厚生労働科学研究費補助金（がん臨床研究事業）
分担研究報告書

70遺伝子シグネチャ（MammaPrint®）の経済評価

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研究要旨　ホルモンレセプター陽性早期乳がん患者において、再発リスクの評価と化学療法の効果予測に基づいて、術後化学療法の適応を示し、効果の見込めない化学療法を回避することによって、毒性軽減につながる多遺伝子検査（multigene assay）のなかで、国際的に実用化され、日本での臨床的検証研究も報告されている70遺伝子シグネチャ（MammaPrint®）の日本の保健システム下での経済評価を行った。推定された増分費用効果比は、N－，Her2－で¥3,873,922/QALYであったが、感度分析では必ずしも頑健な結果とはいえなかった。したがって費用対効果に優れる可能性があり、公的医療制度でのこの検査の提供が社会的に受け入れられる可能性もあることが示唆されたといえる。

A．研究目的

ホルモンレセプター陽性早期乳がん患者において、再発リスクの評価と化学療法の効果予測に基づいて、術後化学療法の適応を示し、効果の見込めない化学療法を回避することによって、毒性軽減につながる多遺伝子検査（multigene assay）のなかで、国際的に実用化されている70遺伝子シグネチャ（MammaPrint®）の日本の保健システム下での経済評価を行った。

70遺伝子シグネチャは、米国食品医薬品局（FDA）で承認され、国際的な乳癌治療ガイドライン（St Gallen consensus 2009）に含められ、日本での臨床検証研究が報告され（Ishitobi M et al (2010) Jpn J Clin Oncol. 40(6):508-512.）、日本乳癌学会の科学的根拠に基づく乳癌診療ガイドラインにも取り上げられている治療方針決定のための遺伝子診断であるが、費用が高い（日本では38万円）ことが特徴のひとつである。ただし、たとえば、多遺伝子検査によらない従来の診断情報に基づいて、術後化学療法の適応とされた患者について、多遺伝子検査によって費用が高い（最大100万円程度）化学療法が回避される場合もあり、医療財源の効率的使用という観点からは、適切な経済評価が求められる。

B．研究方法

日本でのホルモンレセプター陽性早期乳がん患者での遺伝子シグネチャの経済評価としては、研究分担者らが、21遺伝子シグネチャに関して先行研究を報告してきている（Kondo M et al (2008) Breast Cancer Res Treat. 112(1):175-187.; Kondo M et al (2011) Breast Cancer Res Treat. 127(3):739-749.）。ただし、これらの研究では、21遺伝子シグネチャの適応としてN±，Her2±を検討してきている。70遺伝子シグネチャもN±，Her2±への適応が考えられるが、本研究では、日本への保険適応を考える上で中核的な患者集団となると考えられるN－，Her2－を対象として、21遺伝子シグネチャの経済評価結果と比較検討を可能とするため再発以降のモデルを共通化した。

（倫理面への配慮）

本研究は、文献による経済モデリングであり疫学研究の倫理指針や臨床研究の倫理指針にかかるものではない。ただし、経済評価の対象である医療技術は商業的に提供されているものである。そこでその商業的提供者に関して利益相反がないことを確認した。

C．研究結果

Table 1が主要な検証研究の結果による70遺伝子シグネチャによる治療方針の変更と予後である。日本での検証研究では同様な結果が報告されていないので、欧米の成績によっている。70遺伝子シグネチャを用いずにSt Gallenガイドラインに従った治療方針をとると約90%の患者が術後補助化学療法を受けるのに対して、70遺伝子シグネチャに従った治療方針をとると、術後補助化学療法を受ける患者が約46%になる。1～6年と6～10年の再発予後も70遺伝子シグネチャに従った治療方針をとることによって改善している。

Figureが判断樹とマルコフモデルからなる経済モデルである。またTable 2は、経済モデルに組み込んだ確率とクオリティ・オブ・ライフの調整のための効用値、さらに、費用である。

Table 3が経済評価の結果である。増分費用効果比は、¥3,873,922/QALYであった。

Table 4は感度分析の結果である。増分費用効果比は治療方針の確率やそれに伴う予後などに大きく依存し、全体としては必ずしも頑健であるとはいえなかった。

直接的な比較の解釈は難しいが、21遺伝子シグネチャで同じ患者集団を対象とした場合の増分費用効果比¥434,096/QALYと比較すると70遺伝子シグネチャの方の値が大きかった。

D．考察

医療の技術革新を公的医療制度のもとで普及させる際の医療経済的効率性の基準としては、おおよそ、1QALYを獲得するために500万円程度までは、社会

は支払う意思があるとされている。この基準と比較すると、Table 3に示された結果は、費用対効果に優れ、公的医療財源でその費用をまかなうことが正当化できるということになる。しかし、感度分析では結果の不確実性も示された。

E．結論

国際的に実用化されている70遺伝子シグネチャ（MammaPrint®）の日本での臨床導入は、経済評価によって費用対効果に優れる可能性が示された。この結果は、医療の経済評価の枠組みからは、公的医療制度でのこの検査の提供が社会的に受け入れられる可能性があることが示唆されるものである。

