系によって細胞が処理されることになります。

戸井　では笹野先生，病理学的にはいかがでしょうか。

笹野　そもそも細胞の形がどうみえるのかという観察から，アポトーシスとネクローシス，オンコーシスの概念が生まれました。1940～1950年代にかけて，電子顕微鏡を含む細胞の形態学的観察から，同じ死んだ細胞でも自分で死んでいく細胞と外から殺される細胞に分けました。単純化しますと，最初に起きる変化が細胞の中のどこなのかということから，核で変化が始まればアポトーシス，細胞膜や細胞質ならばネクローシスあるいはオンコーシスというように分けられました。

戸井　通常の光学顕微鏡では区別はなかなか難しいですね。

笹野　さまざまな方法を使えば推察はできますが，アポトーシスとオンコーシスはあくまで電子顕微鏡で細胞をみた観察に基づいた定義です。電子顕微鏡は現在では使っていない施設が多いのですが，こういった重要な情報を得ることができ，現代でも細胞の研究には欠かすことができません。

がんにおける細胞死

戸井　がんの増殖のなかでも細胞死は常に起きていますが，そのメカニズムはどのようなものでしょうか。

笹野　未治療のがんでは，腫瘍細胞が増殖する際に正常よりも非常に大きなエネルギーが必要です。そのため生体の宿主に働きかけて，たくさん栄養素をよこせといった情報を出し，血管新生が起こります。ところが，同時にこのような腫瘍では vasohibin などの血管新生を抑える因子が次々にみつかっています。それらの血管新生抑制因子によって，たとえば血流が十分でなくなるとがん細胞は栄養がなくなりますから，その生存に非常に不利な状態になります。すると，そこで細胞膜が破れることもありますし，ストレスを感じる細胞内情報伝達系がまだがん細胞の中にある場合には，アポトーシスを起こすような酵素や細胞質の中の系を活性化させて死に至らせることもあります。このように，生体と同じで自分の生存に不利だとなれば細胞は死んでいきます。

このほか，種々の外的因子や免疫因子も関与します。いずれにしろがん細胞自身の成育に必要な栄養が届くか届かないのかということが宿主とがん細胞との関係に最も影響してくると思います。

戸井　がんの悪性度によって細胞死に違いはありますか。

佐谷　増殖能の高い腫瘍ほど細胞死がよくみられます。われわれが行っている研究では，多くのがん細胞において分裂期チェックポイントに異常のあることがわかっています。細胞が分裂するとき，チェックポイントの異常があるままで増殖していくと，DNA あるいは染色体の損傷をもったままで細胞が回転し，染色体の数や構造に変化のある細胞が出現します。極端に染色体の数が変わった細胞は生きる能力が低いので，死んでいくということがよく観察されます。悪性度が高く，染色体の不安定性が強い腫瘍ほど，たとえば脳腫瘍の神経膠芽腫などでは，組織中に染色体が凝集したような分裂像を呈して死んでいく細胞が多くみられます。

つまり，腫瘍には血流が不足することによって死ぬという死に方と，あまりに早く回転して，チェックポイントの異常があるままで増殖するために染色体に異常を起こし，自ら生活能力を失って死んでいく死に方があります。このような細胞死が起こるために，悪性腫瘍ほど細胞が多く死んでいるという，逆説的な病理像がよく観察されるのだと思います。

戸井　がんのなかでもホルモン依存性腫瘍とアポトーシスとの関係はいかがでしょうか。

笹野　エストロゲン受容体陽性の乳がんはエストロゲンがなければ増殖しません。そして，その増殖しない状態が続くと，アポトーシスを起こして死んでいきます。車のエンジンに対するガソリンのように，エストロゲンが全くなければ生存すら難しいような腫瘍細胞系が存在しているわけです。したがって，エストロゲン生成を阻害するような薬剤，たとえばアロマターゼ阻害剤を使うと，ほとんどのがん細胞がアポトーシスを起こし，結局は死んでしまいます。そして，マクロファージがそれを貪食して生体内から排除してしまいます。術前補助療法において病理学的完全寛解（pathological complete response；pCR）が認められる

