●原 著●

乳癌周術期化学療法の現状およびSupportive Careの工夫
—JBCRG01試験アンケートより—

Japan Breast Cancer Research Group (JBCRG)

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Summary

We carried out a survey of supportive care at institutions that participated in the JBCRG01 study (FEC followed by docetaxel) as neoadjuvant therapy for operable breast cancer. The purpose was to share the information of supportive care for the treatment effect of perioperative intensive chemotherapy among institutions.

Appropriate supportive care for nausea, vomiting, edema and febrile neutropenia (FN) is important with respect to the safety of chemotherapy. According to the results of the questionnaire, support from the family and the relationships with doctors, nurses and pharmacists familiar with the chemotherapy were important. The equipment and service for outpatients' cancer chemotherapy center are also important.

This multicenter study enhances the exchange of information among institutes. The results of this survey suggest that adequate supportive care makes anthracycline and taxane chemotherapy manageable in the outpatient setting.

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要旨 乳癌周術期の intensive な化学療法施行の際に，治療効果を最大限に期するための工夫が各施設にて行われている。FEC＋docetaxel の術前化学療法の臨床試験（JBCRG01）参加施設の supportive care の工夫を施設間で共有することを目的にアンケート調査を実施した。化学療法を安全に遂行するには悪心・嘔吐や浮腫，発熱性好中球減少症などの有害事象に対する適切な supportive care が重要である。それには医師のみでなく，癌薬物療法に詳しい看護師や薬剤師をはじめとする医療スタッフのかかわり，外来化学療法センターなどのハード面の整備，家族の支えなどが重要なポイントであることがアンケート調査から判明した。本グループのように多施設臨床試験を遂行することで施設間の情報交換も進む。今回のアンケートで得られた工夫が十分に行われていれば，anthracycline 系ならびに taxane 系薬物療法は，外来ベースで管理可能な薬物療法であると考えられた。

はじめに

近年，乳癌の治癒をめざし，エビデンスやガイドラインに準拠した化学療法レジメンの遂行が重要視されている。よりよい成果を得るためには，適格なレジメンの選択と同時に，予定されている投与サイクル，投与量を計画的に実施することがポイントである。化学療法には悪心・嘔吐，全身倦怠感などをはじめ数々の副作用を伴うことから，その予防と管理が重要である[1,2]。

Japan Breast Cancer Research Group（JBCRG）では，2002～2004 年に FEC（5-FU 500 mg/m^2，epirubicin 100 mg/m^2，cyclophosphamide 500 mg/m^2）を 4 コース後，docetaxel（DOC）75 mg/m^{2}を 4 コース行う術前化学療法の臨床試験（JBCRG01）を実施した。79 例の中間解析では，完遂率は FEC 97.5%，DOC で 92.3%とコンプライアンスは良好であり，臨床的効果は 71%であった[3]。

今回われわれは intensive な化学療法施行の際に，治療効果を最大限に期するための工夫を施設間で共有することを目的に，JBCRG01 試験参加施設を対象に周術期化学療法の現状および各有害事象に対する supportive care の現状についてアンケート調査を行い検討した。

Ⅰ．対象と方法

2005 年 6 月に，JBCRG01 試験に参加した 13 施設を対象に，周術期化学療法の現状ならびに各有害事象に対する supportive care の現状に関するアンケート調査（図 1）を，e メールもしくは FAX による回答形式で実施した。回収率は 100%であった。

本調査参加施設は，国立病院機構大阪医療センター，東京都立駒込病院，関西労災病院，聖路加国際病院，愛知県がんセンター中央病院，国立病院機構九州がんセンター，広島市立安佐市民病院，旭川医科大学，東京医科大学病院，国立病院機構名古屋医療センター，昭和大学附属豊洲病院，岩手医科大学，熊本大学の 13 施設である。

Ⅱ．調査結果

1．JBCRG 参加施設における周術期化学療法の現状

周術期化学療法の各施設の現状を，1）原発乳癌症例数，基本レジメンについて，2）術前・術後化学療法の初回コース治療開始状況，3）外来化学療法システムの整備，4）クリニカルパスの導入，5）informed consent（IC）の工夫，としてまとめた。

1）原発乳癌症例数，基本レジメンについて

1 年間の乳癌初発症例数（2004 年度）は，中央値で 160（20～500）例であった。術前化学療法施行数は，中央値で 16（3～150）例，13 施設での平均施行率は 15.8（4～40）%であった。その際，通常よく使用する術前化学療法レジメンとして，表 1 に示すレジメンが列挙されたが，多くは anthracycline 系と taxane 系の逐次併用レジメンであった。

2）術前・術後化学療法の初回コース治療開始状況

6 施設が全コース外来実施を基本としていた。しかし，このうち 5 施設においては，JBCRG01 試験参加前には 1 コース目の化学療法は入院にて行っていた。

外来実施が可能な主な理由として，① 治療内容，目的，起こり得る副作用とその対策についての十分な説明をチーム医療で行っている，② 緊急時の対応が可能（検査・入院体制），③ 看護師をはじめスタッフが有害事象に関してもよく理解し慣れている，などがあげられた。

一方，7 施設が 1 コース目は入院，2 コース目以降は外来で行う体制をとっていた。1 コース目に入院にて治療を行うメリットは，① 十分な説明と理解のための時間の確保ができる（患者への対応や教育の充実），② 不安解消・副作用出現時の対策をとりやすい，③ 個人差の掌握が可能，④ 病床稼働率・平均入院日数減少への貢献，などをほぼ共通してあげていた。デメリットは，医療費の高騰，患者負担の増加（生活制限・入院費），ベッドコントロールの煩雑さがあげられた。

周術期化学療法に関するアンケート

周術期化学療法の現状についてお答えください。再発例の場合は除いてお考えください。

- 施設と症例数・基本治療レジメについて
  - ① 1年間(2004年)の乳癌患者数(新患原発例)
  - ② 術前化学療法施行症例数(2004年)と代表的レジメ(3つ)
- 患者さんの治療形態について
  - ① 術前ないし術後の化学療法の施行場所について
    全コース外来を基本 or 1コース目は入院で2コース目以降は外来 or 施行毎に入院 or その他
  - ② 全コース外来を基本とされている施設への質問
    ◆昔、FEC やタキサン系治療を最初にはじめられたときでも、入院治療の経験はありませんか？
    最初は入院治療で経過をみたり、WBC などを測定したり、[ ]などの経験を踏んだ上で、今は外来治療スタートが可能となった or 最初から、外来治療で安心と考え、入院での治療経験はない
    ◆全コース外来治療が可能な理由
  - ③ 入院治療を行う施設の先生方へ質問。その理由をお聞かせください。
  - ④ 入院治療を行うメリット・デメリットは何でしょうか？
- 外来通院型化療療法施行の際の工夫について

1. ハード面の整備として外来化学療法センターの整備はありますか？
   有りのご施設は以下の①～⑦の質問にお答えください。無しのご施設は2. 以降の質問にお答えください。
   - ① 外来化学療法センターの開設時期
   - ② ホームページや外来等で化学療法室について紹介していますか？
   - ③ スタッフについて：専任医師＿＿＿名・専任薬剤師＿＿＿名・専任看護師＿＿＿名
   - ④ 設備について
     ベッド/救急カート/EKG モニター/ナースコール/リクライニングチェア/トイレ/洗面台/ テレビ/DVD プレイヤー/冷蔵庫/図書/BGM /プライベート空間確保のためのカーテンなど
   - ⑤ 稼働日・稼動時間について
   - ⑥ 業務について：ルート確保及び薬剤の調合は誰が行いますか？
2. 化学療法に関し、クリニカルパスは導入していますか？
   導入している場合各レジメ毎に作成？何種類あるか？
   導入していない場合他に何か工夫をしていますか？
3. 化学療法に関する患者さん向け文書を利用しているか？
4. ソフト面での工夫(一般的に使用するレジメを想定してお書きください)
   - ① 治療効果をあげる目的では、治療の完遂が大切かと思います。そのために工夫されているICのポイントなどを列記してください。
   - ② 患者を支える家族へのアプローチには何か工夫がありますか？
   - ③ 悪心・嘔吐対策・予防
   - ④ 口内炎などの粘膜障害への対策・予防
   - ⑤ 血管炎、血管痛対策・予防
   - ⑥ 血管外漏出に対する予防⇒実際に起きたときを想定してマニュアルを作成していますか？
   - ⑦ FN(好中球減少性発熱)への対策・予防(予防的抗生物質を処方する場合にはその使用基準もお示しください)
     ⇒実際に熱がどのようになれば、救急受診など含めて来院するように指導していますか？
     ⇒実際に、クール途中で、好中球などを測定しますか？その理由も。
     ⇒あるクールで、FNを確認した場合、次クールでの対策は？
     ⇒G-CSF について、その使用基準をお教えください。
     ⇒FN の際に、使用する抗菌剤・抗生物質について、使用薬剤・投与期間・使用基準・抗菌剤使用の有無
   - ⑧ 爪の変形・色素沈着などの皮膚障害への対策・予防
   - ⑨ 神経毒性(しびれ、感覚異常など)への対策・予防
   - ⑩ 関節痛・筋肉痛への対策・予防
   - ⑪ 浮腫対策・予防
   - ⑫ 脱毛対策・予防