G．研究発表

1．論文発表

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2．学会発表

なし。

H．知的財産権の出願・登録状況

1．特許取得

なし。

2．実用新案登録

なし。

3．その他

なし。

厚生労働科学研究費補助金
分担研究報告書

術前化学療法における治療効果と予後に関するCD24とCD44の意義に関する研究

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研究要旨
ACOSOG Z0011（Z11）試験において，臨床的に腋窩リンパ節転移を認めない乳房温存術を行うT1-2症例で，術後乳房照射が行われる場合，センチネルリンパ節（SLN）転移が1-2個では腋窩リンパ節郭清（ALND）の追加は生存に関与しないと報告された．一方で，腋窩リンパ節転移4個以上の症例は予後不良で，化学療法と術後鎖骨上下窩へのリンパ節領域への術後照射が勧められ，補助療法により局所再発のみならず，生存率を有意に改善させることが示されている．このため，4個以上のリンパ節転移の存在を把握することは必要と考えるが，SLN摘出個数が4個を越えることはほとんどない．Z11の結果との比較を含め，SLN転移が1-2個の症例に対して腋窩郭清の省略が可能か，当院におけるSLN転移陽性例で4個以上の腋窩リンパ節転移が存在した症例について後方視的に検討した．

A.　研究の目的

ACOSOG Z0011（Z11）試験において，臨床的に腋窩リンパ節転移を認めない乳房温存術を行うT1-2症例で，術後乳房照射が行われる場合，センチネルリンパ節（SLN）転移が1-2個では腋窩リンパ節郭清（ALND）の追加は生存に関与しないと報告された．一方で，腋窩リンパ節転移4個以上の症例は予後不良で，化学療法と術後鎖骨上下窩へのリンパ節領域への術後照射が勧められ，補助療法により局所再発のみならず，生存率を有意に改善させることが示されている．このため，4個以上のリンパ節転移の存在を把握することは必要と考えるが，SLN摘出個数が4個を越えることはほとんどない．Z11の結果との比較を含め，SLN転移が1-2個の症例に対して腋窩郭清の省略が可能か，当院におけるSLN転移陽性例で4個以上の腋窩リンパ節転移が存在した症例について後方視的に検討した．

B.　研究方法

2001年1月～2011年3月まで，当院でSLNBを施行した1425例で検討を行った．

SLNBの適応は 触診，超音波，CTで明らかな腋窩リンパ節転移を認めない（cN0）浸潤癌，およびC領域にかかる浸潤部の存在を疑う非浸潤性乳管癌（DCIS），乳腺全摘を必要とするDCISとした。SLN同定困難（23例），化学療法後（12例）の症例は除外した。

（倫理面への配慮）
今回検討を行った患者情報は集積解析され，個人名と直接結びつくことはない．

C.　研究結果

全SLNB1425例中SLN転移は394例（27.6%）で認められ，356例（90.0%）で追加の腋窩郭清が施行され，59例（16.6%）で総リンパ節転移個数が4個以上であった．

SLN転移が1個のみであった257例中，総リンパ節転移個数が4個以上の症例は21例（18%）で認められた．SLN転移個数が2個以上は99例あり，このうち総リンパ節転移個数が4個以上は38例（38%）であった．総リンパ節転移個数が4個以上存在する予測因子として，単変量解析では，腫瘍径2cm以上（p=0.05），脈管侵襲陽性（p=0.04），SLN転移個数2個以上（p=＜0.0001）が挙げられた．多変量解析ではSLN転移個数2個以上（p=＜0.001）が抽出された．

乳房温存術を行うT1-2症例でSLN転移が1-2個，術後乳房照射が行われる症例として検討を行ったZ11試験と同条件症例には164例が該当した．これらに限った場合でも，ALNDの追加により13%でリンパ節転移が4個以上存在した．

観察期間中央値4.5年で，全SLNB症例中19例で遠隔再発を認めた．リンパ節転移1-3個と比較してリンパ節転移4個以上では有意に遠隔再発率が高かった（log-rank　p=0.006）が，乳癌死では差が認められなかった（log-rank　p=0.22）．

D.　考察

リンパ節転移が1-3個以下のホルモン陽性（luminal A）例では化学療法追加の閾値に当てはまないことがあるため（St.Gallen 2009），1-2個のSLN転移陽性例でALNDで省略すると4個以上のリンパ節転移の存在が把握できなくなり，補助療法がおこなわれない可能性がある。

また現時点でリンパ節転移個数による全生存率に

差はないが，再発後の生存期間は一般にホルモン陽性例で長いため，長期的に見れば差が出てくる可能性がある。

E.　結論

リンパ節転移個数は補助療法の決定に必要な予後因子であり，特にHR陽性例においてリンパ節転移4個以上の存在を把握することは必要である．転移個数の把握のため，腋窩郭清の省略に関してはまだ検討の余地があると考えられた。

F.　健康危険情報

なし

G.　研究発表

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H.　知的財産権の出願・登録状況（予定を含む。）

なし

研究成果の刊行に関する一覧表

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Original article

Relationship between body mass index and preoperative treatment response to aromatase inhibitor exemestane in postmenopausal patients with primary breast cancer

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ABSTRACT

Background: Some studies have shown that high body mass index (BMI) is associated with inferior outcome after adjuvant therapy with anastrozole in breast cancer patients. We aimed to investigate predictive effect of BMI on clinical response to neoadjuvant therapy with exemestane in postmenopausal patients with primary breast cancer.

Patients and methods: The study group consisted of 109 patients from the JFMC 34-0601 neoadjuvant endocrine therapy trial. Patients were categorized into three groups according to BMI: low (BMI < 22 kg/m^2), intermediate ($22 \leq$ BMI < 25 kg/m^2) and high (BMI ≥ 25 kg/m^2). Statistical analyses were performed to explore the predictive effect of BMI on clinical response.