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のは，こういった機序によるものと考えられます。

がん幹細胞と組織幹細胞

佐谷　がんの細胞死を考えるうえでがん幹細胞(cancer stem cell/cancer initiating cell)の概念を無視することはできません。がん幹細胞に関する研究を行っていて実感することは，いわゆるがん，特に臨床で扱っている患者さんから採取してきた組織には完全に階層性があるということです。分化した細胞もあれば，きわめて未分化な形で維持されている細胞もあり，さまざまな種類の細胞が混在して成立しているということがわかってきました。

そのなかにがんの根になる細胞が存在しており，これががん幹細胞です。本来の組織幹細胞(体性幹細胞)とは違うものだと考えていますが，非常にゆっくりと回転している点は同じです。ほとんど休止(dormancy)に近いくらいの緩やかな分裂をしています。がん幹細胞からできた前駆細胞は，非常に速く分裂しながら分化します。このように，ゆっくり分裂をしているがん幹細胞を“親分細胞”，そこから作られ高速に成育する細胞を“子分細胞”と考えて話を進めます。“子分細胞”は成育しながら形を変えて分化していきます。また，この“子分細胞”は抗がん剤などには非常に感受性が高く，治療によってかなりの頻度で消失していきます。ところが，がん幹細胞である“親分細胞”は抗がん剤に対して抵抗性であり，生き残ってしまうということが治療の大きな問題になっています。

戸井　“子分細胞”ができるときに環境，たとえば酸素濃度や低酸素などは影響するのですか。

佐谷　さまざまな考察がなされていますが，まだ結論は出ていないと思います。ただいえることは，“親分細胞”つまりがん幹細胞が，がん幹細胞としての性質を維持するためには特定の周辺環境が必要であるということです。これを現在ではniche(ニッチ)と呼んでいます。その環境のもとでがん幹細胞は自分と同じコピーを作る，あるいは時折コピーとは違う“子分細胞”を作るという2つの顔をもち，自分の後継ぎを作っていきます。ですから，低酸素や低血流といった周囲の環境が変化することで，がん幹細胞が突然増殖しだすことも当然あると考えられます。

笹野　そもそも正常な組織を作る幹細胞とがん幹細胞とは分けて考えなければなりません。

佐谷　そのとおりです。名前がよく似ているために，がん幹細胞とは組織幹細胞から発生したものだと考える方が非常に多いのですが，それはきわめてまれで，ほとんどの場合は増殖しながら分化段階にある細胞に変化が生じて，組織幹細胞のように自己複製能を獲得したものががん幹細胞であると解釈されています。笹野先生がおっしゃるように組織幹細胞とがん幹細胞の性質を混同しないほうがよいと思います。そのため，

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最近米国ではcancer stem cellという呼び方を変えて，cancer initiating cellに，すなわちがんを起こす細胞にしたほうがいいと主張する方もいます。

戸井　がん幹細胞にしても，がんのタイプによってアポトーシスの起こりやすさは違うのでしょうか。分化あるいは増殖していくなかでアポトーシスが起きやすくなるのでしょうか。

佐谷　今のところ明らかになっているのは，がん幹細胞は抗がん剤に対して抵抗性が高いということです。それは増殖がゆっくりである，代謝が非常に亢進していて抗がん剤をくみ出してしまう，あるいはnicheと呼ばれる環境に取り囲まれているために抗がん剤がアクセスしにくいなど，さまざまな理由から"親分細胞"は外部のストレスから守られています。さらに，抗がん剤で"子分細胞"を殺すと，"親分細胞"の相対的な比率が増加することになり，それだけ抗がん剤が効きにくくなってくるわけです。組織幹細胞もそうですが，幹細胞と呼ばれるものは，きわめてアポトーシスに対して抵抗性であるといえます。

戸井　では，抗がん剤の耐性を考える際に，がん幹細胞の密度が重要になりますか。

佐谷　私自身はそう思っています。もし組織中のがん幹細胞を正確に同定できる方法がみつかれば，それを算定することによって事前に抗がん剤がどれくらい効くのかを予測することが可能ではないかと考えています。