それでは具体的に・・・今回の術前化学療法における貴施設の基本的な支持療法をお教えください。

JBCRG 01(FEC→Doc) 試験の supportive care に関するアンケート

副作用対策としてレジメごとに工夫されている支持療法を具体的にご記入ください。

- FEC 時
  化学療法投与前の投薬：薬剤名・投与経路・投与期間・総投与量・投与時期・使用理由
  化学療法投与後の投薬：薬剤名・投与経路・投与期間・総投与量・投与時期・使用理由
- Doc 時
  化学療法投与前の投薬：薬剤名・投与経路・投与期間・総投与量・投与時期・使用理由
  化学療法投与後の投薬：薬剤名・投与経路・投与期間・総投与量・投与時期・使用理由

図1 JBCRG01 アンケート概要

表 1 JBCRG 参加施設で施行されている術前化学療法レジメン（施設数）

FEC100×4 コース＋docetaxel×4 コース（n＝13）
AC（adriamycin＋cyclophosphamide）×4 コース＋weekly paclitaxel×12 コース（n＝3）
FEC100×4 コース＋weekly paclitaxel×12 コース（n＝2）
AC×6 コース（n＝2）
ET（epirubicin＋docetaxel）×6 コース（n＝1）
weekly paclitaxel×12 コース（n＝1） など

各施設より主な 3 レジメンが列挙された

表 2 周術期化学療法における IC の工夫

①乳癌について現実を理解してもらう
全身病の性格，具体的な数字を示し再発したら助からないことを理解する
②化学療法のメリットを理解してもらう
治療の目的・目標（治療完遂の重要性），スケジュールを明確にし，治療意欲を惹気する
特に術前化学療法の場合は，
治療効果の確認，pCR の意義，温存術成功の可能性などメリットを明言
オーダーメード医療の一環，translational research への貢献も説明
③化学療法の副作用を理解してもらう
副作用の十分な説明とともに，supportive care を示し，不安を解消する
予防できることできないこと（脱毛など）を明確にする
④緊急時の連絡先，対応などを明示
電話相談，メール相談
⑤精神的なサポート（安心感を与える）
カウンセラー，患者中心の精神サポートグループへの参加を提案
同じ治療を受ける患者間での情報交換，患者どうしの支えあいもポイント
⑥説明時の工夫
説明パンフレットの利用，家族の同席，同意を急がない，生活様式の調整を図る
医療相談室へ紹介し，早期から経済支援方法の検討を提案する
家族のみとの話し合いにより患者の精神的サポートをお願いする

3）外来化学療法システムの整備

整備された外来化学療法センターは 9 施設で稼動している。開設時期は早い施設で 1994 年，1999 年に開設されているが，その他は 2002 年以降であった。9 施設のうち 7 施設が外来やホームページで外来化学療法センターの開設について紹介していた。7 施設が月～金の朝から夕方まで平日はほぼ全日稼動していた。各施設の規模は異なるもののほぼ共通して，ベッド，リクライニングチェア（3～25 床），救急カート，ECG モニター，ナースコール，カーテンなどのプライベート空間確保，洗面台，トイレの設備がされていた。これらの設備に加え，BGM，図書，テレビ，DVD プレーヤー，冷蔵庫など患者が心地よく過ごせるためのアメニティも工夫され，癒しの環境づくりに力を入れている施設があった。

9 施設とも外来化学療法センターの専任医師，専任薬剤師，専任看護師のいずれかが常駐しており，5 施設では専任医師・専任薬剤師・専任看護師すべてが常駐していた。外来化学療法センターの整備に伴い，業務の分担も明確になってきている。13 施設中，調剤は薬剤師が担当するのが 8 施設，点滴ルートの確保も一定の資格を有した看護師が実施する施設は 3 施設あった。

4）クリニカルパスの導入

5 施設で化学療法用のクリニカルパスが導入され，基本レジメン以外に複数のクリニカルパスが作成されていた。一方，未導入の 8 施設でも電子カルテのレジメン登録機能の活用，レジメンの固定，チェック機能の強化，医療スタッフの固定，患者情報を共有するなど工夫を行い，常に同じレベルで患者への対応や指導説明ができる体制をとっていた。

5）Informed consent（IC）の工夫

適格な治療を選択し完遂率を高め，ひいては治癒率を向上させるために informed consent（IC）は大変重要である。各施設の IC の工夫をまとめた結果を表 2 に示す。周術期化学療法に際しては告知はもちろんのこと，具体的に再発リスクを示すなど治療への意欲を引きだす工夫がなされている。患者説明用パンフレットの活用は 12 施設で実施されており，11 施設では製薬メーカーの冊子を応用するだけでなく，院内で独自に作成していた。

2．JBCRG01 試験参加施設における supportive care

各施設で基本的なレジメンを行う上で，各有害事象に対する supportive care の現状を調査した。図 2 は

図 2 JBCRG01 試験における supportive care の工夫
上段は各施設でほぼ共通して行われており，下段は各施設で試行されている工夫を示している。

JBCRG01（FEC-DOC 術前化学療法）時の premedication と supportive care を示す。以下，有害事象別の予防と対策のポイントであった。

1）悪心・嘔吐，便秘

悪心・嘔吐，倦怠感に対しては，図2に示すステロイドと 5-HT_3拮抗剤の前投薬が全施設で共通しており，化学療法後の supportive care も内容の細部は異なるものの，各施設で制吐剤が中心であった。注射剤の 5-HT_3拮抗剤の前投薬以外に，投与の翌日以降から3~5日間経口 5-HT_3拮抗剤を併用するなどの工夫がされていた。予測性嘔吐に対してマイナートランキライザーをあらかじめ内服させる施設もあった。制吐剤によるコントロール以外に，急性期嘔吐と遅発性嘔吐を管理できるように症状経過の予測をあらかじめ説明したり，開始前の外来受診時に化学療法室（治療現場）を見学することで予測性嘔吐を予防したり，食事の工夫，患者との信頼関係の構築，排便コントロールの重要性を啓発するなどの工夫がなされていた。

5-HT_3拮抗剤の使用の影響などで便秘傾向になる場合もあるが，長期間の便秘は悪心・嘔吐の遷延の原因にもなり得る。便秘に対しては，センノシド錠，酸化マグネシウムなどの緩下剤をあらかじめ処方する，水分コントロール・食事の工夫を考え指導する，などがなされていた。

2）口内炎など粘膜障害

予防として，うがいの励行，治療開始前の齲歯治療・口腔内の保清の指導がされていた。化学療法中にエレースアイスボールなど氷片を含み，発現時にはステロイド外用薬（デキサルチン軟膏，プロピオン酸ベクロメタゾン）が処方されていた。

3）血管炎，血管痛

繰り返し同じ血管を用いない，点滴時間を速くし化学療法終了後，生理食塩水によりフラッシュする，点滴中は加温（ホットパックなど），終了後冷庵する，FEC の epirubicin で RTU（水溶液）を用いる時はステロイドとの併用で pH を調整するなど予防の工夫がされていた[4]。実際に血管炎が起きた場合には，ステロイド軟膏を処方する，重症例では一期的に CV ポートを留置するなどされていた。

4）血管外漏出

予防として，確実なルートを確保するために太い血管を選ぶ，関節近傍の血管には挿入しない，採血用のルートと点滴用の血管を変える，抜針前生理食塩水のフラッシュを徹底する，実際に起きた場合はステロイド軟膏・

ステロイド局注を行い，生理食塩水ガーゼで冷罨がされていた。8施設では実際に起こったことを想定してマニュアルを作成し，皮膚科紹介などがされていた。

5）発熱性好中球減少症（FN）

あらかじめニューキノロン系薬剤（ciprofloxacin, ofloxacin）やセフェム系抗生物質などを処方し発熱時の対応を指導する，経口の抗生物質を使用しても遷延（抗菌剤を服用しても熱が下がらない場合，熱が3日以上継続する時）や高熱を認める場合には救急受診するように指導されていた。

