● 原 著 ●

HER2陽性乳癌に対する術前化学療法の臨床的検討

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Summary

Objective: The clinical significance of preoperative chemotherapy, including trastuzumab for HER2-positve breast cancer, was examined based on hormone receptors (HR) to clarify future issues. Subjects: 104 HER2-positive breast cancer patients who completed preoperative chemotherapy and underwent surgery from May 2005 to August 2010. All patients received sequential treatment with taxane±trastuzumab for FEC (5-FU+epirubicin+cyclophosphamide) therapy, and from 2008 they received trastuzumab postoperatively for one year. Results: Concerning the histological effects, the rate of comprehensive pCR (CpCR) in the 104 patients (31 HR-negative administered trastuzumab, 15 HR-negative not administered trastuzumab, 28 HR-positive administered trastuzumab, 30 HR-positive not administered trastuzumab) was 65%, 47%, 21% and 23% for each group, respectively CpCR was a significant factor ($p<0.05$) in prolonged distant disease-free survival (DDFS) in the HR-negative group. Distant metastasis occurred in 14 patients, namely, brain metastasis in 7 patients (4 HR-negative administered trastuzumab, 1 HR-negative not administered trastuzumab, 2 HR-positive administered trastuzumab). The therapeutic efficacy was pINV in 5 of these 7 patients (3 HR-negative administered trastuzumab, 1 HR-negative not administered trastuzumab, 1 HR-positive administered trastuzumab), and 4 of those 5 patients received trastuzumab postoperatively. Discussion: The responsiveness to preoperative chemotherapy including trastuzumab for HER2-positve breast cancer differs between HR-positive and HR-negative. pINV patients seem to be at a high risk for brain metastasis regardless of HR, and it may be difficult to suppress its occurrence only with trastuzumab adjuvant therapy. Key words: HER2-positive breast cancer, Preoperative chemotherapy, Trastuzumab (*Received Mar. 5, 2012/Accepted May. 23, 2012*)

要旨 目的: HER2陽性乳癌に対するtrastuzumabを含む術前化学療法の臨床的意義をホルモン受容体(hormone receptor: HR)別に検討し，今後の課題を明らかにする。対象: 2005年5月～2010年8月までに術前化学療法および手術を完遂したHER2陽性乳癌104例。全例，FEC (5-FU+epirubicin+cyclophosphamide) 療法にタキサン±trastuzumabの逐次治療を実施し，2008年以降は術後にtrastuzumabを1年間投与した。結果: 104例 (HR陰性trastuzumab投与31例，HR陰性trastuzumab非投与15例，HR陽性trastuzumab投与28例，HR陽性trastuzumab非投与30例) における組織学的効果は，comprehensive pCR (CpCR) がそれぞれ65%，47%，21%，23%であった。HR陰性群では，CpCRがdistant disease free survival (DDFS) の延長に有意な因子 ($p<0.05$) であった。14例が遠隔転移を発症し脳転移は7例であった。7例中5例 (HR陰性trastuzumab投与3例，HR陰性trastuzumab非投与1例，HR陽性trastuzumab投与1例) は治療効果がinvasive cancer on pathologic examination (pINV) であり，またその5例のうち4例は術後にtrastuzumabを投与していた。考察: HER2陽性乳癌に対するtrastuzumabを含む術前化学療法はHR陽性と陰性とで反応性が異なる。一方で，HRに関係なくpINVの症例は脳転移のハイリスク群と考えられ，trastuzumabの補助療法のみでは発症を抑制するのは困難な可能性もあると考えられる。

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はじめに

近年の分子生物学の進歩に伴い，乳癌は遺伝子発現パターンによりいくつかのサブタイプに分類され，それぞれのサブタイプの特性に基づいた治療法を選択することが重視されるようになった。実地臨床でよく用いられる分類は，ホルモン受容体（hormone receptor: HR）陽性HER2陰性，HER2陽性，HR陰性HER2陰性の三つに分類する方法である。

術前化学療法は腫瘍縮小効果による乳房温存率の向上の他，近年は薬物感受性を評価することができることから個別化治療の指標として注目されている。術前化学療法により組織学的完全奏効（pathologic complete response: pCR）を得られた症例は，組織学的に浸潤癌の遺残を認める（invasive cancer on pathologic examination: pINV）症例に比べて無再発生存率，全生存率が良好であるといわれる[1]。

しかし，HR陽性乳癌は陰性乳癌に比してpCRを得にくいなどpCRの観点からみた化学療法の反応性はサブタイプによってそれぞれで異なることがわかってきており[2]，pCRの予後予測における意義もサブタイプ別により異なるとの考えが主流になってきた。

HER2陽性乳癌は従来，悪性度が高く予後不良とされてきたが，trastuzumabの登場と化学療法剤の進歩によりその治療成績は著しく改善している[3]。手術を先行した場合でも，原則として5 mmもしくは10 mm以上の腫瘍径を有するHER2陽性乳癌では手術後にtrastuzumabの投与が推奨される。また化学療法の感受性が高くpCRを得やすい性格ゆえに，比較的術前化学療法の適応を受け入れやすいサブタイプである。Buzdarら[4]の報告にみられるように，trastuzumabを併用することでpCR率が上昇し，約60%の確率でpCRを得ることが期待できる。

本稿では，大阪医療センターにおける術前薬物療法を適応したHER2陽性乳癌の特性とその治療成績をretrospectiveに検討することにより，HER2陽性乳癌に対する治療の現状と課題を検討した。

Ⅰ．目　的

HER2陽性乳癌における術前化学療法の臨床的意義について検討する。

Ⅱ．対　象

2005年5月～2010年8月までに，大阪医療センターで術前化学療法および根治手術を完遂したHER2陽性の原発性浸潤性乳癌104例を対象とした。stage Ⅳは除外した。全例，手術先行療法，化学療法，ホルモン療法，その他の治療法を考慮した上で，術前化学療法の適応があるとカンファレンスで合議されることを必須とした。また，適切な臓器機能を有すること，心エコー法で測定したベースラインの左室駆出率が55%以上であること，胸部CT検査もしくは胸部レントゲン写真で明らかな間質性肺炎や肺線維症を認めないことなど，trastuzumabと化学療法の使用の安全性を担保した。全例70歳以下の女性患者で，治療開始前には術前治療への文書同意を取得した。

Ⅲ．方　法

術前化学療法施行前に針生検材料で組織型，ER，PgR，HER2，組織学的グレードを判定した。ER，PgRの判定は免疫組織学法（IHC法）を使用し，J-Scoreの区分を用いて10%未満は陰性とした。HER2はIHC法で3＋を陽性，1＋および0を陰性とし，2＋はFISH法でHER2/CEP17のシグナル比が2.0以上である場合を陽性とした。

治療開始前の腫瘍径は触診径を用い，腋窩リンパ節転移の診断は表在型超音波診断（US）もしくは造影CTの画像所見を主たる手段としTNM分類を行った。

治療レジメンはアンスラサイクリン系とタキサン系の逐次投与とし，trastuzumabはタキサン系薬剤とのみ同時併用した。アンスラサイクリン系はFEC（fluorouracil 500 mg/m^2，epirubicin 100 mg/m^2，cyclophosphamide 500 mg/m^2）を3週間に1回投与で4回，タキサン系はdocetaxel（DTX）75 mg/m^{2}は3週間に1回投与で計4回，paclitaxel（PXL）80 mg/m^{2}は毎週投与で計12回とした。trastuzumabの投与スケジュールは，DTXの場合は初回8 mg/kg/3 week，2回目以降6 mg/kg/3 weekを，PXLの場合は初回4 mg/kg/week，2回目以降2 mg/kg/weekを併用した。

臨床的効果判定はUS，MRI，もしくはCTを用いてRECIST（Response Evaluation Criteria in Solid Tumor）v1.1に従って判定した。病理学的効果判定は乳癌取扱い規約第16版に従い診断し，組織学的奏効はKuroiらの分類[5]に従った。

Ⅳ．結　果

1．症例の背景

年齢の中央値は51（25～70）歳，閉経前49例（47%），閉経後55例（53%）であった。症例の内訳は，HR陰性で術前治療にtrastuzumabを投与した（HR陰性/trastuzumab投与）群31例，HR陰性でtrastuzumabを投与しなかった（HR陰性/trastuzumab非投与）群15例，

Table 1　Patient characteristics

Characteristic	HR−/H+ (n=31)	HR−/H− (n=15)	HR+/H+ (n=28)	HR+/H− (n=30)
Median age (years)	55	54	51	48
Range	(27-69)	(32-70)	(36-69)	(25-69)
Clinical stage				
I	1	2	1	0
IIA/IIB	13/5	5/6	16/7	16/9
IIIA/IIIB	10/2	2/0	3/1	4/1
IIIC・IV	0	0	0	0
Tumor size (cm)				
1.0≦T≦2.0	3	2	1	1
2.0<T≦5.0	20	11	23	24
5.0<T≦7.0	8	2	4	5
Clinical nodal status				
Negative (cN0)	15	6	20	17
Positive [cN (+)]	16	9	8	13
Histological classification (CNB specimen)				
Papillotubular carcinoma	5	1	7	5
Scirrhous carcinoma	17	8	15	19
Solid-tubular carcinoma	8	6	4	4
Special types	1	0	2	2
Histological grade (B & R)				
Grade 1	2	0	4	3
Grade 2	9	5	10	15
Grade 3	20	10	14	12
Taxanes				
Docetaxel (DTX)	15	10	19	28
Weekly paclitaxel (PXL)	16	5	9	2

