID mice (Charles River Laboratories, Sulzfeld, Germany) (100 μl per mouse). Five mice were injected with each clone. The tumor volume was measured daily until day 141 with a caliper rule. The tumors' major and minor diameters were measured and the volume was estimated at $4/3\pi \times (\text{major radius}) \times (\text{minor radius})^2$. Mice were killed when the major diameter of the tumor had reached 2 cm. In all the experiments, the ethical guidelines for investigations in conscious animals were followed and the experiments were approved by the local Ethics Committee for Animal Research.

Deglycosylation

Cells were lysed in a denaturing buffer (PBS, 0.5% SDS, 1% β-mercaptoethanol) and boiled for 10 min. The supernatant was incubated for 1 h with endoglycosidases F (New England Biolabs, Beverly, MA, USA) as recommended by the manufacturer.

Immunoprecipitation

Cells were lysed in a lysis buffer (PBS, 20 mM EDTA, 1 mM PMSF, 1% Elugent (Calbiochem, Darmstadt, Germany)) at 4°C for 30 min and centrifuged at 12 000 *g* at 4°C for 20 min. The supernatant was incubated with anti-HA affinity matrix (Roche Molecular Biochemicals) at 4°C overnight and the matrix was washed with the lysis buffer six times.

Proteolytic Activity of Napsin A

Recombinant proSP-B lacking the entire 102 amino-acid C-terminal domain (SP-$B_{\Delta C}$) was synthesized in the baculovirus system. The sequence encoding residues 1–279 of the human SP-B proprotein (SP-$B_{\Delta C}$) was cloned in-frame with a six-residue C-terminal histidine tag and ligated into pVL1393 (BD Biosciences Pharmingen, San Diego, CA, USA). Recombinant baculovirus was produced by homologous recombination in *Spodoptera frugiperda* cells, as previously described.[22] Fresh monolayers of *Trichoplusia ni* cells were infected with plaque purified recombinant virus at an MOI of 2 and cultured in serum-free media for 72 h. Recombinant SP-$B_{\Delta C}$ was purified from the culture media of infected insect cells by NTA affinity chromatography, under non-denaturing conditions, as previously described.[23] Purified SP-$B_{\Delta C}$ was incubated with immunoprecipitates from the stable transfectants of control, napsin A or either mutant in an incubation buffer (0.1 M sodium acetate, 20 mM EDTA, pH 4.7) at 37°C for 2 h.

Immunofluorescence

Cells grown on coverslips were fixed (4.0% formaldehyde in PBS, pH 7.4, 10 min), permeabilized (0.1% Triton X-100 in PBS, 3 min) and blocked for 30 min in medium containing 5% normal goat serum. After rinsing with PBS, immunostaining was performed by incubating the cells with antibodies in the following order with rinsing between antibodies: anti-HA (2 ng/μl; clone 3F10; Roche Molecular Biochemicals), Cy3-conjugated anti-rat IgG (H + L) (1:100; Jackson ImmunoResearch, West Grove, PA, USA), anti-LAMP-1 (1:200; BD Biosciences, Palo Alto, CA, USA) and fluorescein-conjugated anti-mouse IgG heavy and light chain (1:30; Calbiochem). Coverslips were mounted onto slides with VECTASHIELD Mounting medium with DAPI (H-1200, Vector Laboratories Inc., Burlingame, CA, USA). Cells were visualized by an immunofluorescence microscope (Axioplan 2 imaging, ZEISS). Cells in collagen gels were stained after digestion of collagen with 100 U/ml collagenase (Sigma-Aldrich Sweden AB, Stockholm, Sweden) for 15 min. Cells were fixed (4.0% formaldehyde in PBS, pH 7.4, 15 min), permeabilized (0.5% Triton X-100 in PBS, 15 min), incubated (0.5 mg/ml sodium borohydrate in PBS) and blocked for 30 min in medium containing 5% normal goat serum. After rinsing with PBS, immunostaining was performed as above.