3施設で，個人差把握の目的で実際にコース途中で好中球数を測定していた。10施設では発熱時以外，好中球数は測定していなかった。

G-CSFの投与のタイミングは，発熱後すぐ開始（3施設），FNが続く時のみ使用する（7施設），原則使用しない（1施設），FNがなくても遅延などによるdose intensity低下を防ぐ目的で使用（2施設）と回答が分かれた。経口抗生物質でコントロール可能なFNであった場合，次コース以降の対応は，25%減量し投与するのが7施設，再度減量せず投与するのが4施設，G-CSFを使用しながらdose intensityを維持するのが2施設との回答であった。G-CSFの投与はガイドラインに基づき行われていた[5,6]。

6）爪の変形・色素沈着などの皮膚障害

ビタミンB_6，ケラチナミン軟膏，尿素軟膏，デキサルチン軟膏などの処方がされていた。投与中に冷却パックで冷庵する，爪に対してマニキュアで爪を保護するという施設もあった。

7）神経毒性（しびれ・感覚異常など）

発現時にビタミンB_{12}・B_6剤内服，漢方薬（牛車腎気丸，芍薬甘草湯），胃炎・潰瘍治療剤，非ステロイド性抗炎症薬（NSAIDs）などの処方がされていたが，標準的治療はなかった。

8）関節痛・筋肉痛

ビタミンB_{12}剤内服，漢方薬（牛車腎気丸，芍薬甘草湯），NSAIDsなど鎮痛剤の処方が行われていた。

9）浮　腫

ステロイド剤の予防投与，心・腎機能が正常ならば出現時には早期から利尿剤の処方，治療前から浮腫傾向がある患者には漢方薬として柴苓湯や五苓散が処方されていた。サポーターや弾性包帯の使用，マッサージ，減塩食の指導などの工夫もされていた。

10）脱　毛

予防はできないため，脱毛は抗癌剤使用中に限られる点の理解を得，かつらや帽子，バンダナの紹介を行っていた。

Ⅲ．考　察

JBCRG参加施設における周術期化学療法のレジメンは基本的にanthracycline系とtaxane系の逐次併用療法であり，これらのintensiveな治療を確実に行うためには，外来化学療法センターの設備（アメニティ）などのハード面の工夫と，看護師・薬剤師とのチーム医療の実践，そして有害事象のマネージメントがポイントとしてあげられる。外来での化学療法により，患者はQOLを維持しながら通常の生活を継続することが可能になる。また，化学療法の入院治療から外来治療への移行は，今後，diagnosis-procedure combination（DPC）の採用に伴いますます加速するものと思われる[7]。JBCRGに参加している施設は，初回コースの治療開始状況は外来治療と入院治療に分かれていたが，2コース目以降は全施設で外来治療が行われていた。外来治療でも緊急時に対応できる体制として，専門スタッフの固定，レジメンの固定，クリニカルパスの導入などがされており，外来化学療法にはチーム医療による治療管理体制の構築が重要と思われた。また，患者に化学療法への能動的な気持ちを惹起するために，ICの際に家族のみとの話合いを行い患者の精神的サポートの協力を依頼したりするなど，家族の協力や患者間での情報交換支援が重要なポイントと思われた。

有害事象がいったん生じると患者の化学療法継続への意欲低下，ひいては治癒率の低下に結び付くため，予防に重点をおくことが大切である。JBCRG01試験の中間解析時では，前半のFEC時にはgrade 1，2の悪心・嘔吐が半数以上の患者で発現し，grade 3も認められた。嘔吐は約半数で発現しているが，後半のDOCでは発現頻度は低下していた[3]。基本は予防であり，ガイドラインに従い5-HT_3拮抗剤とステロイドの投与は全施設で実施されていた[8-10]。悪心・嘔吐を理由に治療継続を断念した例はなかったことから，悪心・嘔吐に対するpremedicationやsupportive careにより管理可能と考えられた。ただし，FEC療法中の悪心の発現率は高く，さらに予測性の悪心・嘔吐対策にも心がける必要があると思われた。また，5-HT_3拮抗剤の使用の影響などで便秘傾向になり，長期間の便秘は悪心・嘔吐の遷延の原因にもなり得るので，FEC療法での便秘への対策も必須と考えられた。

また，中間解析の結果では，DOC療法はFEC療法に対し浮腫や筋肉痛・関節痛の発現頻度が高かったがいずれもgrade 1，2であり，重篤なものはなかった。浮腫に対してはステロイドの予防投与や早期からの利尿剤の処方，筋肉痛・関節痛が発現した際にはNSAIDsを服用す

るよう指導がされており，早期のsupportive careにより重篤な副作用が避けられると思われた。FNはDOCに対しFEC療法で発現頻度が高かったが（19% vs 3.8%）[9]，多くの施設で発熱時以外には好中球を測定しておらず，発熱時に抗菌剤を服用するように指導されていた。副作用の発現時の服用を指示する以外に，外来化学療法の施行時には感染予防のためのセルフケア支援なども重要と考えられた[11]。

今回のアンケート調査の結果から，各施設の様々なアイデアを得ることができ，グループ内で共有することができた。本グループのように多施設臨床試験の遂行により，施設間の情報交換が進む。副作用対策に関してもガイドラインに準拠する以外に，今回のアンケートのように施設間で情報交換し，すぐに実践するような姿勢が，高い完遂率と良好な治療成績に結び付くものと思われた。今回の調査で得ることができた様々な化学療法時のsupportive careが十分に行われていれば，FEC療法やtaxane系薬剤による化学療法は，外来ベースで管理可能な薬物療法であると考えられた。

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Intratumoral concentration of sex steroids and expression of sex steroid-producing enzymes in ductal carcinoma *in situ* of human breast

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Abstract

It is well known that sex steroids play important roles in the development of invasive ductal carcinoma (IDC) of the human breast. However, biological significance of sex steroids remains largely unclear in ductal carcinoma *in situ* (DCIS), regarded as a precursor lesion of IDC, which is partly due to the fact that the intratumoral concentration of sex steroids has not been examined in DCIS. Therefore, in this study, we first examined the intratumoral concentrations of estradiol and 5α-dihydrotestosterone (DHT) using liquid chromatography/electrospray tandem mass spectrometry in DCIS. Intratumoral concentrations of both estradiol and DHT were threefold higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, steroid sulfatase, and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1)), and androgen-producing enzymes (17βHSD5 and 5α-reductase type 1 (5αRed1)) were abundantly expressed in DCIS by real-time PCR and immunohistochemical analyses. The intratumoral concentration of DHT was significantly lower in IDC than DCIS, while the expression of aromatase mRNA in carcinoma cells and intratumoral stromal cells was significantly higher in IDC than those in DCIS. Immunohistochemistry for sex steroid-producing enzymes in DCIS demonstrated that 5αRed1 immunoreactivity was positively correlated with Ki-67 labeling index and histological grade and was also associated with an increased risk of recurrence in patients with DCIS examined. Results of our study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. Therefore, estradiol and DHT may play important roles in the development of DCIS of the human breast.

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Introduction

Breast carcinoma is the most common malignant neoplasm in women worldwide. Among breast carcinomas, the incidence of ductal carcinoma *in situ* (DCIS) has been markedly increasing during the past two decades due to advancements in detection by mammographic screening (Li *et al.* 2005) such that DCIS now comprise ~20% of all human breast carcinomas diagnosed (Kepple *et al.* 2006, Tsikitis & Chung 2006). DCIS is regarded as a precursor lesion of invasive ductal carcinoma (IDC) and the risk of IDC developing was reported to be four to ten times higher after the diagnosis of DCIS compared to women without DCIS (Franceschi *et al.* 1998, Warnberg *et al.* 2000). Therefore, it is very important to examine the biological features of DCIS in order to improve clinical outcome of breast carcinoma patients.