Results: Higher BMI correlated with positive progesterone receptor status ($p < 0.01$) and low Ki-67 index ($p = 0.03$). Objective response rates (ORR) were 21.7% in low BMI, 56.0% in intermediate BMI and 60.6% in high BMI, respectively ($p = 0.01$). In a multivariate analysis, low BMI was an independent negative predictor of clinical response.

Conclusion: Low BMI was associated with a decreased ORR to neoadjuvant endocrine therapy with exemestane. Our results may suggest that the predictive effect of BMI varies according to the type of aromatase inhibitor and objective outcome.

Introduction

Aromatase inhibitors have been widely used in postmenopausal patients with hormone receptor-positive breast cancer. Patients treated with third-generation aromatase inhibitors showed lower recurrence rates compared to those treated with tamoxifen in an adjuvant setting.[1–3]

Obesity, which is measured using body mass index (BMI), is a prognostic indicator in patients with primary breast cancer.[4–6] It is assumed that obesity affects the circulating plasma levels of estrogen, insulin, insulin-like growth factor or other hormonal factors, which may lead to tumor growth.[7] In postmenopausal women, the conversion of androgens to estrogens

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in adipose tissue is regarded as a major source of estrogen supply.

The prognostic effect of BMI has also been shown in patients treated with hormonal therapy.[8–10] Several studies have suggested that the efficacy of hormonal therapy on overall and disease-free survival varies according to BMI. In the ATAC trial, anastrozole, a non-steroidal aromatase inhibitor, was significantly less effective in postmenopausal breast cancer patients with high BMI, whereas an equal efficacy of tamoxifen was shown across all BMI levels.[10] However, the relationship between BMI and clinical treatment response to hormonal therapy has not been described. Therefore, we investigated whether BMI is also predictive of treatment response to exemestane, a steroidal aromatase inhibitor, in postmenopausal patients with primary breast cancer who participated in the clinical trial of neoadjuvant hormonal therapy (Neoadjuvant exemestane for 24 weeks in postmenopausal women with hormone receptor-positive Stage II or IIIA breast cancer (JFMC34-0601, UMIN Clinical Trial ID; C000000345).[11]

Patients and methods

Patients and treatment

Postmenopausal women aged 55–75 years with operable, Stage II or IIIA estrogen receptor (ER)-positive primary breast cancers were prospectively included in this clinical trial JFMC 34-0601. Details of the study design, methods, primary objectives and major outcomes have been described previously.[11] This study was performed in accordance with the Declaration of Helsinki and the Ethical Guidelines for Clinical Research of the Ministry of Health, Labour and Welfare of Japan. Approval was obtained from the institutional review board at each study centre. Written informed consent was obtained from all patients before enrollment.

Patients were initially treated with 25 mg exemestane once daily, orally, for 16 weeks and then clinical response was assessed. Patients with progressive disease were withdrawn from the study and the remainder continued the treatment for additional 8 weeks. After 24 weeks of treatment, clinical response was evaluated again and surgery was performed.

Evaluation

Clinical response was evaluated using ultrasonography. We employed two kinds of response evaluation criteria. One was the Response Evaluation Criteria in Solid Tumors (RECIST), wherein target lesions are measured by uni-dimensional measurements (the maximum axial diameter) and the other was the World Health Organization (WHO) criteria wherein target lesions are measured by bi-dimensional measurements (cross-sectional longest diameters).[12,13] Tumor reduction rate from the baseline measurement was subsequently calculated and response was evaluated based on each criteria.

Pathological response was categorized using the modified criteria described previously by Miller et al.[14] and assessed as follows: a complete response, when there was no evidence of malignant cells at the original tumor site; partial response, when histological decrease in cellularity and/or an increase in fibrosis was detected; and non-response, where there was no change.

The status of the ER, progesterone receptor (PgR), human epidermal growth factor receptor 2 (HER2) and Ki-67 were evaluated by immunohistochemical (IHC) analyses, as described before.[11] Patients were defined as positive for ER or PgR when 10% or more nuclear staining was observed. Positive HER2 status was defined as either 3 + or 2 + by IHC with confirmed c-erbB2 gene amplification by the fluorescent *in situ* hybridization test. In this study, the median baseline value of the Ki-67 index was 11%. Therefore, Ki-67 was categorized as positive if the index was 11% or more.

BMI was defined as weight in kilograms divided by the square of the height in meters (kg/m^2).

Statistical methods

First, we evaluated the relationship between baseline clinicopathological characteristics and BMI using the chi-square or Kruskal–Wallis test. Second, we analyzed the correlation between pathological response and tumor reduction rate measured by uni- and bi-dimensional criteria using the Wilcoxon rank-sum test. Finally, we performed univariate and multivariate analyses to evaluate the predictive effect of BMI on clinical response. Clinical response was divided into two categories based on the tumor reduction rate. Objective response for the WHO criteria was defined as patients whose tumors showed 50% or more reduction by the bi-dimensional measurements. Objective response in the RECIST criteria was defined as a 30% or more reduction in the uni-dimensional measurements. The chi-square test was used for the univariate analysis. Multivariate analysis was performed using a multivariate logistic regression model. Variables included in the multivariate analysis were BMI, age, clinical nodal status, PgR status and the Ki-67 index. Tumor size and HER2 status were excluded from the analysis because most patients were clinical T2-stage and HER2 negative (Table 1). Patients with missing values were excluded from the analysis. Analyses were performed using JMP® for Windows (release 8.0.2.2: SAS Institute Inc, Cary, NC, USA).