がんの可塑性と治療

戸井　笹野先生，がん幹細胞と治療についてはどのようにお考えですか。

笹野　がん細胞は非常に多くの形に"化け"ますから，治療にはがんの可塑性(plasticity)を考えたほうがいいと思います。ある時点では有効であっても，さまざまな治療をすると適応能力が高い細胞は変化して治療を無効にしてしまいます。しかし，このように適応しても残る性質があり，それに対して分子標的薬などを使うわけですが，最終的には免疫細胞ができてこないとがんは根治できないと考えています。

たとえば，モノクローナル抗体であるトラスツズマブを例にとりますと，トラスツズマブはその抗体依存性細胞毒性というよりは，HER2/neuを阻害してシグナルが伝わらないようにして増殖を抑えているといわれています。抗体を標的にしているので最初は病理学的にみても腫瘍がほとんどなくなるくらいの効果が得られますが，次第にがんは"化け"てしまいます。

ですから，細胞の表面だけに存在していてどんなにがんが"化け"ても，それだけはずっと残っているといった蛋白が認識でき，それに対してヒト型抗体を使うことができれば，がん幹細胞のコントロールも可能になると思います。

佐谷　正常な体細胞の増殖から分化に至る過程ある

いは幹細胞の性質とがんが最も違う点は，まさにその可塑性だと思います。いわゆる“化ける”という性質が可塑性であり，それがあるために，外的なストレスが入ったときにそれに対して形を変えることができるのです。たとえば，“子分細胞”は通常はそのまま滅んでいく運命にあるとわれわれは考えていますが，“子分細胞”自身にも可塑性がありますので，これが“親分細胞”に成り代わることになります。ですから，笹野先生がおっしゃるようにがん自身が形を変えていくことに対して，それを問題にしないでいいような治療が補助療法あるいはファーストライン治療，セカンドライン治療として行われるべきだと思います。免疫治療に希望があるといわれるのは非常に理解できます。

もう1つ，私が考えているのは先ほどのnicheです。周辺環境を変化させることで，がん幹細胞自身がそれを持続させる能力を失っていく，つまり樹木が土を失うことによって自壊していくように細胞が死にやすい状況を作ること，それががんを退治していく治療になるかもしれないのです。これは周辺の環境に対する治療ですので，がんの可塑性に対してあまり影響しないというメリットがあります。周辺環境を変化させることによってがん幹細胞としての性質を維持するために必要なnicheを失わせ，そしてがん幹細胞を全滅させるというniche療法も1つの考え方だと思っています。

免疫系を考慮した治療へ

戸井　可塑性を規定しているのはがん細胞自身なのでしょうか。

佐谷　可塑性を決定している重要なファクターとしてがん細胞自身のゲノムの不安定性があります。ゲノムの不安定性が基盤となってがん幹細胞ができますので，成立したがん幹細胞もゲノムの安定性が悪いことになります。ですから，どういう攻撃をかけていっても，がん細胞はどんどん形を変えてその攻撃に対抗していくのです。

がん細胞自身に対してではなく周辺環境に対する治療は，がんに対するアプローチよりはマイルドになります。免疫療法を含めて，こういったがん幹細胞自身に直接ストレスをかけない治療法はきわめて重要になってくると思います。

笹野　そのとおりだと思います。細胞情報の伝達系に，たとえば慢性骨髄性白血病(chronic myelogenous leukemia；CML)や消化管間質腫瘍(gastrointestinal stromal tumor；GIST)のc-Kitのように，それがないと腫瘍細胞は増殖できないで死んでしまうというがんは非常にわずかで，多くの腫瘍細胞には迂回するような細胞内情報伝達系がいくらでもあります。では，次にどうすればいいのか。そこで今度は立場を変えてみると，増殖のシグナルを抑える抗原が重要になってくると思います。

がんの抗原に対する方法はワクチンをはじめさまざまなものがありますが，血管新生を抑制することが非常に重要です。周囲にある正常なマクロファージやT細胞，B細胞といったネットワークがあってはじめてがんを抑えることができるわけです。ですから治療方針を立てるには，がん細胞ばかりではなく，がんの周囲の線維芽細胞やリンパ球などの間質細胞を含めて考えたnicheを考えていくべきです。