It is well known that breast tissue is a target for sex steroids. Among the sex steroids, estrogens greatly contribute to the growth of IDC (Vihko & Apter 1989) and anti-estrogens such as tamoxifen, aromatase inhibitors, or luteinizing hormone-releasing hormone (LH-RH) agonists are currently used in patients

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with IDC to block the intratumoral estrogen actions. A great majority of DCIS cases express sex-steroid receptors, i.e., estrogen (ER), progesterone (PR), and androgen (AR) receptors (Selim *et al.* 2002, Baqai & Shousha 2003, Moinfar *et al.* 2003, Barnes *et al.* 2005, Rody *et al.* 2005, Kepple *et al.* 2006), which suggest an important role of sex steroids in both DCIS and IDC. Tamoxifen was reported to inhibit the growth of premalignant mammary lesions and the progression to invasive carcinoma in a transplantable mouse model of DCIS (Namba *et al.* 2005). The National Surgical Adjuvant Breast Project (NSABP) P-1 trial demonstrated that tamoxifen significantly reduced the risk of noninvasive breast cancer by 50% (Dunn *et al.* 2005) and results of the NSABP B-24 trial indicated that adjuvant tamoxifen therapy was clinically effective in ER-positive DCIS and reduced the recurrence of non-invasive breast carcinomas by 27% (Cuzick 2003).

The concentration of the biologically active estrogen, estradiol, is significantly higher in IDC than in the areas considered as morphologically normal (Chetrite *et al.* 2000), and estradiol is locally produced from circulating inactive steroids by estrogen-producing enzymes such as aromatase (conversion from circulating androstenedione to estrone or testosterone to estradiol), steroid sulfatase (STS; hydrolysis of circulating estrone sulfate to estrone), and 17β-hydroxysteroid dehydrogenase type 1 (17βHSD1; conversion of estrone to estradiol) in IDC (Suzuki *et al.* 2005*a*; Fig. 1). Intratumoral concentrations of the biologically active androgen, 5α-dihydrotestosterone (DHT), were also significantly higher in IDC than in plasma (Recchione *et al.* 1995) and androgen-producing enzymes, such as 17βHSD5 (conversion of circulating androstenedione to testosterone) and 5α-reductase (5αRed; reduction of testosterone to DHT) were frequently expressed in IDC (Suzuki *et al.* 2005*a*; Fig. 1). Immunolocalization of aromatase (Zhang *et al.* 2002, Oliveira *et al.* 2006) and 17βHSD1 (Ariga *et al.* 2000) has been previously reported in DCIS, suggesting the possible importance of *in situ* production of sex steroids in DCIS. However, to the best of our knowledge, the intratumoral concentration of sex steroids has not been reported at all in DCIS and no information is available regarding the expression of androgen-producing enzymes in DCIS. Information on sex steroids is very limited in DCIS compared with that in IDC and so the clinical and/or biological significance of sex steroids in DCIS remains largely unclear. Therefore, in this study, we examined the intratumoral concentrations of estradiol and DHT and expression of sex steroid-producing enzymes in DCIS, and compared these findings with those in non-neoplastic breast and IDC tissues. In addition, we immunolocalized sex steroid-producing enzymes in 83 DCIS cases, and correlated these findings with various

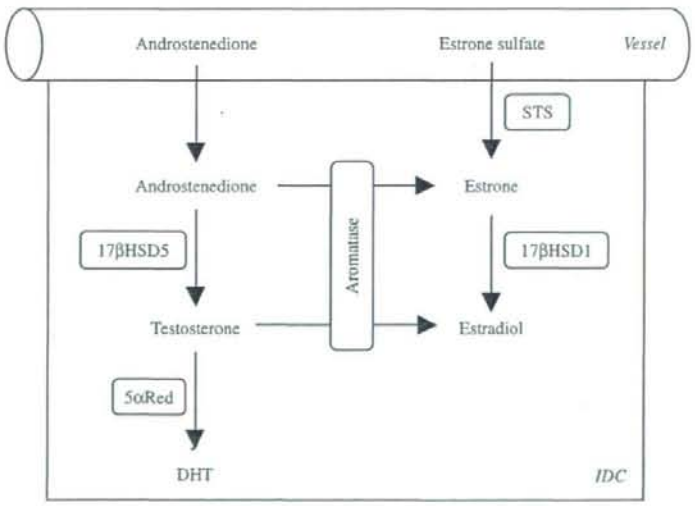

Figure 1 Schema representing intratumoral production of sex steroids in human invasive ductal carcinoma (IDC), which is currently postulated. STS, steroid sulfatase; 17βHSD1, 17β-hydroxysteroid dehydrogenase type 1; 17βHSD5, 17β-hydroxysteroid dehydrogenase type 5; and 5αRed, 5α-reductase.

clinicopathological parameters in order to further examine the significance of sex steroids in DCIS.

Materials and methods

Patients and tissues

Specimens of pure DCIS ($n=12$) and IDC ($n=12$) were obtained from female patients who underwent breast cancer surgical treatment from 2001 to 2004 in the Departments of Surgery at Tohoku University Hospital and Tohoku Kosai Hospital, Sendai, Japan. Non-neoplastic breast tissues were also obtained in 8 out of the 12 IDC patients, who underwent mastectomy and were examined in this study. Specimens for sex-steroid extraction or RNA isolation were snap-frozen and stored at −80 °C and those for immunohistochemistry were fixed with 10% formalin and embedded in paraffin-wax. The histological grade of each specimen was evaluated based on the Van Nuys classification (Silverstein *et al.* 1995) in DCIS and by the method of Elston & Ellis (1991) in IDC. Informed consent was obtained from all patients prior to their surgery and the examination of the specimens used in this study.

Eighty-three pure DCIS specimens were obtained by surgical excision from 1990 to 2005 in Department of Surgery, Tohoku University Hospital, Sendai, Japan. The mean age of the patients was 57.0 years (ranges 30–80 years). All of the patients did not receive irradiation, chemotherapy, or hormonal therapy prior to the surgery. Disease-free survival data were available in 78 patients, and the mean follow-up time was 54 months (ranges 8–117 months). All specimens were fixed with 10% formalin and embedded in paraffin wax.

Research protocols for this study were approved by the Ethics Committee at both Tohoku University School of Medicine and Tohoku Kosai Hospital.

Liquid chromatography/electrospray tandem mass spectrometry (LC–MS/MS)

Concentrations of estradiol and DHT were measured by LC–MS/MS analysis in Teizo Medical Co. (Kawasaki, Japan), as described previously (Miki *et al.* 2007, Suzuki *et al.* 2007, Yamashita *et al.* 2007). Briefly, the weights of the breast carcinoma specimens (32–89 mg for each sample) were measured by an electronic balance to a reasonable accuracy (AEX-200B (Shimadzu, Kyoto, Japan); readability, 0.1 mg; and capacity, 200 g), and these were then homogenized in 1 ml distilled water. After addition of 100 pg estradiol-$^{13}C_4$ (Hayashi Junyaku, Tokyo, Japan) or DHT-$^{2}H_3$ (CDN Isotope, Pointe-Claire Quebec, Canada) as internal standard, steroids were extracted with diethyl ether from the homogenate. The separated organic layer was evaporated, and then dissolved in picolinic anhydride in tetrahydrofran solution (100 μl) with triethylamine (20 μl). After application to a Bond Elut C18 column, steroid derivatives were eluted with 80% acetonitrile solution. The derivative estradiol and DHT fraction were dissolved in the elution solvent of LC.

In this study, we used an LC (Agilent 1100, Agilent Technologies, Waldbronn, Germany) coupled with an API 4000 triple-stage quadrupole mass spectrometer (Applied Biosystems, Foster City, CA, USA) operated with electrospray ionization in the positive-ion mode, and the chromatographic separation was performed on Cadenza CD-C18 column (3×150 mm, 3.5 μm, Imtakt, Kyoto, Japan). The injection volume was 20 μl. The mobile phase consisted of solvents A (0.1% formic acid in water (v/v)) and B (acetonitrile) and delivered at a flow rate of 0.4 ml/min. Total run time was 10 min. We used a mixture of solvents A and B (30:70 (v/v)) as an initial condition. After injection, it was followed by a linear gradient to 100% solvent B for 4 min, and this condition was maintained for 3 min. The system was returned to the initial proportion within 0.05 min, and maintained for the final 2.95 min of each run. The retention times for the derived estradiol and DHT were 5.3 and 5.8 min respectively. Ion spray voltage was 4.5 kV and turbo gas temperature was 450 °C in ionization conditions. For multiple reaction monitoring mode, the instrument monitored the *m/z* 255.3 (I.S.: 258.3) as ion produced from 396.4 (I.S.: 399.4) and the *m/z* 262 (I.S.:268) from 383.3 (I.S.: 487.2) respectively for estradiol and DHT derivatives.