Results

BMI and patient characteristics

A total of 116 patients were enrolled in this study. Of these, 109 patients, whose baseline BMI was available were included in this analysis. Fig. 1 shows the distribution of patients according to BMI. The BMI ranged from 17.4 to 38.8 kg/m^2 (median, 23.7 kg/m^2). BMI was categorized as low (BMI < 22 kg/m^2), intermediate ($22 \leq$ BMI < 25 kg/m^2) and high (BMI ≥ 25 kg/m^2). We employed cut-off points, as they have some clinical meaning (WHO cut-off points or standard body mass in Japan) and thus we could divide the patients into three groups of approximately the same size.

Table 1 shows the baseline patient and tumor characteristics according to the BMI groups ($n = 109$). All patients were ER positive and most patients were clinical T2-stage and HER2 negative. The BMI groups correlated significantly with PgR status and Ki-67 index ($p < 0.01$ and $p = 0.03$, respectively).

Clinical response, pathological response and BMI

Next, we analyzed the correlation between pathological response and tumor reduction rate measured by uni- and bi-dimensional criteria. All of the pathological responders had a partial response, whereas the remainder were non-responders. Information about pathological response and/or clinical response at 24 weeks was not available in 29 patients. Therefore, 24 pathological responders and 56 non-responders were included in the analysis. Pathological responders showed a significantly higher reduction rate based on the bi-dimensional measurements ($p = 0.04$). In contrast, no significant difference in reduction rate was observed based on the uni-dimensional measurements and pathological response (Fig. 2).

Fig. 3 shows the objective response rate (ORR) based on the WHO criteria at 24 weeks according to the BMI groups. The ORR in the low BMI group decreased significantly compared with that of

Table 1
Baseline patient and tumor characteristics by Body Mass Index (BMI) category.

Characteristic	Status	Total		Body Mass Index (BMI)[c]						p-value*
				Low		Intermediate		High		
		No.	%	No.	%	No.	%	No.	%	
Number	–	109	100	33	30.3	34	31.2	42	38.5	–
Age	Median (min–max)	64	(55–79)	63	(55–76)	67	(56–79)	66	(55–77)	0.12
T	2	104	95.4	31	93.9	32	94.1	41	97.6	0.68
	3	5	4.6	2	6.1	2	5.9	1	2.4	–
N	0	81	74.3	21	63.6	26	76.5	34	81	0.22
	1–2	28	25.7	12	36.4	8	23.5	8	19	–
Clinical stage	IIA	80	73.4	20	60.6	26	76.5	34	81	0.24
	IIB	25	22.9	12	36.4	6	17.6	7	16.7	–
	IIIA	4	3.7	1	3	2	5.9	1	2.4	–
ER[a]	Positive	109	100	–	–	–	–	–	–	–
PgR[a]	Positive	73	67	15	45.5	23	67.6	35	83.3	<0.01
	Negative	36	33	18	54.5	11	32.4	7	16.7	–
HER2	Positive	3	2.8	1	3	2	5.9	0	0	0.31
	Negative	95	87.2	30	90.9	29	85.3	36	85.7	–
	Unknown	11	10.1	2	6.1	3	8.8	6	14.3	–
Ki-67 index[b]	Positive	49	45	18	54.5	18	52.9	13	31	0.03
	Negative	47	43.1	10	30.3	12	35.3	25	59.5	–
	Unknown	13	11.9	5	15.2	4	11.8	4	9.5	–

Abbreviations: ER, estrogen receptor; PgR, progesterone receptor; HER2, human epidermal receptor type-2.
*Chi-square test or Kruskal–Wallis test.
[a] ER, PgR positive was defined as 10% and more stained cells by immunohistochemistry.
[b] Ki-67 positive was defined as 11% or more stained cells by immunohistochemistry.
[c] BMI was categorized as following: Low (BMI<22), Intermediate (22 ≤ BMI<25), High (BMI≥25).

the intermediate and high BMI groups (21.7% vs. 56.0% vs. 60.6%, $p = 0.01$). The same tendency was observed in a response evaluation at 16 weeks ($p = 0.11$). However, no correlation was found between BMI groups and ORR based on the RECIST criteria (data not shown). Univariate and multivariate analysis showed that the intermediate and high BMI groups were independent predictors for objective response based on the WHO criteria (Table 2).

Discussion

We explored the predictive efficacy of BMI on clinical response to neoadjuvant hormonal therapy in postmenopausal patients with primary breast cancer. The ORR decreased significantly in the low BMI group compared with the intermediate and high BMI groups. A multivariate analysis revealed that low BMI was an independent negative predictor of clinical response based on the WHO criteria.