戸井　術後補助療法として，抗がん剤と分子標的薬，たとえば抗体療法を併用すると，3ヵ月間の治療でも治ってしまうがんが少なからず存在します。こういった抗体療法を一緒に使った場合，免疫系の関与はどのくらいになるのでしょうか。

笹野　抗がん剤で免疫が完全に抑えられてしまうなど，抗がん剤は免疫療法にとって非常にデメリットが大きいものです。たとえpCRであったとしても，組織をよくみることが重要です。がん細胞が残っていたとしても線維芽細胞に置き換わっている症例もあるはずです。また，これだけ免疫細胞にとっては厳しい環境であっても，リンパ球が浸潤してきます。最終的に増殖を抑える，あるいはそれを完全除去するのは，免疫系なのです。

こうした現象が宿主のなかには必ず存在しています。今まではがん細胞に目を向けていましたが，今後は完全寛解(complete response；CR)にならない症例は周囲の組織との相互作用を検索することを，臨床的には非常に難しいですが考慮すればいいのではないか

と思っています。

佐谷　宿主の免疫系とがんの関係は抗がん剤による治療を行うにあたって実に複雑です。抗がん剤はがん細胞のみならず，それを処理するために働くはずの免疫細胞まで殺してしまったり，機能を抑えてしまい，逆に治療の効果を下げることもありえます。

反対に腫瘍の中に浸潤しているマクロファージは，実は善玉として働いているのではなく悪玉として働いており，腫瘍関連マクロファージ(tumor associated macrophage；TAM)と呼ばれています。TAMに対しては，その働きをブロックすることによって，治療効果を高めることができます。

笹野先生の最初のお話にありましたように，まさにバランスです。免疫系が腫瘍に対して助ける方向に働いているものもあれば，逆にそれを抑える方向に働いているものもあります。バランスがとれたときに最適の治療が行えると思います。

分子標的薬と抗体療法を組み合わせていく，あるいは従来の細胞傷害性抗がん剤治療と抗体療法を組み合わせていくと，よく効く場合があります。私の想像ですが，抗体療法はがん幹細胞のような“親分細胞”に作用し，従来の化学療法剤あるいは増殖に対する低分子薬はどちらかというと“子分細胞”に対して作用します。そして，“親分細胞”に対しての作用と“子分細胞”に対しての作用がうまく組み合わされることで，良好な効果が得られるのではないかと思います。これからは同じ治療を行いながら，効いた症例と効かなかった症例とでは何が違うかを明確に分析すること，そういったアプローチが基礎と臨床の協力のもと必要だろうと思います。

戸井　確かにそうですね。

笹野　1つ付け加えますと，ホルモン療法は抗がん剤とは若干違います。ホルモン療法はホルモン受容体をもっているがんには強く作用しますが，性ステロイドの作用をほとんど受けない周りの間質には非常に優しい治療法です。たとえば，下垂体からの性腺刺激ホルモン分泌を抑えたり，局所でのアロマターゼ活性を抑えたり，核内受容体に結合するステロイドを抑えたりしても，がんに対しては非常に効きアポトーシスに傾いていきますが，周囲の線維芽細胞や免疫細胞は十分に機能しています。腫瘍と間質の関係において，ホルモン療法は抗がん剤などの化学療法とはかなり違うことを，腫瘍内科の先生方に認識していただければと思います。

予測とモニタリング

戸井　では最後に，予測が難しい場合のモニタリングについて，今後どういう方向に進めればいいのでしょうか。

佐谷　乳がんは解析をするのにきわめて理想的だと思っています。多くの場合，治療前に生検を行いますから，治療前の標本が入手できます。実際に手術をすることになれば，術前に化学治療を行い，それから腫瘍を摘出します。つまり，化学療法を行う前と行った後で標本を採取することができるわけです。

他の腫瘍でも最近では抗がん剤による術前補助療法を行ってから腫瘍の摘出を行うことがありますので，1人の患者の治療前と治療後の標本を使って，慎重にさまざまな角度から比べることにより，がんがどういう性質なのかをある程度予測していけるのではないかと思います。