In our present study, the lower limit of quantification (LLOQ) was 0.2 pg for both the estradiol and the DHT. It was determined by combination of determination validation, reproducibility, accuracy, and precision. The reproducibility was evaluated by intra- and inter-assays ($n=3$) and their coefficient variations (CVs) were 11.8 and 2.4% for estradiol, and 15.0 and 13.4% for DHT respectively. The accuracy and precision were measured using five different concentrations (0.2, 0.5, 1.0, 10, and 50 pg) of estradiol or DHT and five determinations for each concentration. The LLOQ did not exceed 20% of the CV. The recovery for estradiol and DHT was 80–85%.

Real-time PCR

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies Inc.), and a reverse transcription kit (SUPERSCRIPT II Preamplification system (Gibco-BRL) was used in the synthesis of cDNA. The Light Cycler system (Roche Diagnositics GmbH)

Table 1 Primer sequences used in real-time PCR in this study

cDNA (gene symbol; accession no.)	**Sequence** (position in cDNA)	**Size** (bp)	**Reference**
Aromatase[a] (aromatase; X13589)	FWD: 691–712 REV: 786–806	116	
STS[a] (STS; M16505)	FWD: 1550–1569 REV: 1683–1702	153	
17βHSD1[a] (HSD17B1; NM000413)	FWD: 1300–1321 REV: 1604–1625	326	
17βHSD5[a] (AKR1C3; NM003739)	FWD: 969–992 REV: 1052–1071	103	
5αRed1[a] (SRD5A1; NM001047)	FWD: 658–677 REV: 796–815	158	
5αRed2 (SRD5A2; NM000348)	FWD: 500–520 REV: 794–814	315	Suzuki *et al.* (2001)
RPL13A (RPL13A; NM012423)	FWD: 487–509 REV: 588–612	125	Vandesompele *et al.* (2002)

[a]Oligonucleotide primers used in this study were designed in the different exons.

was used to semi-quantify the mRNA expression levels by real-time PCR (Dumoulin *et al.* 2000). Characteristics of the primer sequences used in this study were summarized in Table 1 (Suzuki *et al.* 2001, Vandesompele *et al.* 2002). Settings for the PCR thermal profile were as follows: initial denaturation at 95 °C for 10 min, followed by 40 amplification cycles of 95 °C for 10 s, annealing at 60 °C (17βHSD1), 64 °C (17βHSD5 and STS), 68 °C (aromatase, 5αRed1, 5αRed2 and ribosomal protein L 13a (RPL13A)) for 10 s, and elongation at 72 °C for 12 s. To verify amplification of the correct sequences, PCR products were purified and subjected to direct sequencing. Negative control experiments lacked cDNA substrate to check for the possibility of exogenous contaminant DNA. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A. The results of real-time PCR analyses were similar when we used other internal standards, such as glyceraldehyde-3-phosphate dehydrogenase (Suzuki *et al.* 2005*b*) and β-actin (Suzuki *et al.* 2001), instead of RPL13A as used in this study (data not shown).

Laser capture microdissection (LCM)/real-time PCR for aromatase

Seven specimens of non-neoplastic breast, eight of DCIS and nine of IDC were available for LCM/real-time PCR analysis in this study. LCM was conducted using the Laser Scissors CRI-337 (Cell Robotics Inc., Albuquerque, NM, USA) and ~5000 epithelial cells or stromal cells were collected under the microscope from frozen sections of breast tissues. Total RNA was extracted according to a RNA microisolation protocol described by Niino *et al.* (2001). The real-time PCR protocol for aromatase was described above.

Immunohistochemistry

The characteristics of primary antibodies for steroidogenic enzymes, such as aromatase (Miki *et al.* 2007), STS (Suzuki *et al.* 2003), 17βHSD1 (Suzuki *et al.* 2000), 17βHSD5 (Suzuki *et al.* 2001), 5αRed1 (Suzuki *et al.* 2001), and 5αRed2 (Suzuki *et al.* 2001), used in this study were described previously. Monoclonal antibodies for ERα (ER1D5), PR (MAB429), AR (AR441), and Ki-67 (MIB1) were purchased from Immunotech (Marseille, France), Chemicon (Temecula, CA, USA), DAKO (Carpinteria, CA, USA) and DAKO respectively.

A Histofine Kit (Nichirei, Tokyo, Japan), which employs the streptavidin–biotin amplification method was used for immunohistochemistry in this study. Antigen retrieval for ERα, PR, AR, and Ki-67 immunostaining was performed by heating the slides in an autoclave at 120 °C for 5 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). The dilution of primary antibodies used in this study was as follows: aromatase; 1/6000, STS; 1/9000, 17βHSD1; 1/500, 17βHSD5; 1/1000, 5αRed1; 1/1000, 5αRed2; 1/1000, ERα; 1/50, PR; 1/30; AR; 1/100, and Ki-67; 1/50. The antigen–antibody complex was visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris–HCl buffer (pH 7.6), and 0.006% H_2O_2) and counterstained with hematoxylin. As a negative control, normal rabbit or mouse immunoglobin G (IgG) was used instead of the primary antibody.

Immunoreactivity of steroidogenic enzymes was detected in the cytoplasm and cases that had more than 10% of positive carcinoma cells staining were considered positive (Suzuki *et al.* 2007). Immunoreactivity of ERα, PR, AR, and Ki-67 was detected in the nucleus. These immunoreactivities were evaluated in more than 1000 carcinoma cells for each case

and subsequently the percentage of immunoreactivity, i.e., labeling index (LI), was determined (Suzuki *et al.* 2007).

Statistical analysis

The statistical analyses between two groups were performed using a Mann–Whitney *U* test, and *P* values <0.05 were considered significant. The relative ratio between two groups was evaluated by their median values. Disease-free survival curves were generated according to the Kaplan–Meier method and the statistical significance was calculated using a log-rank test.

Results

Intratumoral concentration of sex steroids in DCIS

We first examined the tissue concentration of sex steroids in the non-neoplastic breast, DCIS, and IDC tissues by LC–MS/MS. The median with min–max value of tissue concentration of estradiol was 16 (5–83) pg/g in non-neoplastic breast, 52 (10–494) pg/g in DCIS, and 206 (11–1586) pg/g in IDC. The median value in DCIS was 3.3-fold higher than that in non-neoplastic breast tissues (Fig. 2A). The intratumoral concentration of estradiol was 4.0-fold higher in IDC than DCIS, but no significant association was detected ($P=0.20$).

The median with min–max value of tissue concentration of DHT was 100 (63–128) pg/g in the non-neoplastic breast, 323 (140–1593) pg/g in DCIS, and 162 (41–990) pg/g in IDC. The tissue concentration of DHT was 3.2-fold higher in DCIS than non-neoplastic breast (Fig. 2B). The intratumoral concentration of DHT was significantly higher in DCIS than IDC ($P=0.04$ and 2.0-fold).

The intratumoral concentration of estradiol in DCIS was 2.2-fold higher in premenopausal women (93 (10–494) pg/g ($n=5$)) than in postmenopausal women (42 (13–70) pg/g ($n=7$)), but no significant association was detected ($P=0.46$). The median of the intratumoral concentration of DHT in DCIS was 260 (253–380) pg/g in premenopausal women and 326 (140–1593) pg/g in postmenopausal women ($P=0.52$).

mRNA expression of sex steroid-producing enzymes in DCIS

We next examined mRNA expression of sex steroid-producing enzymes in non-neoplastic breast, DCIS, and IDC using real-time PCR. As shown in Table 2, mRNA levels of aromatase, STS, 17βHSD1, 17βHSD5, and 5αRed1 were significantly higher in DCIS than non-neoplastic breast ($P=0.03$ and 4.0-fold in aromatase, $P=0.01$ and 9.5-fold in STS, $P=0.04$ (relative ratio could not be evaluated) in 17βHSD1, $P=0.01$ and 18-fold in 17βHSD5, and $P=0.02$ and 5.1-fold in 5αRed1). The expression level of 5αRed2 mRNA was negligible and no significant difference was detected between DCIS and non-neoplastic breast ($P=0.67$). The expression level of aromatase mRNA was significantly higher in IDC than DCIS ($P=0.046$ and 5.9-fold), but mRNA levels of other sex steroid-producing enzymes were not significantly changed between these two breast carcinoma groups.