RESULTS

Lack of Napsin A mRNA Expression in Renal Cell Carcinoma

Napsin A mRNA expression was examined in normal and cancerous kidney tissue using *in situ* hybridization (Figure 1). In normal kidney tissue, napsin A mRNA expression was observed in proximal convoluted and straight tubules and collecting ducts in agreement with previous reports (Figure 1a and b).[12,19] Twenty-nine renal carcinomas were

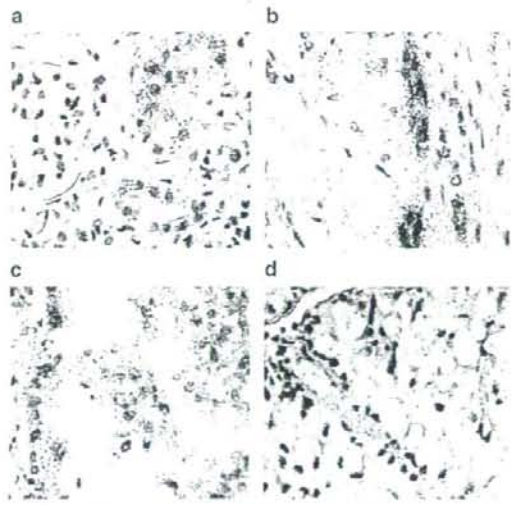

Figure 1 Analysis of napsin A mRNA expression in kidney tissues by *in situ* hybridization. In normal parts of the kidney, proximal convoluted tubules (**a**), proximal straight tubules and collecting ducts (**b**) showed napsin A expression. One positive case of renal cell carcinoma, which expressed napsin A mRNA (a grade II tumor), is shown (**c**). The majority of renal carcinomas studied did not express napsin A mRNA (**d**). Magnification × 40.

examined (10 grade I, 12 grade II and 7 grade III tumors). Only one of these tumors (a grade II tumor) showed detectable napsin A mRNA expression (Figure 1c), whereas the remaining 28 renal cell carcinomas examined did not show napsin A signals above background (Figure 1d).

Generation and Characterization of Cells Expressing Wild-Type and Catalytically Defective Napsin A

The observation of downregulation of napsin A in kidney tumors prompted us to generate kidney tumor cells that stably express napsin A. HEK293 is a tumorigenic cell line originally derived from human embryonic kidney cells by transfection of adenovirus type 5 DNA.[24] This cell line does not express a detectable amount of napsin A.[12] An HA-tagged cDNA napsin A construct (Figure 2a) was generated and transfected into HEK293 cells. Three clones that were stably expressing napsin A (napsin 1, napsin 2 and napsin 3) were isolated, together with two clones transfected with empty vector as a control. We also generated a mutation in the catalytic site by changing Asp283 into an Asn (napsin^{D283N}) (Figure 2a). The mutant was transfected into HEK293 cells and clones stably expressing the mutant were selected (clones napsin^{D283N} 21 and napsin^{D283N} 22). The level of expression of wild-type and mutated napsin A was comparable in these clones (Figure 2b). Napsin A expression was also analyzed by immunofluoresence staining and we found >99% of the cells to stain positive. Cells expressing the mutant had similar population doubling times as napsin A transfectants and significantly ($P<0.001$) longer time than control cells (25.3 ± 1.3 and 24.9 ± 1.5 h for control 1 and 2; 29.2 ± 0.4 and 27.2 ± 1.6 h for napsin 1 and 2; 29.4 ± 3.5 and 31.4 ± 1.2 h for napsin^{D283N} 21 and 22).