HR−: hormone receptor negative, HR+: hormone receptor positive, H: trastuzumab

HR陽性でtrastuzumabを投与した（HR陽性/trastuzumab投与）群28例，HR陽性でtrastuzumabを投与しなかった（HR陽性/trastuzumab非投与）群30例であった。

FECとタキサンの両レジメンを完遂できたのは100例（96%）であった。2例はDTXによる嘔気症状のためにそれぞれ2回，3回のみで中止し，1例はアナフィラキシー反応のために1回のみで投与を中止した。さらに1例はDTXを3回投与した時点でPDと判断して中止した。保険承認に伴い，2008年2月以降は術後にtrastuzumabを1年間投与することを原則としたが，3例は術前化学療法後に心機能の低下を認めたため，また1例は希望されなかったためにtrastuzumabの術後治療が不可能であった。投与できた80例では，その施行中に心機能の低下を認めなかった。全症例の背景をTable 1に示す。

2．術前治療の効果

臨床的効果は全体でCR 59例，PR 37例，SD 7例，PD 1例であり，奏効率は92%であった。HR別では，HR陰性46例中CR 32例（trastuzumab投与25例，非投与7例）（70%），一方HR陽性群58例中CR 27例（trastuzumab投与18例，非投与9例）（46%）であり，HR陰性群でCRの割合は有意に高かった（$p<0.05$）。さらにtrastuzumabの投与有無でみると，trastuzumab投与群ではCRがHR陰性25例（81%），HR陽性18例（64%），非投与群ではCRがHR陰性7例（47%），HR陽性9例（30%）であり，trastuzumabを投与することでHR陰性，陽性ともにCRの割合は有意に上昇した（$p<0.05$）（Table 2）。

乳房温存率はいずれの群でも70%以上であった。

組織学的効果は，comprehensive pCR（CpCR）がHR陰性27例(trastuzumab投与20例，非投与7例)(58%)，HR陽性13例（trastuzumab投与6例，非投与7例）(22%）でHR陰性群が有意に良好であった（$p<0.05$）。trastuzumabの投与別では，HR陽性/trastuzumab投与群21%に対してHR陽性/trastuzumab非投与群23%，HR陰性/trastuzumab投与群65%に対してHR陰性/trastuzumab非投与群47%であり，HR陰性例に対してtrastuzumabの追加効果が大きい傾向を認めた。一方で，quasi pCR（QpCR）でみるとHR陽性群もQpCR率が非投与群で33%，投与群が57%であったことから，trastuzumabの追加効果を認めた（Table 3）。

Table 2 Clinical response

	HR−/H+ (n=31)	HR−/H− (n=15)	HR+/H+ (n=28)	HR+/H− (n=30)
CR	25 (81%)	7 (47%)	18 (64%)	9 (30%)
PR	4	6	9	18
SD	1	2	1	3
PD	1	0	0	0
Response rate (%)	94	87	96	90
Breast-conserving surgery (%)	78	87	82	70

HR−: hormone receptor negative, HR+: hormone receptor positive, H: trastuzumab

Table 3 Pathological response

	HR−/H+ (n=31)	HR−/H− (n=15)	HR+/H+ (n=28)	HR+/H− (n=30)
SpCR	12	4	3	3
pCRinv	8	3	3	4
CpCR (SpCR+pCRinv)	20	7	6	7
CpCR rate (%)	65	47	21	23
Near pCR	5	1	10	3
QpCR (CpCR+near pCR)	25	8	16	10
QpCR rate (%)	81	53	57	33

SpCR (Strict pCR): disappearance of all tumor cells, pCRinv (pCR with *in situ* carcinoma): only *in situ* tumor residues, CpCR: comprehensive pCR, Near pCR: only focal invasive tumor residues, QpCR: Quasi pCR, HR−: hormone receptor negative, HR+: hormone receptor positive, H: trastuzumab

Table 4 Effect to lymph nodes

	HR−/H+ (n=31)	HR−/H− (n=15)	HR+/H+ (n=28)	HR+/H− (n=30)
cN−→pN0	15	6	20	15
cN−→pN+	0	0	0	2
cN+→pN0	16	4	3	5
cN+→pN+	0	5	5	8

HR−: hormone receptor negative, HR+: hormone receptor positive, H: trastuzumab

リンパ節転移に対する治療効果を Table 4 に示す。リンパ節転移の陰性化（cN+→pN−）率は HR 陰性/trastuzumab 投与群 16 例（100%），HR 陰性/trastuzumab 非投与群 4 例（44%），HR 陽性/trastuzumab 投与群 3 例（38%），HR 陽性/trastuzumab 非投与群 5 例（39%）であり，リンパ節に対する効果も HR 陰性群のほうが trastuzumab の追加効果が高かった（$p<0.05$）。

3．再発部位と予後

観察期間の中央値 1,114（408〜3,053）日において，104 例のうち 14 例（13%）が遠隔転移を認めた。12 例は pINV であり，このうち 5 例において初発再発部位が脳であった。HR 陰性，陽性の両群とも trastuzumab 投与群で脳転移例が多くみられた（Table 5）。さらに，5 例のうち 4 例には術後に trastuzumab を投与していた。

組織学的効果と予後の関係を検討してみた（Fig. 1, 2）。104 例全体では，CpCR を得た 40 症例は得られなかった症例に比べて DDFS が有意に良好（$p<0.05$）であったが，OS には差が生じなかった。HR 別にみたところ，HR 陽性群では DDFS と OS のいずれも差は認めなかったのに対して，HR 陰性群では DDFS に差が生じた（$p<0.05$）。

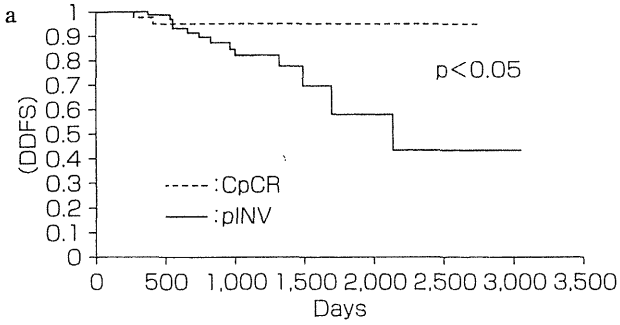
V．考　察

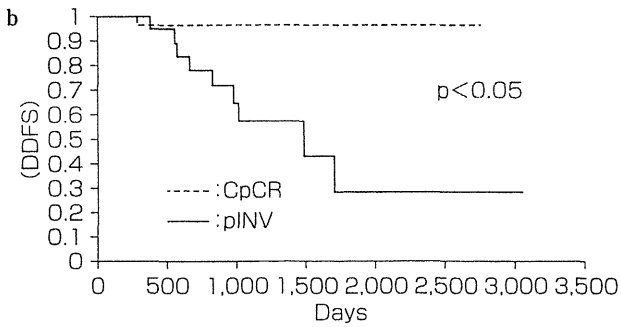
HER2 陽性乳癌に対して術前化学療法を施行した効果と臨床的意義を検討した。HER2 陽性乳癌は悪性度が高く予後不良のサブタイプであるが，化学療法の適切な使用と trastuzumab の登場によりその予後の改善が得られている[5]。周術期における trastuzumab の適応，およ

Table 5 Recurrence sites and pathological effects

	HR−/H+ (n=31)	HR−/H− (n=15)	HR+/H+ (n=28)	HR+/H− (n=30)
Median follow-up (days)	1,257	1,140	921	1,053
Except brain				
pINV	1	3	0	3
CpCR	0	0	0	0
Brain				
pINV	3	1	1	0
CpCR	1	0	1	0

pINV: invasive cancer on pathologic examination, HR−: hormone receptor negative, HR+: hormone receptor positive, H: trastuzumab





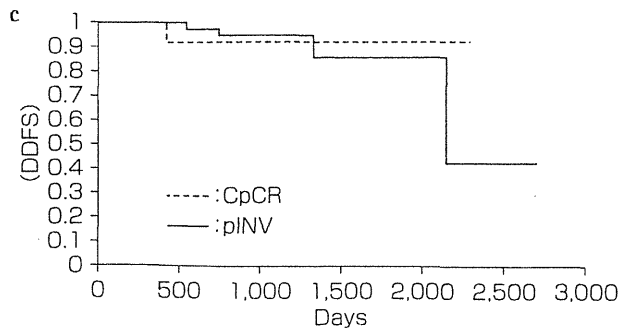
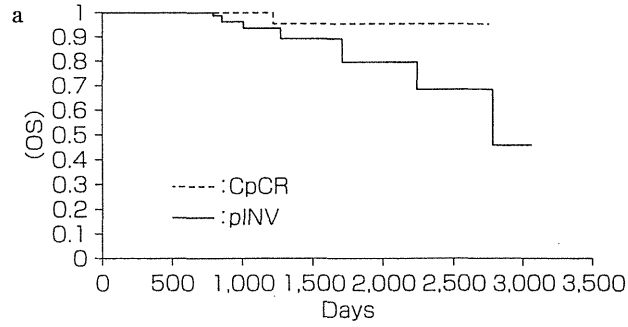
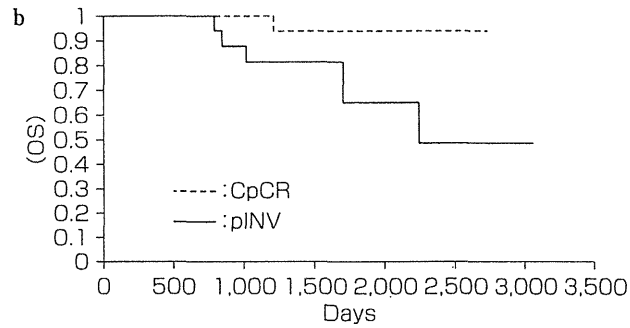


Fig. 1 CpCR was a significant factor ($p<0.05$) in prolonged distant disease free survival (DDFS) in the whole study and in the HR negative group.
a: all patients.
b: HR negative group.
c: HR positive group.