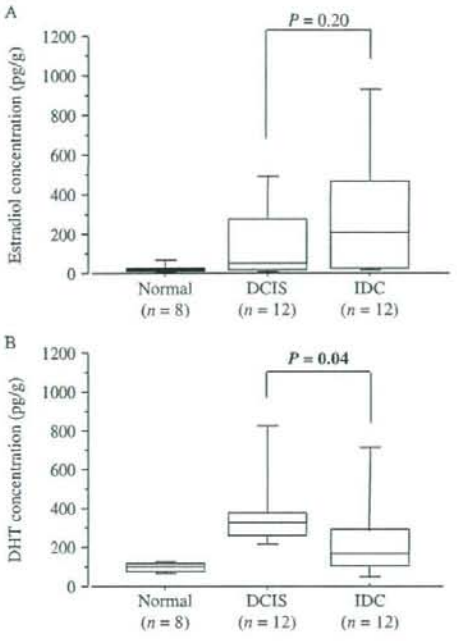

Figure 2 Tissue concentrations of (A) estradiol and (B) DHT in the non-neoplastic breast (normal), DCIS, and IDC tissues. Data are represented as box and whisker plots. The median value is shown by a horizontal line in the box plot and the gray box denotes the 75th (upper margin) and 25th percentiles of the values (lower margin). The upper and lower bars indicate the 90th and 10th percentiles respectively. The statistical analyses were performed between breast carcinoma (DCIS and IDC) groups by a Mann–Whitney *U* test. *P* values <0.05 were considered significant and are indicated in boldface.

Previous studies demonstrated that aromatase was immunolocalized in carcinoma and stromal cells in human breast carcinoma (Zhang *et al.* 2002, Suzuki *et al.* 2005*a*). Therefore, we further examined the expression of aromatase mRNA according to cell type in the non-neoplastic breast, DCIS, and IDC using LCM/real-time PCR. In the epithelial cells, the median

Table 2 mRNA expression of sex steroid-producing enzymes in non-neoplastic breast, ductal carcinoma *in situ* (DCIS), and invasive ductal carcinoma (IDC) tissues

	mRNA expression level (median (min–max)) × (%)			***P* value**	
Enzyme	**Normal ($n=8$)**	**DCIS ($n=12$)**	**IDC ($n=12$)**	**DCIS versus normal**	**DCIS versus IDC**
Estrogen-producing enzymes					
Aromatase	3.5 (0.0–8.0)	14.1 (1.5–113.3)	82.5 (0.0–528.5)	**0.03**	**0.046**
STS	0.4 (0.0–1.5)	3.8 (0.0–93.1)	13.6 (0.3–241.3)	**0.01**	0.13
17βHSD1	0.0 (0.0–0.5)	0.6 (0.0–4.0)	1.5 (0.0–3.9)	**0.04**	0.07
Androgen-producing enzymes					
17βHSD5	0.6 (0.0–3.1)	10.8 (1.0–57.3)	10.9 (0.0–39.6)	**0.01**	0.91
5αRed1	11.5 (0.0–19.8)	58.6 (3.0–223.6)	34.2 (0.0–438.4)	**0.02**	0.60
5αRed2	0.0 (0.0–15.9)	0.2 (0.0–49.0)	0.2 (0.0–58.8)	0.67	0.83

Normal; non-neoplastic breast tissues. The statistical analyses between two groups were performed using a Mann–Whitney's *U* test. *P* values <0.05 were considered significant and are indicated in boldface. The mRNA level of the steroidogenic enzymes was summarized as a ratio (%) of that of RPL13A.

with min–max value of aromatase mRNA was $0.00\ (0.00–0.30)\times10^{-2}\%$ in epithelial cells of non-neoplastic breast, $0.06\ (0.00–0.29)\times10^{-2}\%$ in carcinoma cells of DCIS, and $1.00\ (0.01–3.00)\times10^{-2}\%$ in carcinoma cells of IDC and its expression level was significantly higher in IDC than non-neoplastic breast ($P=0.01$ and relative ratio could not be evaluated) or DCIS ($P=0.03$ and 17-fold; Fig. 3A). The expression level of aromatase mRNA in the stromal cells was $0.00\ (0.00–0.04)\times10^{-2}\%$ in non-neoplastic breast, $0.04\ (0.00–0.10)\times10^{-2}\%$ in DCIS, and $4.00\ (1.00–6.23)\times10^{-2}\%$ in IDC, and the expression level was significantly higher in IDC than non-neoplastic breast ($P=0.001$, and relative ratio could not be evaluated) or DCIS ($P=0.001$, and 100-fold; Fig. 3B).

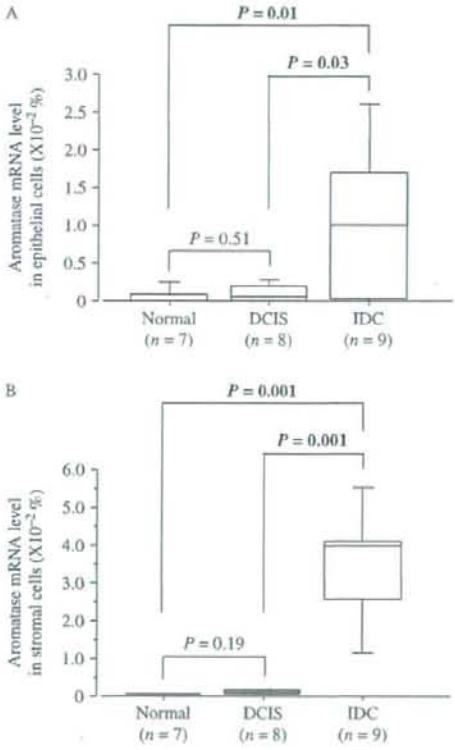

Figure 3 Expression of aromatase mRNA in (A) epithelial cells or (B) stromal cells in non-neoplastic breast (normal), DCIS, and IDC tissues. Epithelial cells and the adjacent stromal cells in non-neoplastic breast tissues, or carcinoma cells and intratumoral stromal cells in DCIS and IDC were collected separately by LCM. Data are represented as box and whisker plots. The statistical analyses were performed using a Mann–Whitney *U* test between indicated two groups. *P* values <0.05 were considered significant and are indicated in boldface.

Immunoreactivity of sex steroid-producing enzymes in DCIS

Aromatase immunoreactivity was detected in the cytoplasm of carcinoma cells in 45 out of 83 DCIS cases (54%; Fig. 4A) and was also detected in some intratumoral stromal cells (Fig. 4B). Immunoreactivity of other sex steroid-producing enzymes was detected in the cytoplasm of carcinoma cells and the number of positive cases was as follows: STS; 45/83 (54%; Fig. 4C), 17βHSD1; 54/83 (65%; Fig. 4D), 17βHSD5; 59/83 (71%; Fig. 4E), 5αRed1; 52/83 (63%; Fig. 4F), and 5αRed2; 13/83 (16%). Associations between immunoreactivity of sex steroid-producing enzymes and clinicopathological parameters in the 83 DCIS cases are summarized in Tables 3 and 4. Among the estrogen-producing enzymes, STS immunoreactivity was significantly associated with the histological grade (Van Nuys classification; $P=0.01$), while no significant association was detected between aromatase or

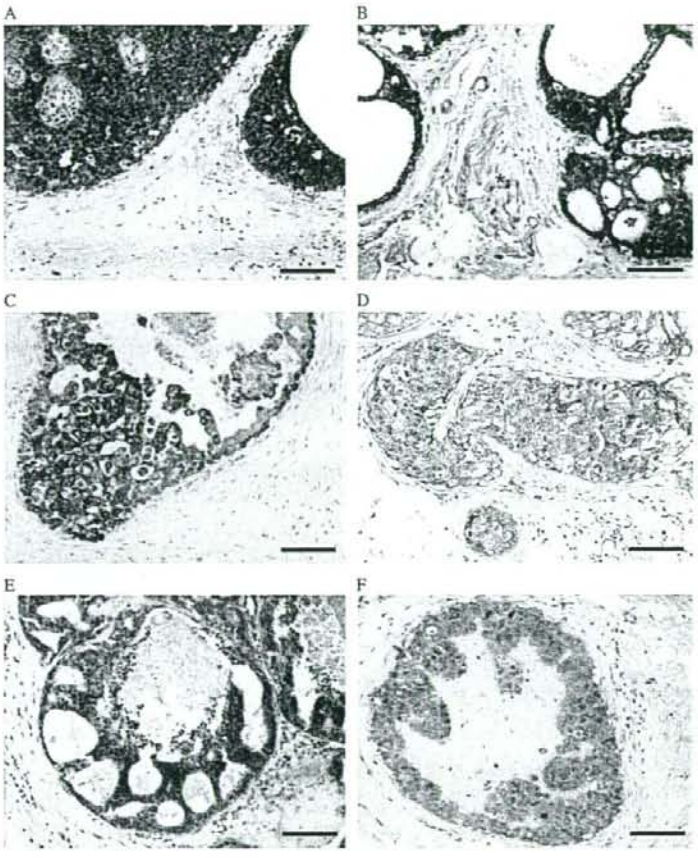

Figure 4 (A and B) Immunohistochemistry for aromatase, (C) STS, (D) 17βHSD1, (E) 17βHSD5, and (F) 5αRed1 in DCIS. Aromatase immunoreactivity was mainly detected in the cytoplasm of carcinoma cells in (A) DCIS, but it was also positive in some intratumoral stromal cells (*) (B) STS, 17βHSD1, 17βHSD5, and 5αRed1 immunoreactivities were detected in the cytoplasm of carcinoma cells in DCIS. Bar=100 μm.