Although we hypothesized that high BMI might be also a negative predictor of neoadjuvant hormonal therapy, our results suggest that low BMI is a negative predictive factor in patients treated preoperatively with exemestane. One possible explanation for this discordance is that the distribution of BMI in our patients was different from previous studies. In the ATAC trial, the proportion of obese patients was about 27% (mean BMI, 27.4 kg/m^2), whereas it was only 8% in our study (mean BMI, 24.3 kg/m^2). The negative

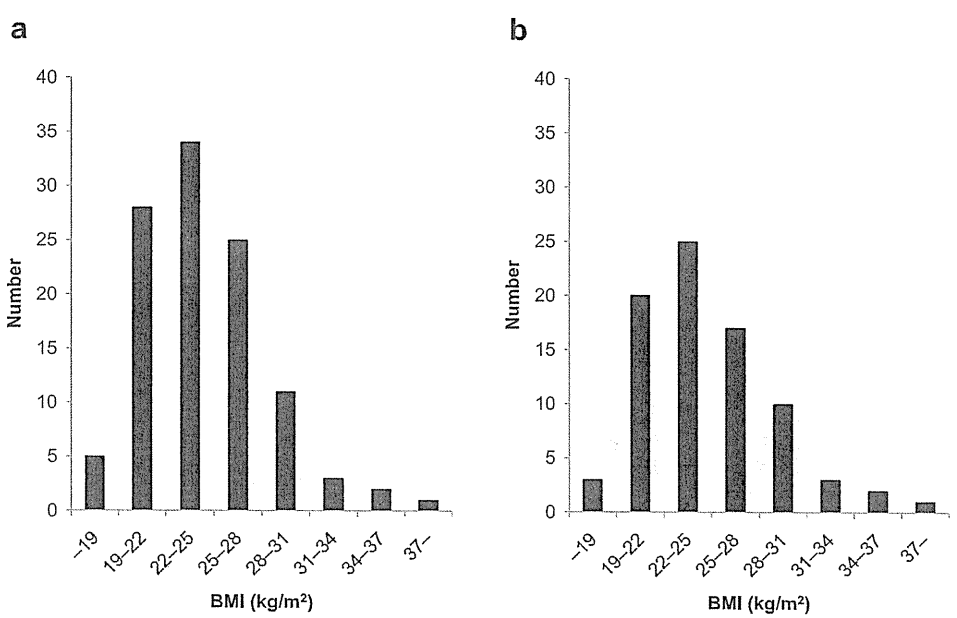

Fig. 1. Distribution of body mass index (BMI). This figure shows the distribution of patients according to baseline BMI. a) Whole patients with information of BMI ($n = 109$). BMI ranged from 17.4 to 38.8 kg/m^2 (median, 23.7 kg/m^2). b) Patients included in the multivariate analysis ($n = 81$). BMI ranged from 17.4 to 38.8 kg/m^2 (median, 23.9 kg/m^2). The distribution of BMI between the two dataset was not significantly different ($p = 0.58$, Wilcoxon rank-sum test).

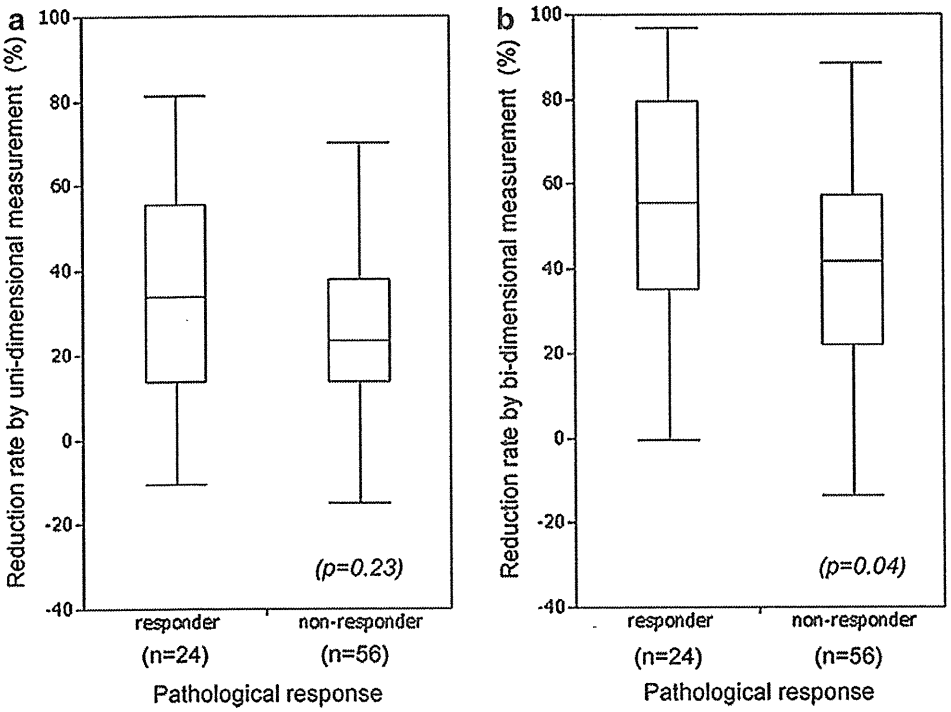

Fig. 2. Correlation between pathological response and tumor reduction rate (n = 80). a) No significant correlation was found in tumor reduction rate by uni-dimensional measurements at 24 weeks based on the pathological response. b) Tumor reduction rate using bi-dimensional measurements. Pathological responders showed a significantly larger reduction rated by bi-dimensional measurements compared to pathological non-responders (p = 0.04, Wilcoxon rank-sum test).