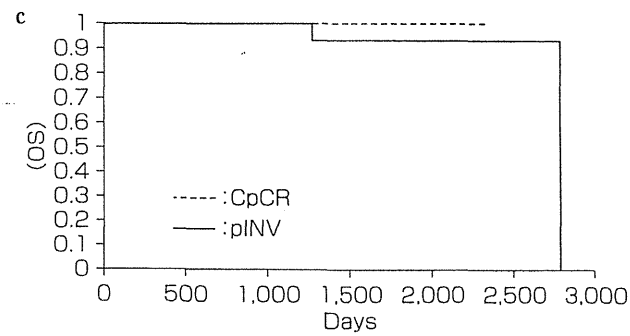


Fig. 2 CpCR was not a significant factor in prolonged over survival (OS) in any group.
a: all patients.
b: HR negative group.
c: HR positive group.

び化学療法の必要性が十分認識されるようになり，術前治療への応用が広まった。そして，trastuzumab は 2011 年 11 月からは本邦でも HER2 陽性乳癌に対する術前治療として化学療法との併用が認可された。術前治療の意義目的には腫瘍の縮小効果による乳房温存の可能性や腋窩手術の縮小に加えて，治療効果から薬物への感受性が把握でき，個別化治療の展開が考えられる。

HER2 陽性乳癌は化学療法の感受性が高く pCR を得やすいサブタイプであることはよく知られているが，今回の検討でも諸家の報告と同じように CpCR 率は全体で 38%と良好な成績であった。一方で，HER2 陽性乳癌のなかでも細胞内シグナル伝達のネットワークから鑑みて，HER2 陽性乳癌を少なくとも HR 陽性（double positive タイプ）と HR 陰性（pure HER2 陽性タイプ）に大別した治療選択の構築が必要とも考えられている[5]。本検討で CpCR 率からみると，HR 陰性群 58%，陽性群 22%で陰性群が有意に高率であった（$p<0.05$）。

また，HER2 陽性乳癌は trastuzumab を追加することで pCR 率が上昇すると Buzdar ら[4]は報告しているが，今回の検討では HR 陰性群ではその傾向は認められたが，HR 陽性群では trastuzumab の追加効果は認められなかった。しかし，QpCR 率で検討してみると，HR 陽性群においても追加効果が認められる。このことから腫瘍量の縮小効果は期待でき，そして本検討でもみられたように術前薬物療法の目的の一つである乳房温存術の可能性も高まることが期待される。

主病巣と転移リンパ節への化学療法の効果の相関は必ずしも一致しないが，HER2 陽性乳癌を対象とした今回の検討では，主病巣と同じように HR 陰性群で trastuzumab を併用した症例の陰性化率が高かった。

HER2 陽性乳癌に対して術後に trastuzumab を 1 年間投与することで，また術前化学療法で pCR を得ることで予後が改善されるといわれている。今回の検討では，104 例全体において CpCR を得た 40 症例の DDFS は有意な延長を認め（$p<0.05$），観察期間約 3.6 年間で再発例は 2 例であった。2 例はともに脳転移であった。一方で脳以外の再発が抑制され，初発再発部位が脳転移の症例が増加しているといわれるように[6]，今回の検討でも再発症例 14 例のうち 7 例が脳転移であり，HR 陰性，陽性にかかわらず術前に trastuzumab を投与している症例が多かった。また，そのうち 4 例は術後に trastuzumab を投与していたが脳転移を発症していることから，脳転移に関しては HR の発現状況よりも術前治療で pINV であることがハイリスクであると考えられ，また術後の trastuzumab の補助療法のみでは発症を抑制するのは困難な可能性もあると考えられた。

脳転移の危険因子を同定し，さらなる個別化治療の開発が HER2 陽性乳癌の治療成績向上のために重要であると考えられた。

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ORIGINAL ARTICLE – BREAST ONCOLOGY

Clinical Implications of Occult Metastases and Isolated Tumor Cells in Sentinel and Non-Sentinel Lymph Nodes in Early Breast Cancer Patients: Serial Step Section Analysis with Long-Term Follow-Up

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ABSTRACT

Background. This study was designed to clarify retrospectively the clinical significance of occult metastases in both sentinel lymph nodes (SLNs) and non-SLNs in patients with early breast cancer.

Methods. A total of 109 (80.1%) of 136 women with breast cancer who had consecutively undergone SLN biopsy (176 lymph nodes) were intraoperatively diagnosed as being free of SLN involvement. SLNs were routinely examined by hematoxylin–eosin (HE) staining of one to four frozen sections per node. Sixty-four (58.7%) of these patients also underwent backup axillary dissection. For the 109 patients, all formalin-fixed, paraffin-embedded tissues of SLNs and non-SLNs were entirely cut into 5-μm-thick sections. All serial step sections at 85-μm intervals were stained with HE and immunohistochemistry with pancytokeratin.

Results. Occult metastases in SLNs and non-SLNs were detected in 25 (23%) and 10 (16%) patients, respectively. The presence of occult SLN metastasis was marginally correlated with T-factor ($P = 0.06$), and predictive factors for occult non-SLN metastases were tumor nuclear grade ($P = 0.039$). With a median follow-up of 86 months, disease-free survival ($P = 0.3$) or overall survival ($P = 0.8$) did not differ between the patients with and without occult SLN metastases, regardless of backup axillary lymph node dissection.

Conclusions. SLN or non-SLN occult metastases detected by serial step sections at 85-μm intervals did not have significant prognostic implications.

The presence and number of axillary lymph node metastases are the most important prognostic indicators in breast cancer.[1] Sentinel lymph node (SLN) navigation surgery is an important technique for the assessment of axillary lymph node status and treatment decisions.[2,3] Axillary lymph node dissection (ALND) is usually performed when SLN metastases are detected intraoperatively.[4] In the seventh edition of the TNM classification by the American Joint Committee on Cancer (AJCC), cancer cells found in regional lymph nodes, including SLNs, are defined as follows: (1) ITC (staged as pN0(i+)) when there is a tumor mass ≤ 0.2 mm in diameter, (2) micrometastasis (staged as pN1mi) when there is a tumor mass > 0.2 mm but ≤ 2 mm in diameter, and (3) macrometastasis if the tumor mass > 2.0 mm in diameter.[5]

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The impact of occult metastasis on disease-free survival (DFS) and overall survival (OS) has been controversial.[6–9] Hansen et al. reported that patients with pN0(i+) or pN1mi apparently have the same 8-year DFS and OS as those with pN0(i−) in SLNs.[10] On the other hand, de Boer et al.[11] reported ITCs or micrometastases in SLNs to be associated with a reduced 5-year DFS in women with early-stage breast cancer, regardless of whether they had received adjuvant therapy. Giuliano et al.[12] recently reported that among patients with limited SLN metastatic breast cancer treated with breast conservation and systemic therapy, the use of SLN biopsy alone compared with ALND did not result in inferior survival.

Some percentage of SLN negative patients will have locoregional recurrence or distant metastasis in the future. One of the reasons for this phenomenon may be explained by the occult metastases.[1,13,14] Underestimation of the extent of local tumor spread at the time of primary surgery might result in an insufficient treatment for patients. For the accurate evaluation of local tumor spread, it may be effective to assess SLN involvement precisely by means of serial sectioning with appropriate immunohistochemistry for detecting micrometastases and isolated tumor cells (ITCs).[10,11] In the current study, we examined the prevalence of occult metastasis in SLNs and non-SLNs by using the most laborious method ever, and investigated the clinicopathological and prognostic significances of occult metastases in SLNs and non-SLNs in patients with early breast cancer.

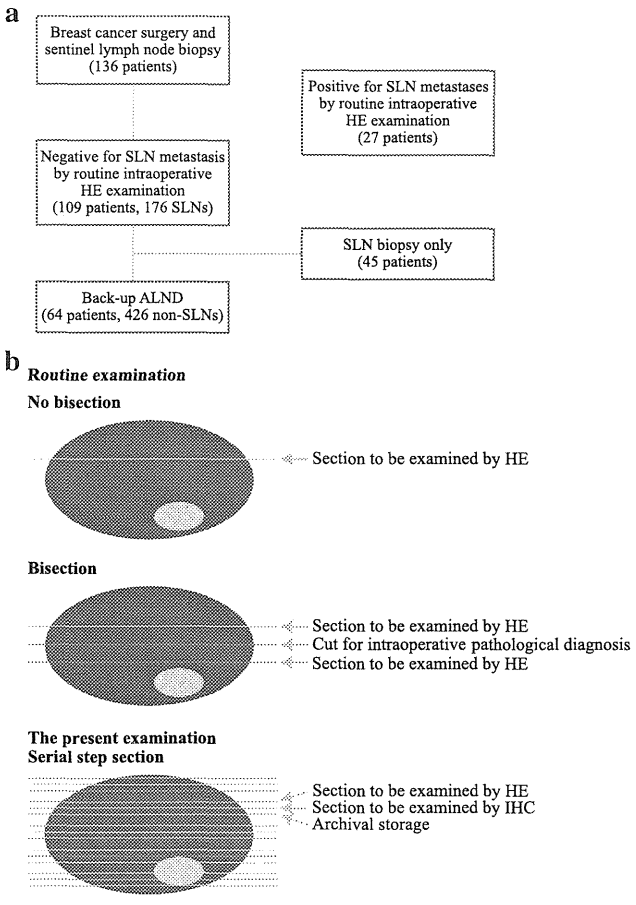

FIG. 1 **a** Schematic presentation of the protocol of the present study. **b** Schematic presentation of specimen preparation for routine histopathological examination (*upper* and *middle*) and for the serial step sectioning employed in the present study (*lower*)

PATIENTS AND METHODS

The study was reviewed and approved by the Institutional Review Board of the National Defense Medical College. Informed consent was obtained from all patients who participated in the present study. Between January 2000 and December 2003, a total of 136 patients with clinically T1N0M0 or T2N0M0 primary breast cancer received a full explanation of the protocol and agreed to enrollment in the current study.[15] The SLN navigation surgery protocol was previously described in detail.[15] Patients were excluded from the protocol if multiple primary breast tumors were present or there was clinical suspicion or confirmed presence of abnormal axillary nodes as determined by ultrasonography.[15] In addition, any case with an intraoperative SLN frozen section positive for metastasis was excluded from the present study.