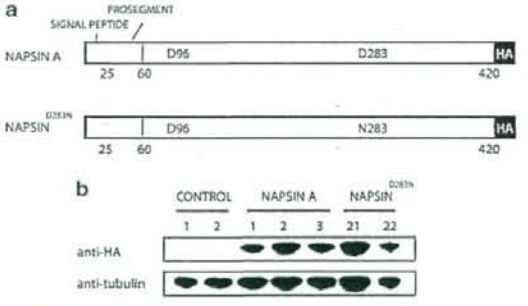

Figure 2 (**a**) Structures of wild-type and mutant napsin A cDNA constructs. The first amino acid (Met) predicted from the full cDNA sequence is numbered as 1. Deduction of the initial residue of mature protein is based on the result by Schauer-Vukasinovic *et al*.[25] In napsin^{D283N}, Asp^{283} in the catalytic site was changed to an Asn by site-directed mutagenesis. Napsin cDNA constructs have HA tags in their C-termini. (**b**) Protein expression levels of each clone. Clones that express napsin A or the catalytically inactive napsin^{D283N} protein were analyzed together with control clones transfected with empty vector. The expression level is shown by western blotting using an anti-HA antibody with tubulin as control.

We examined whether the D283N mutation resulted in loss of catalytic activity and whether the mutation altered the processing and intracellular localization of the enzyme. The N-terminal propeptide of proSP-B has been reported to be a physiological substrate of napsin A.[16] Mutation of the catalytic aspartic acid residue D283 resulted in loss of the ability of napsin to cleave a truncated form of recombinant proSP-B ($SP\text{-}B_{AC}$) (Figure 3a). Napsin A is synthesized in a proform and processed into the mature form by removal of its N-terminal prosegment. The mature protein is glycosylated in some or all of the three potential N-linked oligosaccharide attachment sites.[25] Wild-type napsin A and napsin^{D283N} both migrated as 41 kDa proteins in SDS-PAGE (Figure 3b). Deglycosylation by endoglycosidase F resulted in a decrease in the molecular weight to 38 kDa (Figure 3b), in agreement with the estimated molecular weight (38.9 kDa) of mature napsin A. These results suggest that wild-type and mutant napsins were glycosylated in the same way. As inactivation of the catalytic site did not affect processing, maturation of napsin A is not dependent on intramolecular autocatalysis in HEK293 cells.

Finally, to examine if the D293N mutation affects the intracellular localization of napsin A, we generated an expression vector expressing wild-type napsin A fused with GFP. This vector was transfected into cells expressing either wild-type or mutated napsin A containing an HA tag. As shown in Figure 3c, mutated napsin A (stained with a Cy3-labeled antibody) colocalized with wild-type napsin A (GFP), suggesting that mutation in the catalytic site did not affect the localization of napsin A.

Napsin A Expression Leads to Tube Formation and Reduced Colony Formation in Soft Agar Independent of Its Catalytic Activity

The phenotype of the HEK293 cell clones expressing wild-type or mutant napsin A was examined in detail. The cells of different clones were grown in collagen gels for 2 weeks. Phase-contrast microscopy showed that napsin A-transfected cells formed tube-like structures with branching morphology in the collagen gels whereas control cells did not (Figure 4a). Cells expressing napsin^{D283N} also formed tube-like structures in collagen (not shown). The difference in the organization of the cells in the collagen gels was more clearly discerned after DAPI staining of cell nuclei (Figure 4b). Napsin A-expressing cells formed cyst-like structures whereas control cells did not (Figure 4b and c). Interestingly, napsin A staining was observed in the center of the cyst-like structures (Figure 4c). These results suggest that napsin A promotes differentiation of HEK293 cells.