17βHSD1 immunoreactivity and the clinicopathological factors examined (Table 3).

Among the androgen-producing enzymes, 5αRed1 immunoreactivity was positively associated with the Van Nuys classification ($P=0.001$) or Ki-67 LI ($P=0.02$), but 17βHSD5 immunoreactivity was not significantly correlated with the clinicopathological parameters examined (Table 4). No significant association was detected between 5αRed2 immunoreactivity and the clinicopathological parameters examined (data not shown). There was no significant association among the immunoreactivity of these five sex steroid-producing enzymes in DCIS. The significant correlations described above were confirmed in increased rankings of the positivity to three groups (0–9, 10–49, and 50–100% positive cells; STS and Van Nuys classification, $P=0.04$; 5αRed1 and Van Nuys classification, $P=0.01$; and 5αRed1 and Ki-67 LI, $P=0.04$).

As summarized in Fig. 5, the status of 5αRed1 immunoreactivity was associated with an increased risk of recurrence in 78 DCIS patients examined, although P value was not available because no patients were associated with clinical recurrence in a group of 5αRed1-negative breast carcinomas. On the other hand, no significant association was detected between the status of other steroidogenic enzyme immunoreactivity and risk of recurrence in these DCIS patients (aromatase, $P=0.87$; STS, $P=0.47$; 17βHSD1, $P=0.83$; 17βHSD5, $P=0.98$; and 5αRed2, $P=0.45$).

Table 3 Association between immunoreactivity of estrogen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma *in situ* (DCIS) tissues

	Aromatase immunoreactivity			STS immunoreactivity			17βHSD1 immunoreactivity		
Value	+ (n=45)	− (n=38)	P value	+ (n=45)	− (n=38)	P value	+ (n=54)	− (n=29)	P value
Age[a] (years)	60 (34–80)	56 (30–77)	0.37	54 (34–77)	61 (30–80)	0.43	57 (30–80)	61 (34–77)	0.70
Menopausal status									
Premenopausal	13 (16%)	14 (17%)		16 (19%)	11 (13%)		14 (17%)	13 (16%)	
Postmenopausal	32 (39%)	24 (29%)	0.59	29 (35%)	27 (33%)	0.68	40 (48%)	16 (19%)	0.08
Van Nuys classification									
Group 1	14 (17%)	13 (16%)		8 (10%)	19 (23%)		17 (20%)	10 (12%)	
Group 2	21 (25%)	19 (23%)		25 (30%)	15 (18%)		26 (31%)	14 (17%)	
Group 3	10 (12%)	6 (7%)	0.75	12 (14%)	4 (5%)	0.01	11 (13%)	5 (6%)	0.93
ERα LI[a]	69 (0–97)	82 (0–97)	0.20	67 (0–96)	80 (0–97)	0.13	78 (0–97)	66 (4–94)	0.13
PR LI[a]	52 (0–92)	33 (0–91)	0.12	33 (6–92)	47 (0–93)	0.63	38 (0–93)	31 (0–92)	0.60
AR LI[a]	56 (0–97)	55 (5–94)	0.54	53 (0–93)	59 (4–97)	0.39	56 (2–97)	55 (0–93)	0.86
Ki-67 LI[a]	16 (2–35)	13 (2–35)	0.22	17 (2–35)	12 (2–32)	0.23	13 (2–35)	17 (3–33)	0.45

[a]Data are presented as median with min–max values and were evaluated by a Mann–Whitney U test. All other values represent the number of cases and percentage.

Discussion

To the best of our knowledge, this is a first report that demonstrates intratumoral concentrations of sex steroids in DCIS. Median values of both estradiol and DHT concentrations were (3.3-fold in estradiol and 3.2-fold in DHT) higher in DCIS than those in non-neoplastic breast tissues. Results of our present study also demonstrated that mRNA expression of both estrogen (aromatase, STS, and 17βHSD1)- and androgen (17βHSD5 and 5αRed1)-producing enzymes was significantly higher in DCIS than the non-neoplastic breast tissues. Previous studies demonstrated that aromatase immunoreactivity was detected in the carcinoma and stromal cells (Zhang *et al.* 2002) in 70% of DCIS tissues (Oliveira *et al.* 2006) and 17βHSD1 immunoreactivity was positive in the carcinoma cells in 63% of DCIS cases (Ariga *et al.* 2000). In our present study, we detected aromatase and 17βHSD1 immunoreactivities in 54 and 65% of DCIS cases and these frequencies and localization were consistent with the previous reports. On the other hand, expression of STS, 17βHSD5, and 5αRed has not been reported in DCIS. Our present results showed that immunoreactivity for STS, 17βHSD5, 5αRed1, and 5αRed2 was positive in 54, 71, 63, and 16% of DCIS cases respectively and these frequencies were similar

Table 4 Association between immunoreactivity of androgen-producing enzymes and clinicopathological parameters in 83 ductal carcinoma *in situ* (DCIS) tissues

	17βHSD5 immunoreactivity			5αRed1 immunoreactivity		
Value	+ (n=59)	− (n=24)	P value	+ (n=52)	− (n=31)	P value
Age[a] (years)	53 (30–80)	61 (42–69)	0.19	55 (30–80)	61 (39–77)	0.43
Menopausal status						
Premenopausal	23 (28%)	5 (6%)		19 (23%)	8 (10%)	
Postmenopausal	36 (43%)	19 (23%)	0.18	33 (40%)	23 (28%)	0.44
Van Nuys classification						
Group 1	20 (24%)	7 (8%)		11 (13%)	16 (19%)	
Group 2	26 (3%)	14 (17%)		26 (31%)	14 (17%)	
Group 3	13 (16%)	3 (4%)	0.43	15 (18%)	1 (1%)	**0.001**
ERα LI[a]	71 (0–97)	87 (0–94)	0.12	73 (0–97)	79 (0–97)	0.48
PR LI[a]	53 (0–92)	57 (12–87)	0.96	32 (0–92)	43 (0–93)	0.14
AR LI[a]	73 (16–97)	83 (0–93)	0.10	54 (2–93)	62 (0–97)	0.12
Ki-67 LI[a]	20 (2–35)	13 (7–24)	0.07	18 (3–35)	11 (2–32)	**0.02**

P values <0.05 were considered significant and are indicated in boldface.
[a]Data are presented as median with min–max values and were evaluated by a Mann–Whitney U test. All other values represent the number of cases and percentage and were statistically analyzed using a cross-table using the χ^2 test.

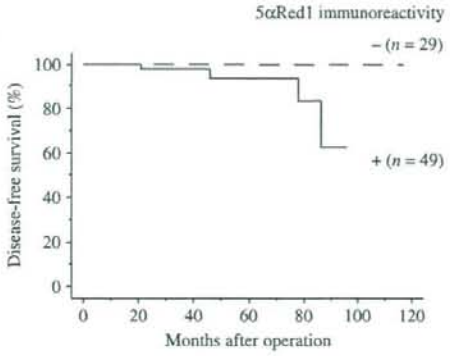

Figure 5 Disease-free survival curve of 78 DCIS patients according to 5αRed1 immunoreactivity (Kaplan–Meier method).

to those reported in IDC (59–88, 53, 58, and 15% respectively; Suzuki *et al.* 2005*a*). 5αRed activity is considered to be mainly mediated by 5αRed1 in IDC, because 5αRed2 expression was shown to be very low (Suzuki *et al.* 2005*a*). Results of our present study all suggest that both the estradiol and the DHT are locally produced from circulating inactive steroids by sex steroid-producing enzymes, which results in increased intratumoral concentrations of these steroids in DCIS in a similar manner to IDC. Recently, Faratian *et al.* (2005) reported that proliferation of DCIS was reduced by aromatase inhibitors, such as letrozole and anastrozole. Therefore, endocrine therapies, such as anti-estrogens, aromatase inhibitors, and/or LH-RH agonists, may be clinically effective in a selective group of DCIS patients.