そのときに細胞だけをみるのではなく，周りの間質の組成や免疫系細胞の種類などをみることは重要です。それらが治療を行うための判断基準になれば，非常に良いトランスレーショナルリサーチになります。さらに，抗がん剤の組み合わせの決定にも影響してくるのではないかと考えています。

戸井　笹野先生，いかがでしょうか。

笹野　がん細胞の特性は血管内皮の炎症性細胞からもみることができるかと思います。炎症ががんの周囲だけで起きていれば，免疫系のさまざまな蛋白が高感度に測定できます。さらに，血管内皮の中にどれだけ免疫細胞を引きつける分子が出てくるか，活性化したリンパ球のマーカーがどのくらいあるか，あるいはマクロファージにも食べるマクロファージとサイトカインなどを分泌して引き寄せるマクロファージの2つがあり，それぞれがどれくらいあるのかといったこともわかるでしょう。今後そういったものを臨床試験に組

み入れることが，1つの方向ではないかと思います。

佐谷　確かに質量分析法がきわめて進んできたので，体液や血液からでも微量な分子量を検出できるようになっています。抗がん剤を投与したときにどういった物質が血液中に出てくるかということで薬剤の感受性が予測できれば，非常に有用だと思います。

笹野　いわゆるプロテオミクスの時代ですので，多くの方ががん細胞の分泌しているものを測ろうとされています。それに加えて，全身にある組織にがんが浸潤したときに分泌されるようなもの，あるいはがんが浸潤したときに生体反応として線維芽細胞が分泌するようなものにも着目し，もし血液中でみることができれば，CRになるのかどうかの1つの指標にもなりえます。血液からの血清蛋白を解析することで，将来的にはプロファイリングができるのではないかと思います。

戸井　ありがとうございました。細胞死から，がんの可塑性，がんの周辺環境やnicheの重要性など，多岐にわたるお話をうかがいました。結論としましては，がん細胞とともに宿主および間質系の細胞にも注目すべきであること，予測性あるいはモニタリングに関しても，今後さらに進めていくべきであるということだと思います。本日は誠にありがとうございました。

PERGAMON

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Celecoxib anti-aromatase neoadjuvant (CAAN) trial for locally advanced breast cancer: preliminary report☆

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Abstract

Anti-aromatase therapy is important in the treatment of breast cancer in postmenopausal women. Cyclooxygenase-2 (COX-2) inhibitors have been shown to be effective in chemoprevention in animal and clinical studies. A proof of principle study was performed to investigate the efficacy of combing anti-aromatase therapy (exemestane) and COX-2 inhibitors neoadjuvantly in hormone-sensitive postmenopausal breast cancers. The initial results are reported. The patients were randomly assigned to receive exemestane 25 mg daily and celecoxib 400 mg twice daily (group A), exemestane 25 mg daily (group B) and letrozole 2.5 mg daily (group C). The analysis was based on 20 patients who received at least one cycle of treatment. Fourteen patients completed two cycles and 12 patients three cycles. All groups showed clinical response and there was decrease in tumor area in each group. However, complete clinical response was only observed for group A patients. There was also progressive decline in blood CEA and CA15.3 levels but the differences between the three groups were not significant. The results of the preliminary analysis are encouraging but definitive conclusion could only be drawn after the completion of the study.

Keywords: CAAN; COX-2; Breast cancer

1. Introduction

Breast cancer is the leading cause of death among women between the ages of 30 and 60 years. In the United States, an estimate of about 180,000 new cases of breast cancer are diagnosed each year and roughly 40,000 women will die from it each year. The incidence of breast cancer in Asia is also shown to be increasing recently, especially in the more affluent cities [1]. Breast cancer has become such a major health care issue that researchers and clinicians all over the world have shown great efforts in the past decades to find out a safe, cheap and effective treatment option.

Breast cancer is commonly associated with female hormones exposure. Growth stimulation of human breast cancer by sex hormones has been known for more than 100 years. In fact, Beatson demonstrated that oophorectomy could lead to shrinkage of breast tumors in premenopausal women in 1896 [2]. It is commonly accepted now that most breast cancers are hormone dependent. Our previous study showed that about 55% of patients possessed hormonal receptors and the frequency of hormonal receptor positivity increased with advancing age [3]. This special feature of breast cancer provides an opportunity for researchers to investigate the usefulness of endocrine therapy in the management of breast cancer. Current researchers are focusing their efforts on the use of aromatase inhibitors.