A total of 109 (80.1%) of 136 women were intraoperatively diagnosed as being free of SLN metastases and were analyzed for occult metastasis. Backup ALND had been performed for 64 (58.7%) of 109 patients without SLN metastasis, who are included in the present non-SLN analysis (Fig. 1a).

In total, 176 SLNs (range, 1–4 nodes; mean, 1.65) from the 109 patients were biopsied. Intraoperative frozen section diagnosis of SLNs was routinely performed as follows: each SLN was bisected from the hilum to the periphery, embedded in OCT compound (Sakura Finetek Japan, Tokyo), snap frozen, and cut into sections for histopathological diagnosis. If the SLN was smaller than 2 mm in diameter, it was embedded without bisection and subjected to frozen section diagnosis. Residual SLN tissue was fixed in formalin, embedded in paraffin, and sectioned for routine hematoxylin and eosin (HE) staining. Dissected axillary lymph nodes were formalin-fixed and paraffin-embedded without bisection and were sectioned for routine HE staining.

Of the 64 patients who underwent ALND, two showed metastasis in non-SLNs, whereas the other 62 were free of metastasis in non-SLNs by routine histopathological examination. Each histopathological diagnosis was confirmed using routinely processed formalin-fixed, paraffin-embedded sections of SLNs and non-SLNs by two pathologists (HT and OM).

Serial Step Sectioning of SLNs and Non-SLNs

All SLNs and non-SLNs were entirely cut into 5-μm sections until residual tissue in the block disappeared, and three serial sections at every 85-μm interval for all lymph nodes were used for the present histopathological and immunohistochemical investigations. One of each set of the three serial sections was stained by HE, another was immunohistochemically with an anti-pancytokeratin antibody (AE1/AE3, Dako, Glostrup, Denmark), and the third was stored as an archival specimen (Fig. 1b). HE staining was manually performed. Immunohistochemistry (IHC) for pancytokeratin was automatically performed using an autostainer (Dako) based on the process of proteinase K treatment through inhibition of the nonspecific peroxidase reaction, incubation with the primary antibody, incubation with Envision plus (Dako), and finally reaction with 3,3′-diaminobenzidine, according to the manufacturer's instructions. Counter-staining of the sections with hematoxylin was manually performed. All sections were examined by H.T. with previous screening for all IHC slides by Y.O. The definitions of lymph node metastasis in SLNs/non-SLNs were in accordance with the TNM classification, 7th edition, i.e., macrometastasis (pN1), micrometastasis (pN1mi), and ITCs (pN0(i+)).[10]

Patient Follow-Up

Patients were periodically examined at the National Defense Medical College Hospital or affiliated hospitals. The patients were observed every 3 months for 5 years and every 1 year thereafter. Follow-up consisted of a complete skin/lymph node evaluation, with laboratory and imaging studies obtained as clinically indicated. Recurrence was defined as positive findings by physical examination and/or by imaging diagnosis. Date of first recurrence was defined as the first notation in the medical record indicating the nodal or distant recurrence. Vital status and cause of death were obtained for every patient from the medical record, direct patient/family contact, and correspondence with referring physicians.

Statistical Analysis

The χ^2 test and Fisher's extract test were used to assess baseline differences between binary variables. For DFS and OS, Kaplan–Meier methods were used to estimate survival rates, and differences between survival curves were evaluated by the log-rank test. The P values <0.05 were considered a significant result. Cox proportional hazards models were used to assess the relative risk of occult metastases by univariate and multivariate analyses. All reported P values are two-sided, and confidence intervals (CIs) are at the 95% level. All analyses were performed by using SPSS version 11.0.1 (SPSS Inc., Chicago, IL).

RESULTS

Patient Population

The clinicopathological characteristics of the 109 patients are listed in Table 1. Median age was 57 (range, 27–82) years. One patient died of gastric cancer, five of breast cancer relapse, and 94 (86.2%) were disease-free at the last follow-up. Median duration of follow-up was 86

TABLE 1 Clinicopathological characteristics of the 109 patients

Characteristic	No. of cases (%) ($N = 109$)
Age at operation (year)	
≤50	37 (34)
>50	72 (66)
Tumor size (cm)	
Tis	10 (9)
≤2	50 (46)
>2	49 (45)
Nuclear grade	
1	20 (18)
2	44 (40)
3	41 (38)
Unknown	4 (4)
Histological type	
Invasive ductal	88 (81)
Invasive lobular	4 (4)
Other	17 (16)
No. of SLNs harvested	
1	61 (55)
2	30 (28)
3	14 (13)
4–5	4 (4)
Axillary lymph node dissection	
Yes	64 (59)
No	45 (41)
Type of surgery	
Breast-conserving surgery	60 (55)
Mastectomy	49 (45)
Systemic adjuvant therapy	
No therapy	35 (32)
Hormonal therapy	49 (45)
Chemotherapy	16 (15)
Both	9 (8)

SLN sentinel lymph node

(range, 6–123) months. Only three patients (2.8%) were lost to follow-up within 20–38 months after the operation.

Occult Metastases in SLN

Metastases were detected in 25 (23%) of 109 patients: 5 (20%) by routine permanent HE examination, 9 (36%) by serial step HE section examination, and 11 (44%) by serial step IHC sections. The largest cluster size of the occult metastases was classified as pN0i+ in 8 (32%), pN1mi in 13 (52%), and pN1 in 4 (16%) patients. Among the clinicopathological parameters, the presence of occult SLN metastases was marginally correlated with pT-factor: 35% (17 of 49) in pT2/pT3 cases and 13% (8/60) in pTis/pT1 cases ($P = 0.06$; Table 2). The occult SLN metastasis was more frequently detected in cases with invasive lobular carcinoma (3/4, 75%) than in those with invasive ductal carcinoma (21/88, 24%; $P = 0.15$). There was no significant association between pathological parameters and the largest diameters of occult SLN metastatic foci (Supplementary Table).

Occult Metastases in Non-SLN

Metastases were detected in 10 (16%) of 64 patients: 2 (20%) by routine HE examination, 3 (30%) by serial step HE section examination, and 5 (50%) by employing serial step IHC sections. Of these ten patients, four had occult SLN metastases. The largest cluster size of the occult metastases was classified as pN0(i+) in two (20%), pN1mi in five (50%), and pN1 in three (30%). The occult metastases in non-SLN were more frequently found in the patients with high-grade primary tumor ($P = 0.039$; Table 2). The status of occult SLN metastasis did not significantly affect the incidence of occult non-SLN metastasis ($P = 0.27$). There was no significant relationship between pathological parameters and the diameter of the largest non-SLN occult metastatic foci (Supplementary Table).

TABLE 2 Correlations of occult SLN and occult non-SLN metastasis with clinicopathological parameters

Parameter	No. of cases (%)			No. of cases (%)		
	Total ($N = 109$)	Occult SLN metastasis Positive (%) ($n = 25$)	P	Total ($N = 109$)	Occult non-SLN metastasis Positive (%) ($n = 10$)	P
Age at operation (year)						
≤50	37	5 (14)	0.23	17	3 (18)	1
>50	72	20 (28)		37	7 (19)	
Tumor size (cm)						
Tis, ≤2	60	8 (13)	0.06	30	3 (10)	0.5
>2	49	17 (35)		34	7 (21)	
Nuclear grade						
1,2	64	13 (20)	0.55	36	2 (5)	0.039
3	41	12 (29)		26	8 (31)	
Histological type						
Invasive ductal	88	21 (24)	0.15	53	9 (17)	0.49
Invasive lobular	4	3 (75)		3	1 (33)	
No. of SLNs harvested						
1	61	10 (16)	0.22			
≥2	48	15 (31)				
Systemic adjuvant therapy						
No therapy	35	7 (20)	0.87	35	3 (9)	1.00
Therapy	74	18 (24)		74	7 (9)	
No. of lymph nodes dissected						
≤8				38	3 (8)	0.099
>8				26	7 (27)	
Occult SLN metastasis						
Positive				15	4 (27)	0.27
Negative				49	6 (12)	

SLN sentinel lymph nodes

Survival Analysis

Disease-free survival and OS curves for occult SLN metastasis-positive and negative patients are shown in Fig. 2. The detection of occult SLN metastasis tended to be associated with a reduced DFS rate (80 vs. 89.3%), although the difference was not statistically significant ($P = 0.2$). The DFS rate was highest (93.8%) in the subgroup in which SLNs were negative for occult metastases and ALND had been performed (−SLN w/ ALND), with marginally significant difference from the subgroup with occult SLN receiving ALND (+SLN w/ ALND; $P = 0.09$). There was no difference between the subgroups in which ALND was omitted with and those without occult SLN metastases (+SLN w/o ALND vs. −SLN w/o ALND; $P = 0.8$; Fig. 2a). OS for these subgroups did not differ significantly (Fig. 2b).