To determine whether the change in phenotype was associated with an altered ability for anchorage-independent growth, the ability of these cell lines to form colonies in soft agar was determined. All three clones of napsin A-expressing cells formed fewer colonies in soft agar than control clones (Figure 5). Cells expressing napsin^{D283N} formed a similar

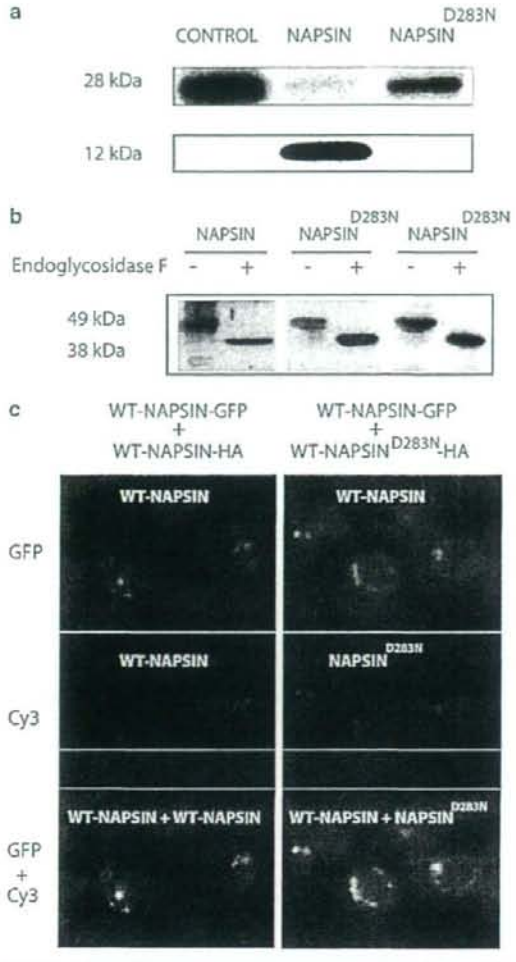

Figure 3 Functional analysis of wild-type and mutated napsin A proteins. (**a**) Recombinant SP-$B_{\Delta C}$ was synthesized in the baculovirus system, and napsin A and its mutant were expressed in HEK293 cells. Recombinant SP-$B_{\Delta C}$ was incubated with napsin A or its mutant at pH 4.7 at 37°C for 2 h. Napsin A cleaved SP-$B_{\Delta C}$ whereas napsin^{D283N} had lost the catalytic activity. (**b**) Deglycosylation of wild-type and mutated napsin A proteins. Napsin A and Napsin^{D283N} migrated as 41 kDa proteins and both these proteins migrated as 38 kDa proteins after deglycosylation by endoglycosidase F. Stable HEK293 clones expressing napsin A were analyzed; napsin-1 (expressing wild-type napsin) and clones napsin^{D283N} 21 and napsin^{D283N} 22 (expressing catalytically inactive napsin A). (**c**) Colocalization of wild-type and mutant napsin A proteins. A vector for wild-type napsin A fused with GFP was transfected into cells expressing HA-tagged napsin A or the napsin^{D283N} mutant. HA-tagged napsin A and the D283N mutant were visualized with Cy3-labeled anti-HA antibody (red). Wild-type napsin A fused with GFP (green) was colocalized with napsin^{D283N} with HA tag (red) (lower panel).