Aromatase is an enzyme complex consisting of a cytochrome P-450 hemoprotein and a flavoprotein. Its function is to convert 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 tissues like fat and muscle. Aromatization could also occur in breast cancers and the activity is mainly happening in the epithelial cells [4].

The cyclooxygenase enzymes are important for the conversion of arachidonic acid to prostaglandins, and their metabolites play a pivotal role in multiple physiologic and pathophysiologic processes. The inducible isoform, COX-2, is commonly over-expressed in breast cancer. Recent work suggests that COX-2-derived metabolites may contribute at multiple points throughout tumorigenesis, including premalignant hyperproliferation, transformation, maintenance of tumor viability, growth, invasion, and metastatic spread. It may also promote tumor-specific angiogenesis, inhibit

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apoptosis and induce proangiogenic factors such as VEGF, inducible nitrogen oxide synthetase (iNOS) promoter, IL-6, IL-8 and TIE-2 [5].

The clinical efficacy and tolerability of anti-aromatase agents have been demonstrated. Recent clinical trials show that these agents, whether type I or II, could improve the objective response (OR) rates by about 3–10% and the median survival by about 3 months for metastatic breast cancer even after tamoxifen failure [6]. Celecoxib (CXB) is a selective cyclooxygenase-2 inhibitor. It has chemopreventive and chemotherapeutic properties in rodent models of breast cancer.

The present trial is a proof of a principle study to evaluate the treatment efficacy of exemestane in combination with CXB in the neoadjuvant setting for postmenopausal hormonal receptor positive patients. As both drugs are well tolerated, most patients would be able to complete the treatment and we would be able to show the objective response rate in a documented fashion.

2. Patients and methods

Local Ethics Committee approval was obtained for this trial. Ninety patients are intended to be recruited. They should be postmenopausal with proven breast cancer. They should have a ECOG performance status of 3 or less of a Karnofsky score of 70 or above. They should be able to give written consent and follow instructions well. The clinical size of the tumor should be 3 cm or greater.

The diagnosis of breast cancer was confirmed by mammographic examinations and cytological examinations of the fine needle aspirates. Routine staging workup, including chest X-rays, ultrasound of the liver and bone scan, was performed. Core biopsy was taken for histological typing as well as determination of the status of the molecular markers and hormonal receptors. If there were no reasons for exclusion (Table 1), the nature and purpose of the trial was explained to the patients and informed consent was obtained for inclusion in the trial.

Blood was drawn from the patients the day before treatment was started and on completion of each cycle of treatment. The blood tumor markers including CEA and CA15.3 were determined.

Table 1
Exclusion criteria

Negative estrogen receptor status
Known sensitivity to anti-aromatase drugs or celecoxib
Major cardiac disease or LVEF <50%
Coronary artery disease
Active liver disease
Renal impairment
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

The patients were randomized into one of the three groups according to the preset random number. Group A patients were given exemestane 25 mg daily and celecoxib 400 mg twice daily. Group B patients were given exemestane 25 mg alone. Group C patients were given letrozole alone. The treatment will be given as monthly cycles. Each patient will be treated for 3 months and surgery will be performed within 7 days after the last cycle. Therefore, the total duration of treatment was 3 months or up until operation was performed. If operation was refused, the last end-point assessment for inclusion into study should be 90th day after commencement of treatment.

The assessment of tumor diameter was performed before starting treatment and also monthly until completion of neoadjuvant therapy. The clinical assessment will be performed by measurement with calipers. The response was defined according to the standard UICC criteria. Partial response was taken as a 50% or greater reduction in the products of the two maximum perpendicular diameters. Complete response was taken as complete resolution of the tumor.

2.1. Statistical analysis

Parameters were compared using the SPSS for Window release 9.0 (SPSS Inc., USA). One-way ANOVA tests were used to compare means in each group. Fisher's exact test or chi-square test were used to compare the number of events between groups. All values were expressed as mean and standard error of mean (S.E.) unless otherwise stated. $P < 0.05$ was considered as statistically significant.