The univariate analysis revealed that the histological type of the tumor was associated with DFS (Table 3). The risk of recurrence was significantly higher in patients with invasive lobular carcinoma (risk ratio, 5.0; 95% CI, 1.1–23; $P = 0.036$) than in those with invasive ductal carcinoma. It was significantly higher in patients with high nuclear grade (risk ratio, 3.1; 95% CI, 1.1–9.4; $P = 0.04$) than in those with low nuclear grade. Tumor size tended to be associated with DFS rates, but the differences were not statistically significant. Systemic adjuvant therapy was not correlated with DFS in the present series. No parameters were found to be predictive of OS (Table 3). Because no patients died in the group with tumors of grades 1 or 2 and invasive lobular carcinoma, statistical analyses for OS were not possible for these parameters.

When six parameters [age ($\leq$50 vs. $>$ 50), invasive tumor size ($\leq$2 cm vs. >2 cm), number of SLNs removed ($\leq$8 vs. >8), nuclear grade (1, 2 vs. 3), histological type (invasive ductal carcinoma vs. invasive lobular carcinoma vs. others) and occult SLN metastasis (absent vs. present)] were included in the multivariate analysis, nuclear grade (grade 3, risk ratio, 3.5; 95% CI, 1.1–12; $P = 0.04$) and histological type (invasive lobular carcinoma, risk ratio, 6.7; 95% CI 1.4–32; $P = 0.016$) were identified as independent risk factors for recurrence. None of the factors examined, including occult SLN metastases, were associated with a significant reduction in OS.

DISCUSSION

The prognostic significance of occult SLN metastases has been a source of ongoing debate.[6–11] The conventional HE examination underestimates metastatic involvement in SLNs and non-SLNs, because only one or two central cross-sections of the nodes are examined. In the present

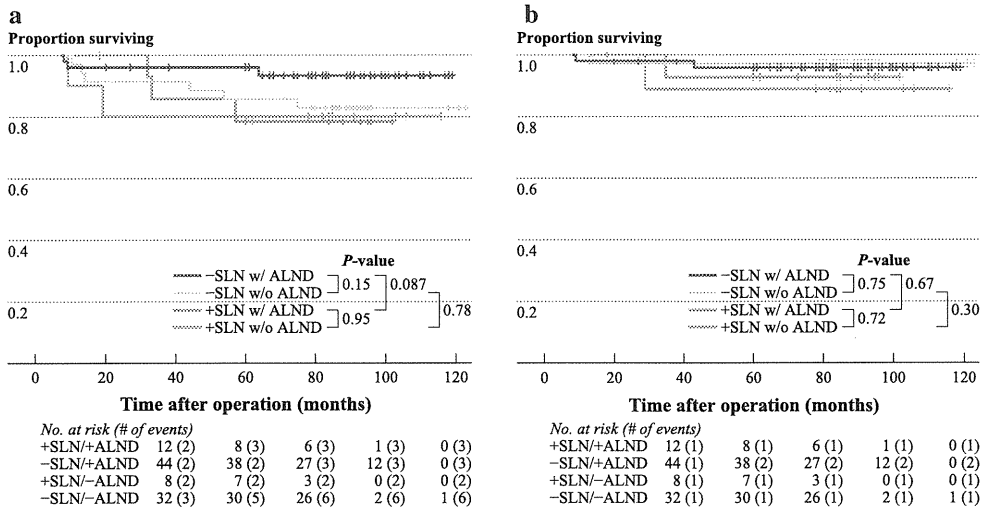

FIG. 2 **a** Disease-free survival curves for patient subgroups. The subgroup without occult SLN metastasis that received ALND (−SLN w/ ALND) ($n = 49$), the group with occult SLN metastasis that received ALND (+SLN w/ ALND) ($n = 15$), the subgroup without occult SLN metastasis in which ALND was omitted (−SLN w/o ALND) ($n = 35$), and the subgroup with occult SLN metastasis in which ALND was omitted (+SLN w/o ALND) ($n = 10$). The 10-year DFS rate was highest (93.8%) in the −SLN w/ ALND subgroup, with marginally significant difference between the +SLN w/ ALND and −SLN w/ ALND subgroups ($P = 0.087$). There was no difference between the +SLN w/o ALND and −SLN w/o ALND subgroups ($P = 0.78$). There were no statistically significant differences among these four subgroups ($P = 0.33$). **b** Overall survival curves for patient groups. The subgroup without occult SLN metastasis that received ALND (−SLN w/ ALND) ($n = 49$), the group with occult SLN metastasis that received ALND (+SLN w/ ALND) ($n = 15$), the subgroup without occult SLN metastasis in which ALND was omitted (−SLN w/o ALND) ($n = 35$), and the subgroup with occult SLN metastasis in which ALND was omitted (+SLN w/o ALND) ($n = 10$). There were no statistically significant differences among these four subgroups ($P = 0.74$)

TABLE 3 Impact of clinicopathological variables and occult SLN metastasis on DFS and OS, estimated by Cox univariate analysis

Variable	Total ($N = 109$)	Disease-free survival			Overall survival		
		No. of recurrences	RR (95% CI)	*P*	No. of deaths	RR (95% CI)	*P*
Age at operation (year)							
≤50	37	3	1		1	1	
>50	72	11	1.8 (0.51–6.6)	0.35	4	1.9 (0.21–17)	0.55
Tumor size (cm)							
Tis, ≤2	60	5	1		1	1	
>2	49	9	2.5 (0.82–7.4)	0.1	4	5.2 (0.57–46)	0.14
Nuclear grade							
1,2	64	5	1		0	[a]	
3	41	9	3.1 (1.1–9.4)	0.04	5		
Histological type							
Invasive ductal	88	11	1		5	[a]	
Invasive lobular	4	2	5.0 (1.1–23)	0.036	0		
No. of SLNs harvested							
1	61	7	1		3	1	
≥2	48	7	1.2 (0.43–3.6)	0.67	2	0.8 (0.13–4.8)	0.81
Axillary lymph node dissection							
No	45	6	1		2	1	
Yes	64	8	0.54 (0.18–1.6)	0.26	3	1.1 (0.17–6.4)	0.94
Type of surgery							
Breast-conserving surgery	60	7	1		2	1	
Mastectomy	49	7	1.3 (0.46–3.8)	0.6	3	1.9 (0.32–12)	0.46
Systemic adjuvant therapy							
No therapy	35	5	1		1	1	
Therapy	74	9	0.86 (0.29–2.6)	0.79	4	1.8 (0.21–16)	0.58
Occult SLN metastasis							
Negative	84	9	1		3	1	
Positive	25	5	2.0 (0.67–6.0)	0.21	2	2.4 (0.39–14)	0.34

DFS disease-free survival; *OS* overall survival; *RR* relative risk; *CI* confidence interval; *SLN* sentinel lymph node

[a] Cannot be calculated because there was no event in one side

study, using a serial step sectioning method, we examined the incidence and clinicopathological significance of occult metastases, which have been overlooked by routine HE examination in SLNs and non-SLNs.

To identify occult metastases in patients with pN0 early breast cancer, we employed a laborious method, examining all serial step sections at every 85-μm interval for all SLNs biopsied and non-SLNs dissected. Several groups of breast surgeons have consistently advocated increasing the detection rate of metastatic tumor cells in axillary lymph nodes by additional sectioning and IHC staining of these lymph nodes.[16–20] For example, in the NSABP (National Surgical Adjuvant Breast and Bowel Project) B32 and ACOSOG (American College of Surgeon Oncology Group) Z0010 trials, occult metastases were detected in 15.9 and 10.5%, respectively.[21,22] In our study, occult SLN metastases were detected in a total of 23% of patients (25/109). This figure is higher than those mentioned above. The discrepancy is probably due to differences in the methods used for detecting ITC/micrometastasis.

We confirmed that invasive lobular carcinoma, high-grade tumors, and pathological tumor size of more than 2 cm in the greatest diameter, indicating pT2 and pT3, were significantly or marginally significantly correlated with occult metastases. It is not surprising that metastatic potential to axillary nodes could be supported by the biological features of the primary tumor.

In the present study with a median follow-up of 86 months, the presence of occult SLN metastases detected by serial step sectioning with HE and IHC did not have any impact on OS, regardless of back-up ALND, suggesting that it could not be discriminatory predictor of patients' prognosis. In some clinical trials, including NSABP B31 and ACOSOG Z0011, investigators have reported that the

magnitude of difference in prognosis did not differ between patients' groups with and without detection of ITC and/or micrometastasis.[12,21] Our observations were consistent with these previous study results. Occult SLN metastases detected by employing serial step section did not appear to influence on further axillary treatment.

The present study has limitations. This was a retrospective, single-institute study with a relatively small patient cohort, and 68% (74/109) of our patients had received adjuvant systemic therapies. Taking into consideration the fact that detection of ITC/micrometastasis using a serial step sectioning method was closely associated with histological features of the primary tumor but no independent factor of prognosis, the method does not appear to be clinically useful for patients who receive SLN biopsy, in whom systemic therapy can be recommended on the basis of the baseline characteristics of the tumor.

CONCLUSIONS

Sentinel lymph nodes or non-SLN occult metastases detected employing serial step sections at 85-μm intervals using both HE and IHC did not appear to have significant prognostic implications.