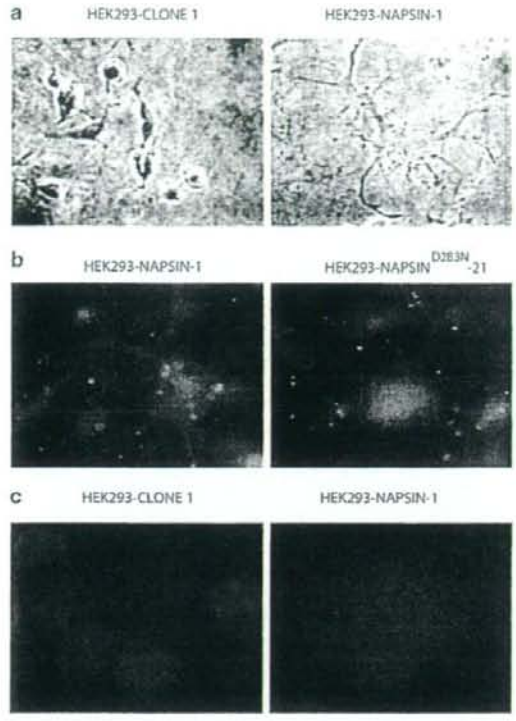

Figure 4 Cyst and branch formation by napsin A-expressing cells. HEK293 cells expressing napsin A and vector-transfected cells were grown in three-dimensional collagen gels for 14 days. (**a, b**) Effect of napsin A on cell morphology. (**a**) Phase-contrast photographs of HEK293 cells in collagen gels. Note that napsin A-expressing cells formed a tube-like structure with branching morphology whereas control cells did not. (**b**) HEK293 cells in collagen gels visualized by fluorescence microscopy. Cell nuclei were stained with DAPI (blue) and cytoplasms were visualized by immunostaining for LAMP-1. (**c**) Staining of napsin A in cells forming cysts. HEK293 cells in collagen gels were visualized by fluorescence microscopy. Cell nuclei were stained with DAPI (blue) and napsin A was visualized by immunostaining (red). Napsin A was expressed within the central portions of the cysts.

number of colonies as cells expressing wild-type napsin, and fewer than control (Figure 5). The catalytic activity of napsin A therefore does not appear to be required for suppression of colony formation in soft agar.

Tumor Growth *In Vivo*

Tumor formation of cells expressing wild-type or mutated napsin A was examined in SCID mice. Injection of HEK293 cells transfected with empty vector resulted in tumors first detected between days 30 and 36 (Figure 6). All injections resulted in tumor formation. The wild-type napsin A-expressing clones formed tumors with delayed onsets (day 70 to day 133). Six injections out of 10 in this group (2 from napsin 1 and 4 from napsin 2) did not result in detectable

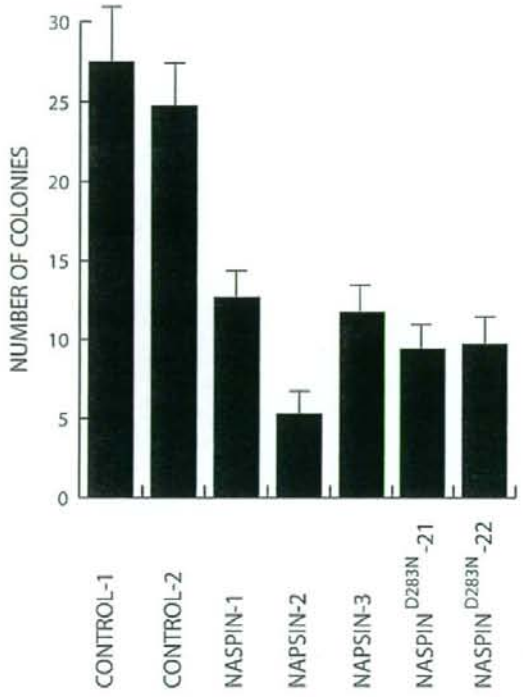

Figure 5 Inhibition of anchorage-independent growth of HEK293 cells by napsin A. Five hundred cells of each clone were suspended in 0.35% soft agar in three replica wells of 12-well plates. After 10 days, colonies with more than 20 cells were counted. All three clones stably transfected with napsin A formed fewer colonies in soft agar than the control cells transfected with vector alone. Both clones expressing napsin^{D283N} formed soft agar colonies with a similar efficiency as cells expressing wild-type napsin A.

tumors during the observation period (141 days). Similarly, the napsin^{D283N}-expressing clones formed tumors with delayed onsets (onset: day 46 to day 102). Five injections out of 10 (3 from napsin^{D283N} 21 and 2 from napsin^{D283N} 22) did not form tumors during the observation period. These data show that expression of wild-type napsin A and catalytically defective napsin A delayed tumor formation of HEK293 cells in SCID mice.