3. Results

Twenty patients were recruited from May to September 2002. All of them were postmenopausal women. The mean age was 64.4 years (S.E. = 2.88). They were randomized according to the preset number. Nine were randomized to group A, four to group B and seven to group C. As the trial is on going, the results presented here are only from a preliminary analysis.

Fourteen patients completed 2 months and 12 completed 3 months of treatment. Of the 12 patients, five were assigned to be in group A, four in group B and three in group C. Seven patients underwent modified radical mastectomy. The analysis is based on the clinical data collected so far.

The initial largest tumor diameters were 4.61 cm (S.E. = 0.68), 4.13 cm (S.E. = 0.67) and 3.76 cm (S.E. = 0.47) for the respective groups of A, B and C. The original areas for the corresponding groups were 22.21 cm^2 (S.E. = 6.52), 14.2 cm^2 (S.E. = 2.32) and 12.79 cm^2 (S.E. = 2.55). The compliance was good and there was no defaulter. The changes in tumor diameter and area were shown in Figs. 1 and 2. All groups showed clinical response and there was a decrease in tumor area in each group. But the differences were not significant between the three groups. When

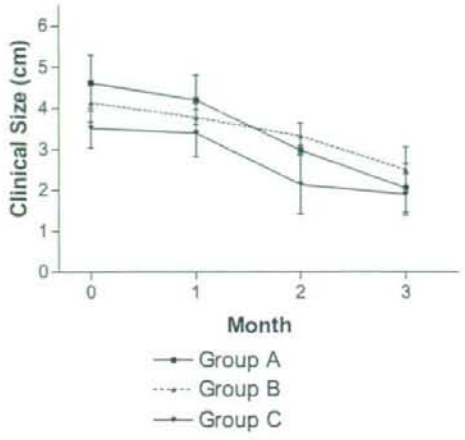

Fig. 1. Reduction in clinical tumor size after three different types of neoadjuvant treatment.

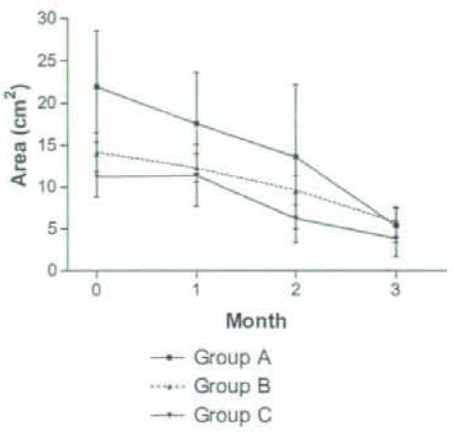

Fig. 2. Reduction in clinical tumor area after three different types of neoadjuvant treatment.

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.

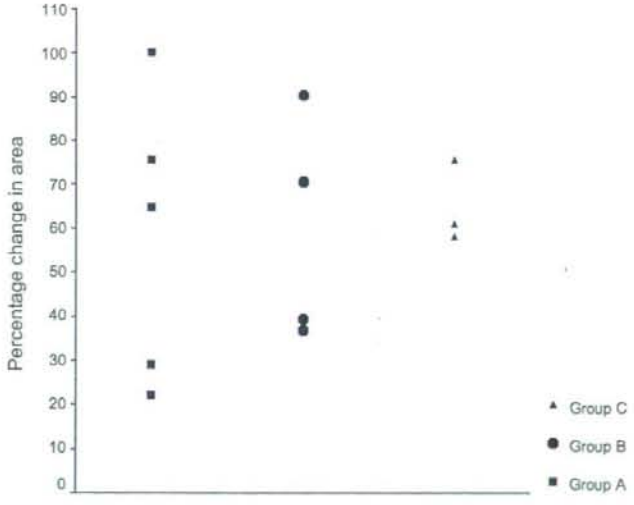

Fig. 3. Percentage reduction from baseline of tumor area for the 12 patients who completed three cycles of neoadjuvant treatment.