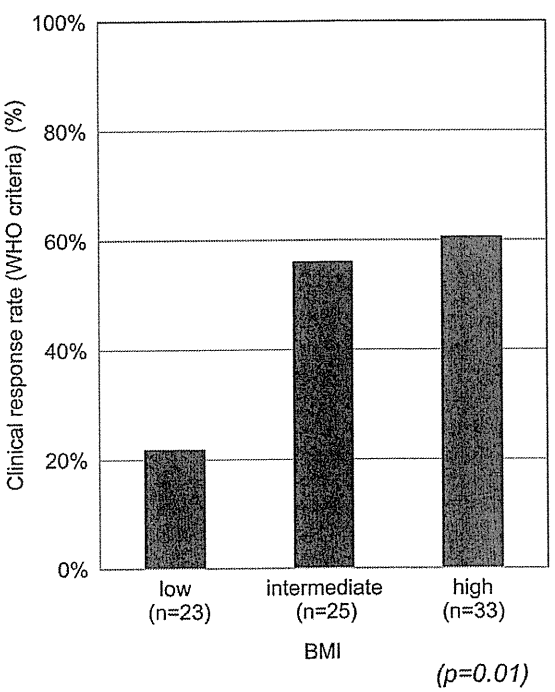

Fig. 3. Correlation between body mass index (BMI) and clinical response (WHO criteria) (n = 81). This graph shows the objective response rate (ORR: CR + PR in WHO criteria) according to BMI group. The ORR in the low BMI group was 21.7%, 56.0% in the intermediate BMI group and 60.6% in the high BMI group (p = 0.01, chi-square test).

effect of BMI on the efficacy of an aromatase inhibitor among obese patients might not be detected in our study because of the small proportion; however, the ORR in obese patients was relatively high (77.8%) in this study.

In the current study, the cases were divided into three groups using BMI cut-off points of 22 and 25 kg/m^2, which were lower than the WHO cut-off point for obesity (30 kg/m^2). WHO expert consultation reviewed BMI in Asian population in 2002 and they concluded that the proportion of Asian people with a high percentage of body fat and a high risk of type 2 diabetes and cardiovascular disease is substantial at BMI levels lower than the WHO cut-off point for overweight (≥25 kg/m^2).[15] A multicenter epidemiologic study for BMI in Japan showed that people with BMI 24 kg/m^2 or more have a high risk of hypertension, hypercholesterolemia, and hyperglycemia compared to those with BMI between 20 and 23.9 kg/m^2. Therefore, BMI 25 kg/m^2 is defined as a cut-off point for obesity in Japan.

Several studies have reported that the levels of endogenous sex hormones in postmenopausal women are associated with BMI.[16–18] The conversion of androstenedione to estrone or estradiol by aromatase in adipose tissue is a major source of estrogen in postmenopausal women. Thus, the standard dose of anastrozole might be insufficient to suppress aromatization in patients with a high BMI.[10] Inhibition of aromatase by a non-steroidal aromatase inhibitor is reversible, whereas that by a steroidal aromatase inhibitor is irreversible.[19] It is presumed that sufficient inhibition of aromatization was achieved in most patients in our study because of the difference in the mechanism of action of aromatase inhibitors. In the TEAM trial, the efficacy of exemestane was the same as that of tamoxifen in overweight patients (data were presented at the San Antonio Breast Cancer Symposium in 2010, #S2-3). It is understandable that the aromatase inhibitor might be less effective in patients with lower BMI because of their lower baseline estrogen

Table 2
Multivariate logistic regression model of clinical factors and odds of clinical response ($n = 81$).

Factor		Univariate				Multivariate			
		OR[a]	95% CI		p-value	OR[a]	95% CI		p-value
Age	<65	Ref							
	≥65	1.05	0.44	2.53	0.91	0.97	0.35	2.63	0.94
BMI	Low	Ref							
	Intermediate	4.58	1.35	17.60	0.01	6.79	1.74	33.00	0.005
	High	5.54	1.74	20.27	0.003	9.14	2.35	45.23	0.001
T[b]	2	Ref							
	3	4034.13	0.00	0.00	0.22	–			
N	0	Ref							
	1–2	0.41	0.12	1.28	0.13	0.35	0.08	1.27	0.11
PgR	Negative	Ref							
	Positive	0.80	0.29	2.16	0.65	0.29	0.07	1.02	0.05
HER2[b]	Negative	Ref							
	Positive	11127.18	0.00	0.00	0.09	–			
Ki-67 index	<11%	Ref							
	≥11%	0.85	0.35	2.04	0.72	1.13	0.42	3.13	0.81

Abbreviations: OR, odds ratio; Ref, reference; BMI, body mass index; PgR, progesterone receptor; HER2, human epidermal receptor type-2.
[a] Odds ratio of overall response (PR vs. SD + PD) by WHO criteria.
[b] T and HER2 were excluded from the multivariate analysis because most patients were clinical T2-stage and HER2 negative.

or aromatization levels. However, we must investigate aromatization or estrogen levels during treatment to evaluate this speculation. Unfortunately, these data were not available in the current study. The relationship between clinical response and long-term survival in patients treated with neoadjuvant hormonal therapy has not been described. We could not evaluate the prognostic effect of BMI, as survival data were not available in this study. Therefore, our results do not indicate the association between low BMI and poor prognosis. However, Moon HG et al. reported that underweight patients showed inferior outcome compared to normal weight patients.[20] Conroy SM et al. also showed higher mortality in both obese and low BMI patients compared to patients with moderate BMI.[21] Further study to investigate the prognostic effect of low BMI in patients treated with hormonal therapy is warranted.