DISCUSSION

Napsin mRNA expression is detected in mesonephric tubules of the mouse kidney at embryonic day 13.[19] Expression is observed in proximal convoluted tubules in the mouse[19] and human (this study). Despite the fact that renal cell carcinomas arise mostly from proximal convoluted tubules,[26] napsin A mRNA was detected only in one of 29 cases of renal cell carcinoma. This result is consistent with a previous report where no staining of napsin A was observed in 15 cases of renal cell carcinoma by immunohistochemistry.[15] A similar pattern of napsin expression during embryogenesis and carcinogenesis is observed in the lung. Napsin expression is detected at the onset of differentiation of type II cells at embryonic day 15[19] and is maintained in type II cells in the adult.[11,12] Napsin A is expressed in well-differentiated lung adenocarcinomas, whereas expression is weak in poorly differentiated tumors.[15,16]

Napsin A has no known role in kidney. The pattern of napsin A expression during development and carcinogenesis raises the possibility that napsin A may have a function in inducing and/or maintaining the differentiated state. We engineered HEK293 kidney cells to express napsin A cDNA. Napsin A is strongly expressed in normal kidney and lung tissue; in the lung, napsin A was originally identified as a strong spot (TA02) on two-dimensional gels[27] and kidney

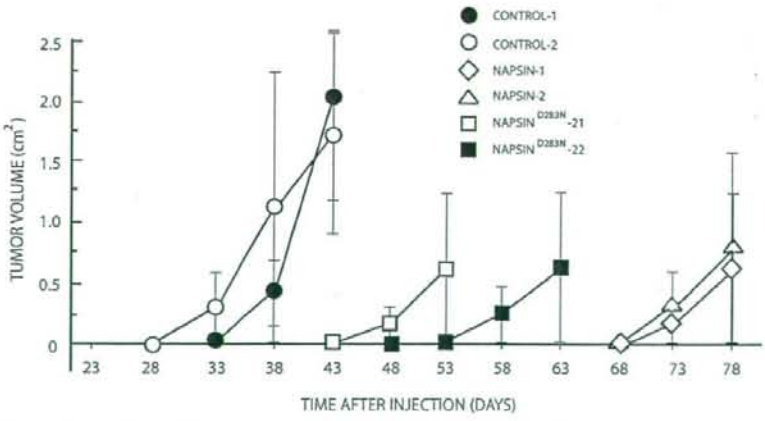

Figure 6 Tumor growth of HEK293 cells in SCID mice. SCID mice were subcutaneously injected with 2×10^6 cells at the dorsal flank. Each group consisted of five mice. Data are mean ± s.e.m. Cells expressing wild-type napsin A and napsin^{D283N} showed reduced growth of tumors compared with control cells. Animals with large tumors were killed in accordance with local ethical committee regulations.

expression has also been reported to be strong.[10] We were therefore not concerned that stably transfected cells would express unphysiological levels of the protein. Napsin A-transfected HEK293 cells had somewhat (10–20%) longer population doubling times compared with vector-transfected cells. Interestingly, when napsin A-expressing cells were grown in three-dimensional collagen gels, they were found to form cyst-like structures. Induction of this phenotype was not dependent on the catalytic activity of napsin A. Although the mechanism(s) involved is presently unknown, this result suggests that napsin A may have a function in the differentiation of kidney epithelial cells.

Napsin A-expressing HEK293 cells showed a reduced capacity for anchorage-independent growth. Furthermore, napsin A-expressing cells formed tumors in SCID mice with a lower efficiency and with a delayed onset. This finding is consistent with an association of napsin A in maintaining a higher degree of differentiation of kidney epithelial cells. The inhibition of anchorage-independent growth and tumor formation was found to be independent of the catalytic activity of napsin.