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

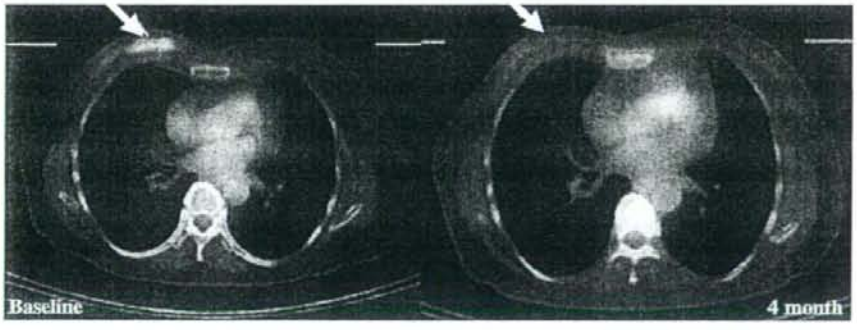

Fig. 1. [^{18}F]-FDG-PET scan obtained in the patient with pathologic complete response at baseline and 4 month after letrozole and imatinib treatment. Significant reduced FDG uptakes at primary tumor was noted in the PET scan.

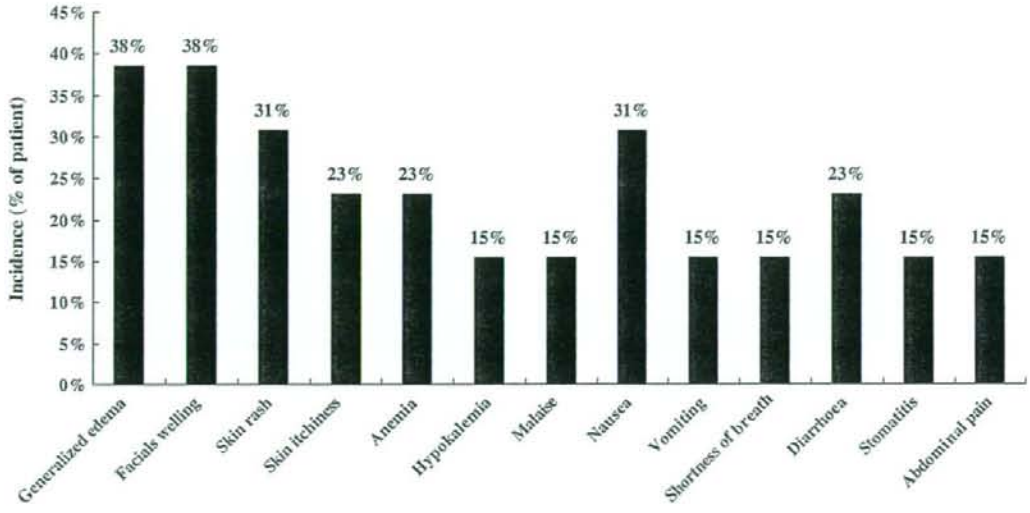

Fig. 2. Grade 1/2 reported adverse events occurring in ⩾10% of patients.

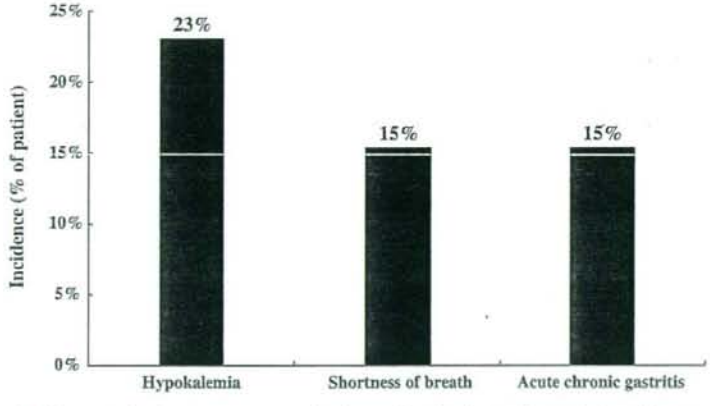

Fig. 3. Grade 3/4 reported adverse events occurring in ⩾10% of patients. No grade 4 toxicity was observed.

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