Clinical response is usually evaluated using RECIST criteria wherein target lesions are measured by uni-dimensional measurements. It is indicated that breast cancers express various morphological patterns of tumor reduction during preoperative systemic therapy. Especially, in case of dendritic or diffuse reduction pattern, response evaluation by uni-dimensional measurement could overestimate residual tumor burden. Therefore, we used both uni- and bi-dimensional measurements to evaluate the clinical response.[12,13] In the multivariate analysis, low BMI was an independent negative predictor of clinical response evaluated by bi-dimensional measurements. The same tendency was observed in the analysis of the relationship between BMI and clinical response evaluated by uni-dimensional measurements. However, we could not show a significant relationship in the multivariate analysis (data not shown). Our results also showed that tumor reduction rate, calculated using bi-dimensional measurements, increased significantly in pathological responders. Recently, Loo et al. showed that change in the largest tumor diameter between baseline MRI and MRI during neoadjuvant chemotherapy does not correlate with pathological response in ER positive/HER2 negative subgroup.[22] Several reports have shown that the RECIST criteria, which used uni-dimensional measurements, was comparable to WHO criteria, which used bi-dimensional measurements.[23,24] Although moderate agreement between the RECIST and WHO criteria was observed in the current study (kappa statistic = 0.51), 20 of 81 cases (24.7%) showed discordance between the RECIST and WHO criteria. This discordance may have resulted in the difference in statistical results. In the multivariate analysis, we excluded 7 patients who were withdrawn from this trial because of disease progression during treatment and who could not have pathological evaluation. If we include these patients into the analysis ($n = 88$), the results will not change. The ORR in the low BMI group decreased significantly compared with that of the intermediate and high BMI groups (20% vs. 50% vs. 57.1%, $p = 0.01$). Multivariate analysis also showed that the low BMI group was an independent negative predictor for objective response.

This study had several limitations. The number of patients was relatively small compared to previous reports. All patients included in the analysis were postmenopausal and most of them were clinical T2-stage, strongly ER positive and HER2 negative. Therefore, our results may be restricted to this subpopulation. 28 cases out of 109 cases were excluded from the multivariate analysis because of missing values in clinical response by ultrasonography ($n = 17$) or Ki-67 index ($n = 11$). However, the distribution of BMI in patients included in the multivariate analysis ($n = 81$, Fig. 1b) was not significantly different from that in the whole patients ($n = 109$, Fig. 1a) ($p = 0.58$, Wilcoxon rank-sum test), nor that in the excluded patients ($n = 28$) ($p = 0.15$). Information about the histological tumor grades was not available, so we could not evaluate confounding effects. The proportion of Ki-67 positive cases in both low BMI group (54.5%) and intermediate BMI group (52.9%) were significantly high compared with that in high BMI group (31%) ($p = 0.03$) (Table 1). Several studies have shown good correlation between tumor grade and Ki-67 index,[25–27] and it was assumed that both low and intermediate BMI group includes more cases with high grade tumor. However, the only low BMI was selected as an independent negative predictive factor in the multivariate analysis. These results may support the independent effect of low BMI as a negative predictive factor of objective response. However, our results require validation study using independent data set which includes information about tumor grade. We also evaluated the predictive effect of BMI on other outcomes, such as pathological response, Ki-67 index after therapy and the reduction rate in the Ki-67 index during therapy. Ki-67 index decreased in most cases and the reduction values of Ki-67 index during the treatment were not statistically different across the three BMI groups ($p = 0.34$). No significant correlation was observed between BMI and other outcomes.

Conclusion

Our results indicate that the steroidal aromatase inhibitor exemestane was less effective in patients with lower BMI than those with intermediate and higher BMI. This is the first report to

show the predictive effect of BMI on clinical response to neoadjuvant hormonal therapy using exemestane. We may have to consider differences in the mechanism of action of aromatase inhibitors when investigating the predictive effect of BMI on treatment response to neoadjuvant hormonal therapy. Our results also indicate that the predictive effect of BMI varies according to objective outcome. However, our results require independent confirmation.

Conflict of interest statement

Dr. Hironobu Sasano has received the educational grant from Pfizer Oncology Japan.

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Trastuzumab Produces Therapeutic Actions by Upregulating miR-26a and miR-30b in Breast Cancer Cells

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Abstract

Objective: Trastuzumab has been used for the treatment of HER2-positive breast cancer (BC). However, a subset of BC patients exhibited resistance to trastuzumab therapy. Thus, clarifying the molecular mechanism of trastuzumab treatment will be beneficial to improve the treatment of HER2-positive BC patients. In this study, we identified trastuzumab-responsive microRNAs that are involved in the therapeutic effects of trastuzumab.

Methods and Results: RNA samples were obtained from HER2-positive (SKBR3 and BT474) and HER2-negetive (MCF7 and MDA-MB-231) cells with and without trastuzumab treatment for 6 days. Next, we conducted a microRNA profiling analysis using these samples to screen those microRNAs that were up- or down-regulated only in HER2-positive cells. This analysis identified miR-26a and miR-30b as trastuzumab-inducible microRNAs. Transfecting miR-26a and miR-30b induced cell growth suppression in the BC cells by 40% and 32%, respectively. A cell cycle analysis showed that these microRNAs induced G1 arrest in HER2-positive BC cells as trastuzumab did. An Annexin-V assay revealed that miR-26a but not miR-30b induced apoptosis in HER2-positive BC cells. Using the prediction algorithms for microRNA targets, we identified *cyclin E2* (*CCNE2*) as a target gene of miR-30b. A luciferase-based reporter assay demonstrated that miR-30b post-transcriptionally reduced 27% ($p = 0.005$) of the gene expression by interacting with two binding sites in the 3′-UTR of *CCNE2*.