In our present study, aromatase mRNA was detected in both carcinoma and intratumoral stromal cells and the expression level was significantly higher in IDC than DCIS in these two cellular components (17- and 100-fold respectively). Previous *in vitro* studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts (Zhou *et al.* 2001), including prostaglandin E2 (Zhao *et al.* 1996), interleukin (IL)-1, IL-6, IL-11, and tumor necrosis factorα (Reed & Purohit 2001, Simpson & Davis 2001). On the other hand, it has been also reported that exogenous growth factors such as epidermal growth factor (Ryde *et al.* 1992), transforming growth factor (Ryde *et al.* 1992), and keratinocyte growth factor (Zhang *et al.* 1998) stimulated aromatase activity in MCF-7 breast carcinoma cells. Very recently, Miki *et al.* (2007) reported that mRNA level and enzymatic activity of aromatase in MCF-7 breast carcinoma cells were significantly increased on coculture with primary stromal cells isolated from human breast carcinoma tissue. Therefore, aromatase expression is suggested to be, at least in a part, regulated by tumor–stromal interactions in breast carcinoma tissues, which may be promoted by invasion of the carcinoma cells into the stroma.

Intratumoral DHT level is associated with the testosterone level in IDC (Mistry *et al.* 1986, Recchione *et al.* 1995) and is considered to be mainly determined by amounts of the precursor. Aromatase catalyzes the conversion of androstenedione and testosterone, which are precursors of DHT, to estrone and estradiol respectively (Fig. 1). Spinola *et al.* (1988) previously showed that treatment with an aromatase inhibitor (4-hydroxyandrostenedione) markedly elevated intratumoral testosterone concentrations in dimethylbenz(a)anthracene-induced rat mammary tumors. In addition, Sonne-Hansen & Lykkesfeldt (2005) reported that aromatase preferred testosterone as a substrate in MCF-7 cells. Very recently, Suzuki *et al.* (2007) demonstrated that aromatase expression was inversely associated with intratumoral DHT concentrations in IDC and aromatase inhibitors suppressed the DHT synthesis from androstenedione in coculture experiments. These findings all suggest that aromatase is a negative regulator of local DHT production in human breast carcinoma. In this study, the intratumoral DHT concentration was significantly lower in IDC than DCIS. On the other hand, aromatase expression was significantly higher in IDC than DCIS, while expression levels of androgen-producing enzymes were not significantly different between these two groups. Therefore, higher expression of aromatase in IDC may increase the conversion of androgens into estrogens with a subsequent decrease of intratumoral DHT concentrations.

Various *in vitro* studies have shown that DHT inhibits the cell proliferation of breast carcinoma cells (de Launoit *et al.* 1991, Lapointe & Labrie 2001, Ando *et al.* 2002) and the proapoptotic effect of DHT was also reported in breast carcinoma cells (Kandouz *et al.* 1999). DHT treatment resulted in a rapid fall in tumor volume of ZR75-1 cells injected into athymic mice (Dauvois *et al.* 1991). However, it is also true that some divergent findings have been reported. For instance, Birrell *et al.* (1995) showed that both DHT and the synthetic non-metabolizable androgen, mibolerone, increased the cell proliferation of MCF-7 and MDA-MB-453 cells. In addition, Zhang *et al.* (2004) demonstrated that DHT-benzoate (DHT-B) induced growth of mouse mammary ductal cells, although it is much weaker than estradiol and

treatment with both estradiol and DHT-B caused more pronounced hyperplasia of mammary ducts and alveoli, compared with the treatment with each hormone alone. In our present study, 5αRed1 immunoreactivity was significantly associated with Ki-67 LI and the Van Nuys classification in 83 DCIS cases and it was also associated with an increased risk of recurrence in the 78 DCIS patients. The Ki-67 antibody recognizes cells in all phases of the cell cycle except the G0 (resting) phase and the Ki-67 LI is closely correlated with the S-phase fraction and mitotic index (Vandesompele *et al.* 2002). The Van Nuys classification is known as a powerful prognostic classification for DCIS and Silverstein *et al.* (1995) reported that the incidence of local recurrence after breast-conservation surgery for DCIS in 238 patients was 4% in Group 1 (non-high-grade DCIS without comedo-type necrosis), 11% in Group 2 (non-high-grade DCIS with comedo-type necrosis), and 27% in Group 3 (high-grade DCIS with or without comedo-type necrosis). ER and PR status in DCIS was inversely associated with the histological differentiation or nuclear grade (Selim *et al.* 2002, Baqai & Shousha 2003). However, AR status was not correlated with ER status in DCIS (Rody *et al.* 2005) and a significant number of poorly differentiated DCIS was reported ER-negative, PR-negative, but AR-positive (Moinfar *et al.* 2003). Results of these previous and our present studies are indicative that DHT may be involved in the development of DCIS. However, no information is currently available on the effects of androgens in DCIS to our knowledge and so further examinations are required to clarify the significance of androgens in human DCIS.

In summary, intratumoral concentrations of estradiol and DHT were higher in DCIS than non-neoplastic breast tissues and estrogen-producing enzymes (aromatase, STS, and 17βHSD1) and androgen-producing enzymes (17βHSD5 and 5αRed1) were highly expressed in DCIS. The intratumoral concentration of DHT was significantly lower in IDC than DCIS and the expression of aromatase mRNA was significantly higher in IDC. Results of immunohistochemistry for the sex steroid-producing enzymes demonstrated that 5αRed1 immunoreactivity was associated with Ki-67 LI, histological grade, and increased risk of recurrence in DCIS patients. Results of our present study suggest that intratumoral concentrations of estradiol and DHT are increased in DCIS, which is possibly due to intratumoral production of these steroids. DCIS frequently expresses ER and/or AR in the carcinoma cells and therefore, both estradiol and DHT may play important roles in the development of DCIS.

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REVIEW

Aromatase in Human Breast Carcinoma as a Key Regulator of Intratumoral Sex Steroid Concentrations

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Abstract. It is well-known that estrogens are closely involved in the growth of human breast carcinomas, and that the great majority of breast carcinoma express estrogen receptors. Recent studies have demonstrated that estrogens are locally produced and act on the breast carcinoma tissue. Among these pathways, aromatase is a key enzyme for intratumoral production of estrogens in breast carcinomas, and aromatase inhibitors are currently used in the breast carcinoma in postmenopausal women as an estrogen deprivation therapy. This review summarizes the results of recent studies on the expression and regulation of aromatase in breast carcinoma tissues, and discusses the potential biological and/or clinical significance of aromatase. Aromatase is abundantly expressed in various cell types, such as carcinoma cells, intratumoral stromal cells, and adipocytes adjacent to the carcinoma, in breast carcinoma tissues. Further, a key regulator for aromatase expression differed according to cell type. In addition, aromatase suppressed *in situ* production of bioactive androgen, 5α-dihydrotestosterone (DHT), in breast carcinoma. Aromatase inhibitors may thus have additional antiproliferative effects through increasing local DHT concentration with estrogen deprivation.

Key words: Androgen, Aromatase, Breast carcinoma, Estrogen

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Intratumoral production of estrogens in the breast carcinoma

IT is well-known that estrogens contribute immensely to the development of hormone-dependent human breast carcinoma, and that estrogen deprivation is an effective treatment for breast carcinoma as an endocrine therapy. Estrogens are mainly secreted from the ovary into plasma in premenopausal women. Ovarian suppression therapies, such as ovariectomy and treatment with gonadotropin releasing hormone (GnRH) agonists, thus are frequently considered in breast carcinoma patients in premenopausal women [1] (Table 1). On the other hand, since the biological effects of estrogens are mediated through the estrogen receptor (ER), antiestrogens such as tamoxifen have been used in breast carcinoma regardless of menopausal status [2] (Table 1).

ER is expressed in a great majority of breast carcinoma tissues, but the great majority of these carcinomas arise after menopause when ovaries are no longer functional. In postmenopausal women and men, estrogens are mainly biosynthesized in various peripheral tissues such as adipose tissue, skin, and muscle, through conversion of circulating inactive androgens from the adrenal cortex or gonads [3]. Increased peripheral conversion of androgen to estrogen might result in elevated serum levels of estrogen, and numerous studies have been performed to study the subtle differences of serum estrogen concentration. However, there is no consistent evidence of increased serum estrogens concentration or other systemic estrogen abnormalities in women with breast carcinoma.

Miller *et al.* and Miller [4, 5] have shown that tissue

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