Previous studies have implicated aspartic proteases in tumorigenesis. Cathepsin D has been shown to stimulate cancer growth *in vitro* and *in vivo*.[28,29] Downregulation of cathepsin D by antisense methods leads to decreased tumorigenicity of breast cancer cells in nude mice.[30] Interestingly, a mutated cathepsin D lacking its catalytic activity stimulates cancer cell proliferation as efficiently as the wild type.[6] It has been hypothesized that stimulation of cancer growth by cathepsin D is mediated by an as yet unknown receptor.[6] As the overall structure of napsin A is similar to that of cathepsin D, one possible mechanism of tumor suppression by napsin A is that napsin A may antagonize the growth stimulatory effect of a hypothetical cathepsin D receptor.

The localization of the catalytic mutant was found to be indistinguishable from that of wild-type napsin A. Furthermore, the mutant enzyme was processed and glycosylated in the same way as wild-type napsin A. Different activation mechanisms have been described for aspartic proteases, including autoactivation (pepsinogen[31]), activation *in trans* by other enzyme(s) (prorenin[32]) and a combination of autoactivation and assisted cleavage (procathepsin D[33]). A previous study showed that purified napsin A does not cleave a 15-amino-acid peptide covering its own propeptide cleavage site.[25] This suggested that napsin A is not activated by autoactivation. Our data directly show that processing of napsin A is not dependent on intramolecular autocatalysis, at least not in HEK293 cells.

In conclusion, we showed that napsin A suppressed tumor growth in HEK293 cells independent of its catalytic activity. Further insight into the mechanim(s) involved may help in defining targets for therapeutic intervention in renal cancer. One possible strategy would be to reintroduce napsin A into cells. Other strategies could involve design of drugs or other agents that mimic the activity of napsin A. It is important to further elucidate the mechanisms of tumor suppression by napsin A to better understand the biology of kidney cancer development.

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Prolonged neutropenia after dose-dense chemotherapy with pegfilgrastim

In the dose-dense (DD) chemotherapy trial result reported by Piedbois et al. [1], they found more hematological toxicity leading to treatment discontinuation in the pegfilgrastim supported DD chemotherapy arm. The manufacturer's product information for pegfilgrastim indicates that it should be used once per chemotherapy cycle and should not be used in the period between 14 days before and 24 h after administration of cytotoxic chemotherapy, which is not practically possible in DD chemotherapy. Although pegfilgrastim has not been approved in Japan, we observed an episode of prolonged neutropenia in a Japanese patient who had undergone DD doxorubicin plus cyclophosphamide (AC) neo-adjuvant chemotherapy in the United States before being referred to us to continue chemotherapy then perform resection.

She was a 48-kg female in her mid-30s who presented at the Ithaca Medical Group clinic (New York) with locally advanced breast cancer. She underwent four cycles of DD AC, with pegfilgrastim 6 mg s.c. on day 2 of each cycle. Her absolute neutrophil count (ANC) was 2350/mm^3, 3650/mm^3, 4150/mm^3, and 7300/mm^3 at the start of the each cycle. After the forth AC cycle, she was referred to us for further chemotherapy. ANC at day 20 of the forth AC cycle was 3300/mm^3 but decreased to 500/mm^3 on day 26. Therefore, we had to postpone chemotherapy. Two weeks later (1 week after the last dose of filgrastim), the patient's ANC recovered to 1500/mm^3 and she received the first cycle of docetaxel (100 mg/m^2). She had received a total of 14 administrations of filgrastim starting from day 3 of the first cycle of docetaxel and had been severely neutropenic from day 7 (300/mm^3) to day 22 (300/mm^3). Her ANC on days 29 and 36 were 600/mm^3 and 1100/mm^3, respectively. Due to prolonged neutropenia, we decided to proceed to surgery. The patient has completed weekly paclitaxel as adjuvant chemotherapy, begun 3 months after the last docetaxel, with no major hematological toxicity.