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ORIGINAL ARTICLE

Loss of heterozygosity on chromosome 16q suggests malignancy in core needle biopsy specimens of intraductal papillary breast lesions

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Abstract It is often difficult to make a definitive diagnosis of papillary breast lesions using core needle biopsy (CNB) specimens. We studied loss of heterozygosity (LOH) on chromosome 16q in order to assess its diagnostic use for papillary breast lesions in CNB specimens. Of 25 patients with intraductal papillary breast tumors, we extracted DNA from paired samples of tumor cells from CNB specimens and non-tumor cells from subsequent excision specimens and analyzed LOH at the D16S419 and D16S514 loci on chromosome 16q. LOH analysis results were compared with final diagnoses based on pathological features of the resected specimens. On the CNB specimens, 21 tumors were histologically diagnosed as indeterminate or suspicious for malignancy, while four tumors were unambiguously malignant. Of the 21 indeterminate or suspicious tumors, 11 were finally diagnosed as benign and ten as malignant, and on these, LOH analyses were informative for 8 of the 11 benign tumors and 7 of the 10 malignant tumors. LOH was also informative on two of the four tumors unambiguously malignant on CNB. None of the eight informative benign tumors showed LOH on 16q. Six of the eleven informative malignant tumors showed LOH on 16q. LOH on 16q was significantly different between CNB specimens of benign and malignant intraductal papillary tumors (P=0.007). Analysis of LOH on 16q may be helpful in making a definitive diagnosis in cases of papillary breast lesions, in both excised and CNB specimens.

Keywords Loss of heterozygosity · Breast · Papilloma · Papillary carcinoma · Core needle biopsy

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Introduction

Preoperative diagnosis of intraductal papillary tumors of the breast is challenging because of the difficulty of differentiating intraductal papillary carcinoma from intraductal papilloma. It is very difficult to diagnose the biological nature of these tumors based on mammography and ultrasonography, unless there is evidence of massive tumor invasion or rapid growth. Although image-guided core needle biopsy (CNB) is a highly reliable method of diagnosing breast lesions, it is often difficult to differentiate between intraductal papillary lesions based on routine pathological examination of CNB specimens. This difficulty arises because intraductal papillary carcinomas tend to be well differentiated, and CNB specimens do not always include a section with pathognomonic features. Therefore, a final diagnosis

can often be made only by histological examination of the surgically resected specimen.

A number of genetic and chromosomal alterations have been identified in sporadic breast carcinomas, and their clinical implications have been investigated. Loss of heterozygosity (LOH) on chromosomes 16q and 17p are frequent in both invasive carcinoma and ductal carcinoma in situ (DCIS), irrespective of differences in the histological types and grades [1–8]. Several studies have reported a striking difference in the incidence of LOH on 16q between DCIS and intraductal papilloma [1, 5, 7] and have suggested that analysis of LOH on chromosome 16q could be helpful in the differential diagnosis of intraductal papillary tumors. In a previous study, we used Southern blot analysis to examine LOH on 16q in intracystic papillary tumors using DNA isolated from frozen, paired, surgically resected samples of tumor and non-tumor tissues [7]. More recently, we reported a polymerase chain reaction (PCR)-based LOH analysis technique using DNA isolated from paraffin-embedded tumor samples [9, 10]. In the study we report here, we used this PCR-based approach to assess its diagnostic utility on CNB specimens of indeterminate or suspicious intraductal papillary breast lesions.

Materials and methods

Samples

We selected tumor samples of 25 women with a preoperative diagnosis of intraductal papillary breast tumor by image-guided CNB, who had undergone surgical resection between 2005 and 2008, from the pathology computer database at the National Cancer Center Hospital, Japan. Image-guided CNB had been performed under sonographic guidance using either a 14-gauge needle or an 11-gauge vacuum-assisted biopsy probe. Twenty-one tumors had been diagnosed as indeterminate or suspicious for malignancy based on the pathological features of the CNB specimens and the lesions had been surgically resected for definitive histological diagnosis. The remaining four tumors had been unambiguously diagnosed as DCIS. The research protocol was approved by the Ethics Committee of the National Cancer Center Hospital, Japan. All patients gave written informed consent for use of their specimens in the study.

Histological criteria of intraductal papillary tumors

The diagnosis of intraductal papillary tumor was based on the presence of epithelial proliferations supported by fibrovascular stalks, with or without an intervening myoepithelial cell layer [11, 12]. All of the hematoxylin and eosin (H&E)-stained slides of the CNB and resected specimens were retrieved and reviewed for diagnostic consistency by the authors using published criteria.

The Japanese reporting form for cytology and core needle biopsy [13] was used to review the CNB specimens. This reporting form records findings and a judgment of whether the specimen is adequate or inadequate. Adequate specimens are categorized as normal or benign, indeterminate, suspicious for malignancy, or malignant.

Intraductal papillary tumors were diagnosed as benign or malignant using the following histological criteria of cytological and structural features [11, 14]. Papillomas or benign papillary tumors were diagnosed in cases showing an arborescent structure composed of fibrovascular stalks covered by a layer of myoepithelial cells with overlying epithelial cells. Intraductal papillary carcinomas or malignant papillary tumors were usually large papillary lesions (mean 2 cm, range 0.4–10 cm) located within a large cystic duct, with thin fibrovascular stalks devoid of a myoepithelial cell layer and a neoplastic epithelial cell population with characteristics of low-grade DCIS. Cases of "papilloma with atypia" with focal atypical epithelial proliferation and low-grade nuclei [15] were categorized as indeterminate in CNB specimens and as benign in resected specimens. For cases in which it was difficult to distinguish between benign and malignant tumors, the diagnosis was made by assessing the architectural features and visualizing the myoepithelial cell layer with immunohistochemical staining. Final diagnosis was made by pathological examination of the excision specimens.

Microdissection of paraffin-embedded tissues and DNA extraction

For all 25 patients, we extracted DNA from paired samples of intraductal papillary tumor cells from CNB specimens and non-tumor cells (normal mammary glands or lymph nodes) from surgically resected specimens, as previously described [9, 10]. Formalin-fixed and paraffin-embedded tissue sections, 5 to 10 μm thick, were cut using a microtome. Sections mounted on PEN foil slides were deparaffinized in xylene for 5 min (twice) and rehydrated using a descending series of ethanol concentrations as follows: 100% for 30 s (twice), 95% for 30 s (twice), 70% for 10 s, and distilled water for 10 s. The sections were stained with Meyer's hematoxylin, washed with water, and then stained with eosin for 1 min (H&E stain). The slides were dehydrated with 100% ethanol, placed in xylene for 10 min, and air-dried. Specific cells of interest were microdissected and selected using a Leica LMD 6000 system in accordance with the manufacturer's instructions (Leica, Narishige Micromanipulator, Wetzlar, Germany). The microdissected cells were placed in 50 μl proteinase K solution (5 mg/ml proteinase K in 10 mM Tris–HCl, 1 mM EDTA, pH 8.0, 1% Tween 20) and incubated for 36–48 h at 55°C. The

proteinase K was inactivated by incubating the samples at 95°C for 10 min, and then subjected to standard phenol-chloroform extraction and ethanol precipitation in the presence of glycogen. The pellets were resuspended in distilled water and the concentration was adjusted to 0.01 μg/μl. The extracted DNA samples were stored at 4°C until further use.

Selection of polymorphic markers

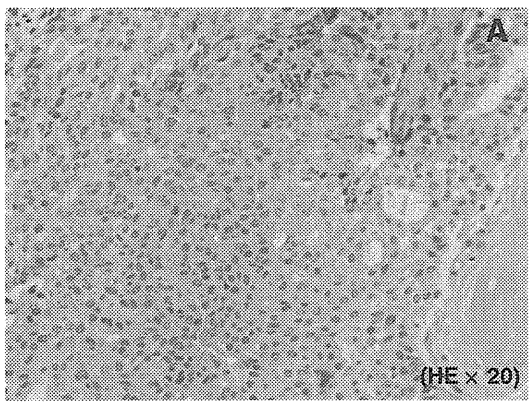
The chromosomal regions and markers used were D16S419 (16q12.2) and D16S514 (16q21). The following primer sequences were used for PCR amplification:

D16S419	Forward	5′-ATT TTT AAG GAA TGT AAA GNA CAC A-3′
	Reverse	5′-GAC GTT AGA CCA GGA GTC AG-3′
D16S514	Forward	5′-CTA TCC ACT CAC TTT CCA GG-3′
	Reverse	5′-TCC CAC TGA TCA TCT TCT C-3′

We selected polymorphic markers located on chromosome 16q based on the following criteria: (1) the markers were localized to regions with frequent DNA polymorphisms and with frequent LOH events reported in intraductal papillary carcinomas, notably low-grade DCIS [1–5, 7, 16], and (2) the amplified fragments were <250 bp, indicating that they could be successfully amplified using DNA from formalin-fixed tissues. Forward and reverse primer pairs for oligonucleotide polymorphic markers corresponding to the sequences retrieved from the UniSTS database (http://www.ncbi.nlm.nih.gov/unists) were synthesized and purchased from Perkin-Elmer (Applied Biosystems, Foster City, CA, USA). The 5′ ends of the forward primers were labeled with 6-carboxyfluorescein (6-FAM).

PCR

Genomic DNA was PCR amplified in a 25-μl reaction mixture containing 2 μl DNA solution corresponding to 20 ng genomic DNA, 0.4 pmol/μl of each primer, and 1× TaqMan Universal PCR Master Mix (Applied Biosystems) using a GeneAmp® PCR system 9600 (Applied Biosystems). The typical PCR cycling conditions included 2 min incubation at 50°C and 10 min denaturation at 95°C, followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. An



$$\frac{\text{Allele 2 peak height (T)} / \text{Allele 1 peak height (T)}}{\text{Allele 2 peak height (N)} / \text{Allele 1 peak height (N)}}$$

$$= \frac{\text{Allele 2 peak height (T)} \times \text{Allele 1 peak height (N)}}{\text{Allele 2 peak height (N)} \times \text{Allele 1 peak height (T)}}$$

$$= \frac{3266 \times 7891}{2749 \times 8266}$$

$$= 1.13\ (0.6 - 1.4) \rightarrow \text{Negative for 16q LOH}$$

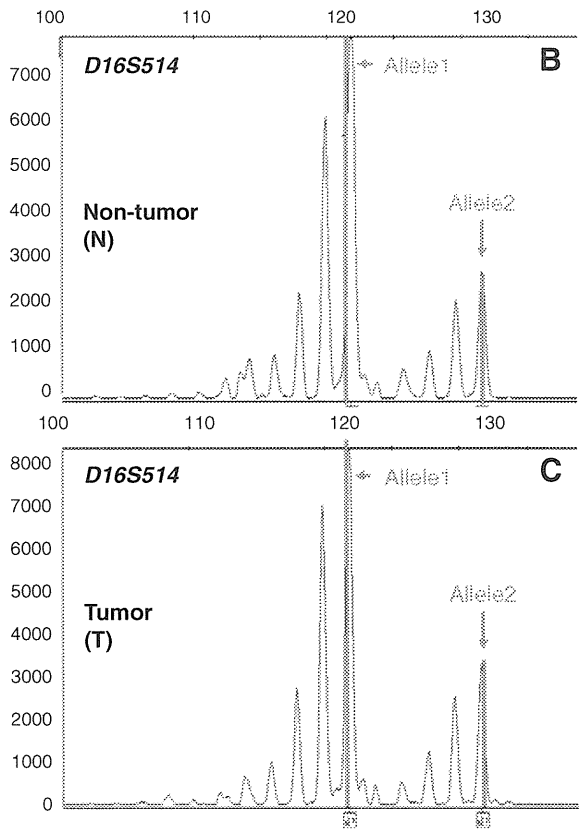


Fig. 1 Analysis of loss of heterozygosity (LOH) in an intraductal papillary tumor (case 4). **a** Based on the pathological features of the excised specimen, the tumor was diagnosed as intraductal papilloma. **b** Electrophoretogram showing constitutional heterozygosity (alleles 1 and 2) at the D16S514 locus in non-tumor DNA. The horizontal axis indicates the size of the DNA fragments (bp), and the vertical axis indicates signal intensity. **c**. Electrophoretogram showing retention of heterozygosity (alleles 1 and 2) at the D16S514 locus in tumor DNA. The axes are the same as in **b**

elongation step at 72°C for 10 min was added to the final cycle. Aliquots of the PCR products were then mixed with size standard and formamide, denatured, and run on an ABI 3130 automated capillary electrophoresis DNA sequencer (Applied Biosystems). The quantity and the quality of the DNA fragments amplified by PCR were confirmed by agarose gel electrophoresis. As a positive control, we used DNA isolated from formalin-fixed, paraffin-embedded tissues of five breast carcinomas in which LOH on 16q had already been detected by Southern blot analysis of fresh frozen tissues [17]. As a negative control, PCR was performed without template DNA.

Assessment of allele loss

The amplified products were assessed for peak height and area using Gene Mapper software (version 3.7; Applied Biosystems). Non-cancerous DNA samples with two different amplified bands were defined as informative cases for LOH analysis. The presence of LOH was determined in accordance with the manufacturer's criteria. LOH was considered to exist if the ratio of the peak heights, which was calculated with the following formula, was <0.6 or >1.4: [peak height of the affected allele (allele A) of the tumor× peak height of the unaffected allele (allele B) of normal cells]/[peak height of allele A of normal cells×peak height of allele B of tumor cells] (Figs. 1 and 2) [17]. If the ratio of the peak height was 0.6 and 1.4 according to the formula, the case was judged to have retention of heterozygosity or absence of LOH.

When the results were questionable, PCR amplification and LOH analysis were performed at least twice to obtain equivalent results. Results were considered non-informative when the normal tissue was constitutionally homozygous and were not evaluated when the tissue lysates were not amplified, that is, PCR was unsuccessful. When either D16S419 or D16S514 showed LOH, the tumor was considered to have LOH. The LOH analysis results were compared with the final diagnoses based on the pathological features of the surgically resected specimens.

Statistical analyses

The χ^2 test was used to determine differences between the benign and malignant groups of intraductal papillary tumors. Differences of $P<0.05$ were considered statistically

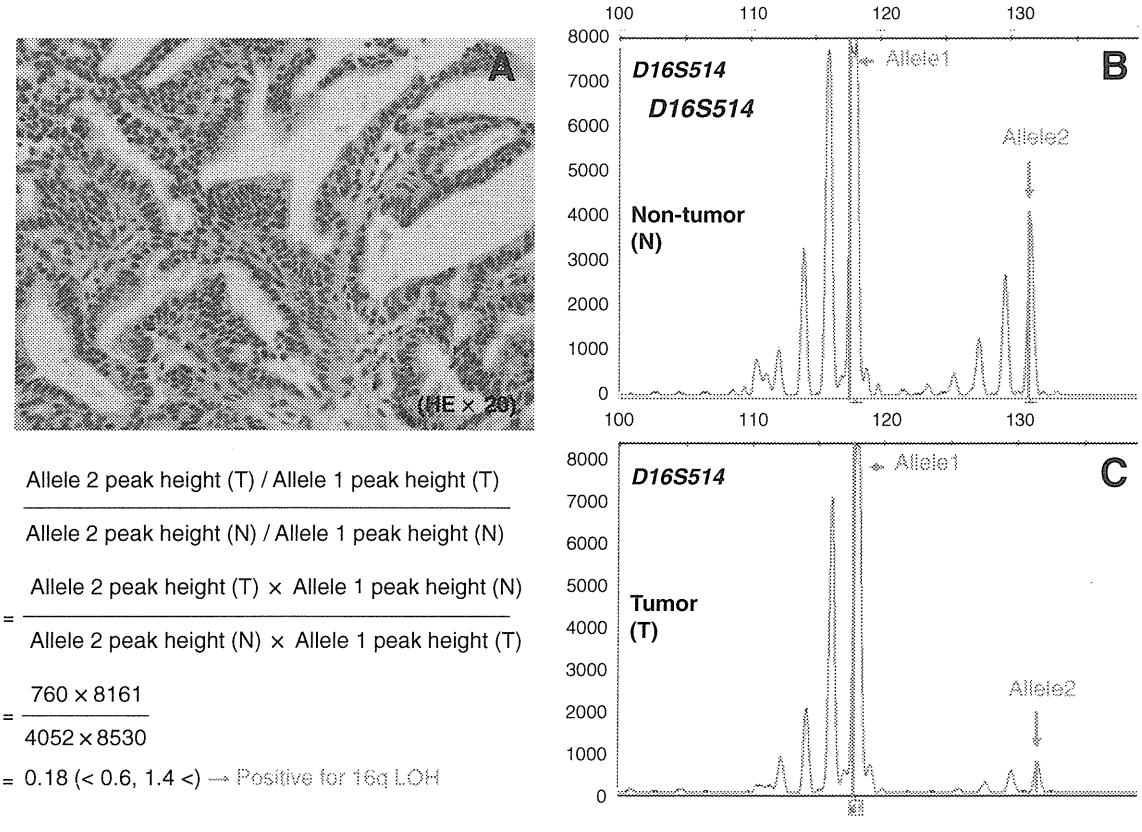


Fig. 2 Analysis of loss of heterozygosity (LOH) in an intraductal papillary carcinoma (case 15). **a** Based on the pathological features of the excised specimen, the tumor was diagnosed as intraductal papillary carcinoma. **b** Electrophoretogram showing constitutional heterozygosity (alleles 1 and 2) at the D16S514 locus in non-tumor DNA. **c** Electrophoretogram showing loss of heterozygosity (loss of allele 2) at the D16S514 locus in tumor DNA

significant. PASW statistics 17 (SPSS, Inc., Chicago, IL, USA) was used for all statistical analyses.

Results

Of the 21 indeterminate or suspicious intraductal papillary tumors, 11 were finally diagnosed as benign and 10 as malignant by microscopic examination of surgically resected specimens (Table 1). The first clinical sign was nipple discharge in 8 (38%) and a palpable mass in 4 (19%) of the 21 cases. Sonographic findings of the papillary lesions included a well-defined solid mass in nine cases (43%), a cystic lesion with solid components in five (24%), and duct dilatation with solid components in seven (33%). Multiple papillary lesions were found in seven cases (33%). The median tumor size on imaging was 1.9 cm (range 0.6–4.0 cm). There were no significant differences in clinical or imaging findings between lesions finally diagnosed as malignant on excisional biopsy specimens and those finally diagnosed as benign (Table 1). Thirteen (62%) of the 21 lesions were biopsied using a 14-gauge needle, and 8 (38%) were biopsied using an 11-gauge vacuum-assisted biopsy probe. The type of percutaneous biopsy was not correlated with postoperative conversion of histopathological diagnosis.

Table 2 shows the final histological diagnoses and 16q LOH results of CNB specimens for each of the 25 intraductal papillary tumors. Eight of the 11 benign tumors were informative, and none of these cases showed LOH on 16q. Nine of the 14 malignant tumors were informative, and these showed frequent LOH on 16q. Out of the total of 25 papillary tumors, seven were considered non-informative (constitutional homozygosity) and one was not evaluated after PCR was unsuccessful. As representative results, case 4 in which 16q LOH was negative is shown in Fig. 1 and case 15 in which 16q LOH was positive is shown in Fig. 2. Case 4 was finally diagnosed as papilloma based on the pathological features of the resected specimen. Figure 1b, c show two peaks of alleles in both the non-tumor and tumor DNA. The ratio of allele 2 peak height to allele 1 peak height in the tumor DNA divided by the ratio in the normal DNA was 1.13.