Serum pegfilgrastim remained elevated in some patients even 14 days after the administration [2] and this seems dependent on weight-adjusted dose [3]. Chemotherapy administration during this period may very well cause more bone marrow suppression. It is possible that a 6-mg dose of pegfilgrastim is too large for Japanese patients in general. In our patient, the elevated ANC (3300/mm^3) on day 20 and grade 4 neutropenia on day 26 suggests that the effect of pegfilgrastim lasted at least 3 weeks. We therefore caution against the routine use of pegfilgrastim in DD chemotherapy until an optimal dose in this setting is ascertained, especially for low-weight patients. We strongly recommend that the European Society of Medical Oncology warns oncologists against the routine use of pegfilgrastim in DD chemotherapy, which is not an indication approved, and in which situation the drug has never been formally tested for optimal dosage. Such a recommendation would be particularly effective if made through the *Annals of Oncology*, given that this journal is well known and influential publication for clinical oncologists worldwide.

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がんバイオマーカー研究の現状と展望

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鶴尾 隆

鶴尾――本日はお忙しい中お集まりいただき、ありがとうございました。これから「がんバイオマーカー研究の現状と展望」という内容で、ヒューマンサイエンス振興財団の座談会を始めさせていただきたいと思います。

バイオマーカー研究は、最近非常に盛んになってきて、その必要性が謳われています。抗がん剤におきましても、その開発あるいは臨床応用の面でも注目されております。また、バイオマーカー研究は、これからの新薬の開発に非常に必要なツールではないかと思っております。このような背景から、本日は近畿大学の西尾先生、京都大学医学部附属病院の戸井先生、それから企業から協和発酵工業株式会社の塩津先生にお集まりいただき、バイオマーカーとはどのようなものであるか、どのような研究が進んでいるか、さらにはその将来の研究、開発におけるインパクト等についてもお話していただければ幸いです。

バイオマーカーとは

鶴尾――まずはバイオマーカーの定義、分類について、西尾先生に話していただきたいと思います。

西尾――バイオマーカーという言葉は古くからありますが、臨床で使われるようになってから特に重要になってきまして、いくつかのグループによって定義の試みがなされてきました。代表的なものがFDA（Food and Drug Administration）のバイオマーカーのdefinition ワーキンググループによる定義で、"バイオマーカーは正常な生物学的過程、発病の過程、もしくは治療介入による薬理学的反応を反映する測定及び評価可能な特性"であると思います。ヒューマンサイエンス振興財団のワーキンググループでも、バイオマーカーを日本において定義しようとしているように聞き及んでおります。

鶴尾――バイオマーカーにはどのようなものがあるかについては、後ほど各論で触れさせていただきたいと思います。

研究開発における バイオマーカー導入の背景

鶴尾――バイオマーカーは薬の研究、あるいは開発に重要であるということは一般的な認識であると思います。バイオマーカーの研究が進んできた、あるいはこのような概念が導入されてきたのはFDAの力が非常にあったからだと思います。塩津先生にバイオマーカー研究の導入の背景について、研究開発における重要性ということも含めましてお話いただければと思います。

塩津――近年、製薬企業がバイオマーカーを導入しようとしてきた背景というのは、薬剤のパイプラインの不足と成功確率の低下が最大の理由ではないかと思います。パイプラインが不足しており、その成功確率も低下しているということがその一つの大きな要因として挙げられます。薬剤開発は、フェーズⅠ、フェーズⅡ、フェーズⅢと進んでいくに従ってコストがかかりますが、臨床開発が後期に進むほど開発コストが上昇するにも関わらず、全体としての成功確率が下がってきたことがバイオマーカー導入の非常に大きな要因ではないかと思います。そのような背景で、2004年にFDAからInnovation or Stagnationというタイトルのレポートが提出され、その後2006年に続報としてCritical Path Opportunity Reportが提出されました。これら二つのレポートの中で、幾つかの問題提言がなされていますが、その大きな方向性として、薬剤開発を合理的に進めるために取り組むべきこととして、具体的には六