Table 1 Clinical and imaging findings in papillary breast lesions

	Total (n=21)	Final histological diagnosis: Benign (n=11)	Malignant (n=10)	P value
First clinical sign				
Nipple discharge	4 (19%)	1	3	0.14
Palpable mass	8 (38%)	4	4	
None	9 (43%)	6	3	
Sonographic findings				
Well-defined solid mass	9 (43%)	4	5	0.31
Cystic lesion with solid components	4 (19%)	2	2	
Duct dilatation with solid components	8 (38%)	5	3	
Mean tumor size on imaging (cm)	1.9±1.0 (0.6–4.0)	1.8±1.0 (0.6–3.0)	2.1±1.1 (0.6–4.0)	0.49
Number of lesions on imaging				
Multiple	7 (33%)	2	5	0.14
Solitary	14 (67%)	9	5	
Method of percutaneous biopsy				
Core needle biopsy (14-gauge)	13 (62%)	8	5	0.27
Vacuum-assisted biopsy (11-gauge)	8 (38%)	3	5	

Table 2 Final histological diagnoses of surgically resected specimens and 16q loss of heterozygosity (LOH) analysis results in core needle (CNB) specimens of papillary breast lesions

Case no.	Final histological diagnosis	Retained alleles on 16q	
		D16S419	*D16S514*
1	Benign	□	□
2	Benign	□	□
3	Benign	□	NI[a]
4	Benign	□	□
5	Benign	NE[b]	NI
6	Benign	□	□
7	Benign	□	NI
8	Benign	NE	□
9	Benign	NE	NI
10	Benign	□	NI
11	Benign	MSI[c]	NI
12	Malignant	■	■
13	Malignant	NI	NI
14	Malignant	NI	NI
15	Malignant	NI	■
16	Malignant	□	■
17	Malignant	□	□
18	Malignant	NI	MSI
19	Malignant	■	□
20	Malignant	□	□
21	Malignant	NI	□
22	Malignant (positive control)	NE	NE
23	Malignant (positive control)	NI	■
24	Malignant (positive control)	NI	■
25	Malignant (positive control)	NI	NI

Filled square loss of heterozygosity (LOH); *empty square* constitutional heterozygosity

NI[a]: not informative (constitutional homozygosity) NE[b]: not evaluated (PCR was unsuccessful)

MSI[c]: microsatellite instability

Therefore, this tumor was considered negative for LOH on 16q. On the other hand, case 15 (Fig. 2) was histologically diagnosed as low-grade DCIS or intraductal papillary carcinoma in the surgically resected specimen. Figure 2b, c shows a difference in the allele 2 peak heights between the normal and tumor DNA, and the ratio of allele 2 peak height to allele 1 peak height in the tumor DNA divided by the ratio in the normal DNA was 0.18. Therefore, this tumor was considered positive for LOH on 16q.

As shown in Table 3, 6 of the 11 (55%) informative malignant tumors showed LOH on 16q, whereas LOH was not detected in benign tumors. The incidence of 16q LOH in CNB specimens of intraductal papillary tumors was significantly different between benign and malignant tumors (P=0.007). Of three malignant tumors which were negative for LOH on 16q, two were histologically diagnosed as intraductal papillary carcinoma associated with papilloma in the surgically resected specimens.

Discussion

The aim of this study was to evaluate the use of LOH on chromosome 16q to make a final diagnosis in case of an indeterminate or suspicous intraductal papillary tumor in a CNB specimen. We found a statistically significant difference in the incidence of 16q LOH between of benign and malignant intraductal papillary tumors on CNB specimens. The results of the present study suggest that analysis of LOH on 16q may be helpful for making a definitive diagnosis of an indeterminate or suspicious papillary breast lesion in CNB and surgically resected specimens.

In our previous studies, we examined LOH on 16q in intracystic papillary tumors by Southern blot analysis using frozen tissue samples [3, 5] and determined that the incidence of LOH on 16q is strikingly different between cases of DCIS and papilloma [1, 7]. In the present study, we performed PCR-based LOH analysis using DNA isolated from formalin-fixed, paraffin-embedded samples from CNB specimens of intraductal papillary tumors. Although we used a different technique and different type of samples than in previous studies, we show that the incidence of 16q LOH is significantly different between CNB specimens of benign and malignant intraductal papillary tumors.

In the present study, LOH was detected at either16q12.2 or 16q21 in 6 of 11 malignant tumors (55%), whereas LOH was not detected in histologically benign tumors. Similarly, our previous data on intracystic papillary breast tumors showed that 12 of 17 intracystic papillary adenocarcinomas (71%) had LOH on 16q, whereas none of 11 intraductal papillomas had this genetic alteration [1]. Di Cristofano et al. [5] documented LOH at locus 16q23.1–16q24.1 in 7 of 11 malignant samples (63.6%), whereas none of the four informative benign samples appeared to be altered. Taken together, LOH on 16q has high specificity and positive predictive value for the diagnosis of malignancy in intraductal papillary tumors of the breast.

None of the benign papillary lesions we examined in any of our studies, including the eight papillomas in the present study, revealed LOH on 16q. In contrast, Di Cristofano et al. [5] found LOH on 16q in benign papillary lesions, with LOH at locus 16q21.1–16q22.2 detected in both malignant and benign lesions, and at 16q23.3–16q24.1 detected only in malignant lesions. Based on these results, the authors concluded that these differences might be due to the use of the novel molecular marker D16S310 which targets 16q21.1–16q22.2, which putatively contains a tumor suppressor gene involved in the genesis/progression of breast carcinomas.

We propose that the differences between results can be explained by the cellular heterogeneity of the intraductal papillary lesions. Atypical proliferative breast lesions are thought to be precursors of breast carcinomas and have frequently been shown to have LOH on 16q [18, 19].

Table 3 Incidence of loss of heterozygosity (LOH) on 16q in core needle biopsy (CNB) specimens of papillary breast lesions

Final histological diagnosis	Number of cases (%)					
	Chromosome 16q				Total	P-value
	LOH		Constitutional heterozygosity		(Informative)	
Benign	0	(0)	8	(100)	8	0.007
Malignant	4	(57)	3	(43)	7	
Malignant (positive control)	2	(100)	0	(0)	2	

Atypical proliferative lesions and carcinomas are considered to be clones and probably originated from a field within these clones [19]. "Atypical papilloma" or "papilloma with atypia" is defined as papilloma with a proliferation of epithelial cells that have cytological and architectural features consistent with atypical ductal hyperplasia (ADH). Page et al. [15] further refined these terms and used atypical papilloma when the ADH focus involved 3 mm or less of the papillary lesion and the term minor DCIS lesion when the atypical focus involved more than 3 mm of the papillary lesion. These definitions were applied to the surgically resected specimens in the present study. In contrast, Tavassoli [20] suggested using the term atypical papilloma if the area of ADH occupies less than 33% of the papillary lesion, and the term carcinoma arising in a papilloma when the area of ADH occupies 33–90% of the papillary lesion. The ratio of atypical epithelial cells to total epithelial cells may have influenced the LOH analysis results.

Papillary lesions in CNB specimens are diagnosed as benign, atypical (indeterminate), suspicious for malignancy, or definitely malignant based on their pathologic features. Papillary lesions which are histologically diagnosed as definitely malignant must be treated as breast carcinomas. Papillary lesions with atypia, i.e., lesions that are histologically diagnosed as indeterminate or suspicious for malignancy in CNB specimens, need to be resected to determine if there is a more significant lesion [21]. Based on the results of our study, we propose that papillary lesions in CNB specimens that are histologically diagnosed as indeterminate or suspicious for malignancy and show LOH on 16q should also be treated as carcinoma. However, absence of LOH on 16q occurred in both papillomas and papillary carcinomas, and the predictive value of absence of LOH for a benign lesion was only 73%. In lesions in CNB judged as indeterminate or suspicious for malignancy, absence of LOH on 16q therefore has no diagnostic significance.

It is still controversial whether lesions diagnosed as papilloma without atypia by CNB need to be resected. From a pathological review of 19 papillary lesions with postoperative conversion from nonmalignant to malignant, Cheng et al. [22] concluded that the causes of diagnostic conversion were borderline atypical lesions (47%), sampling problems (32%), interpretation errors (16%), and an inadequate sample (5%). Based on the results of the present study, we cannot give clear guidelines for the management of papillomas without atypia based on LOH on 16q, but we consider that analysis of LOH on 16q in CNB specimens with an adequate amount of tumor tissue could reduce interpretation errors and be helpful in determining whether a papilloma without atypia needs to be resected.

The following limitations of the present study are worth discussing. First, results of analysis of LOH on 16q are not sufficiently sensitive for detection of malignancy. Absence of LOH cannot guarantee a benign lesion. Second, the number of cases examined in the present study is small. Third, we did not consider the possibility of intratumor heterogeneity, e.g., cases of carcinoma arising within papilloma. To our knowledge, this is nevertheless the first report which confirms that the incidence of LOH on 16q is significantly different between CNB specimens of benign and malignant intraductal papillary tumors. In conclusion, analysis of LOH on 16q may be helpful in making a definitive diagnosis in cases of papillary breast lesions, in both excised and CNB specimens.

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Conflict of interest The authors declare that they have no conflicts of interest.

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