Conclusion: In BC cells, trastuzumab modulated the expression of a subset of microRNAs, including miR-26a and miR-30b. The upregulation of miR-30b by trastuzumab may play a biological role in trastuzumab-induced cell growth inhibition by targeting *CCNE2*.

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Introduction

The overexpression of HER2 has been reported in 20% to 30% of patients with breast cancer. The overall survival and time to relapse for patients whose tumors overexpressed HER2 were significantly shorter [1,2]. The malignant phenotypes are also enhanced with HER2 overexpression. HER2-overexpressing tumors are also more likely to be resistant to treatment with tamoxifen and standard chemotherapy [3–5].

Trastuzumab (Herceptin) was designed to target the extracellular domain of HER2 and block its function, and is currently used in patients with HER2-positive breast and gastric cancers. The application of trastuzumab in the adjuvant and metastatic setting has been shown to prolong the survival of patients with HER2-positive breast cancer [6,7]. The overall response rate was approximately 26–31% for trastuzumab monotherapy [8,9], and 50–61% for trastuzumab-chemo combined regimens [6,7]. Moreover, most patients with an initial response developed resistance to trastuzumab within one year [10]. Therefore, clarifying the molecular mechanisms of trastuzumab treatment will be beneficial to improve the treatment of HER2-positive breast cancer. For example, more fundamental knowledge about the mechanisms responsible for trastuzumab treatment would helpful in developing a monogram for tailoring trastuzumab treatment, and a novel agent for modulating the trastuzumab sensitivity of breast cancer cells.

According to accumulating reports, trastuzumab is thought to induce its therapeutic effects basically via two biological mechanisms: a direct effect by a blockade of the HER2 signal, and an induction of antibody-dependent cell-mediated cytotoxicity (ADCC). In terms of the direct therapeutic effects, trastuzumab binds to the extracellular domain of the HER2 molecule, and represses the signal transduction from the HER2 molecule by inhibiting the homo/hetero dimerization of HER2 and HER family members. Moreover, trastuzumab reduces the amount of HER2 on the breast cancer cell surface by promoting the internalization and cleavage of HER2 molecules. Therefore, trastuzumab blocks the downstream signal pathways from HER2

positive BC cells, including PI3K/Akt, MAPK, and mTOR pathways. However, little is known regarding the biological role of microRNAs in the trastuzumab therapeutic mechanism.

MiRNAs are a class of short, non-coding RNAs 18–25 nucleotides (nt) in length that are found in animal and plant cells. In 1993, the first miRNAs were recognized in *C. elegans*. In 2001, various small regulatory RNAs were discovered in plants and mammals, and were designated as "microRNA"s. As of today, 1921 human miRNAs are registered in the miRBASE database (Release 18, November, 2011). MiRNAs are involved in RNA interference (RNAi) machinery to regulate gene expression post-transcriptionally, and contribute to diverse physiological and pathophysiological functions, among them the regulation of developmental timing and pattern formation, the restriction of differentiation potential, cell signaling, and carcinogenesis.

In the present study, we screened for trastuzumab responsive microRNAs by utilizing microarray-based microRNA profiling. We identified miR-26a and miR-30b, which were induced in breast cancer cells by trastuzumab exposure, and played important biological roles in the trastuzumab therapeutic mechanism.

Materials and Methods

Cell lines and trastuzumab

Human mammary epithelial cells (HMEC, CC-2551, Lonza) were cultured using the medium supplied by the MEGM Bullet Kit (CC-3150, Lonza) at 37°C and 5% CO_2. In this study, we used a total of 11 breast cancer cell lines. Among them, MCF7, MDAMB231,SKBR3, T47D (obtained from the American Type Culture Collection, ATCC), MDAMB453 (RCB1192, RIKEN BioResource Center), HMC-1-8, and MRK-nu-1 (JCRB0166 and JCRB0628 respectively, Health Science Research Resources Bank) were cultured in RPMI 1640 medium (Invitrogen) containing 10% FBS. BT474 (HTB-20, ATCC) Hs578T (86082104, European Collection of Cell Culture), YMB1E (TKG0440, Cell Resource Center for Biomedical Research, Tohoku University, identical to ZR-75-1,) were cultured in DMEM containing 10% FBS. Trastuzumab was kindly provided by Chugai Pharmaceutical Co., LTD. (Tokyo, Japan).

DNA and RNA extraction from cells

The genomic DNA of breast cancer and HMEC cells was extracted using a DNeasy kit (Qiagen, Germany). Small RNA-preserved total RNA samples were extracted by a combination of Isogen reagent (Nippon Gene, Co., LTD. Japan) and a PureLink RNA mini kit (Invitrogen). The amount of DNA and RNA was measured by a Nanodrop spectrophotometer (ND-1), and the RNA quality of the samples was assessed by an Agilent's Bioanalyzer system (model-2100 and RNA 6000 nano kit).

Assessment of HER2 amplification status in cell lines

The genome amplification status at the *HER2* locus in HMEC and the 11 breast cancer cell lines was assessed by quantitative genomic PCR. The amount of amplification at the *HER2* locus was normalized by the average amount of *NLK* and *ACACA* located between *HER2* and the centromere of chromosome 17. The genomic amount of the *HER2* locus relative to that in the HMEC cells represented the amount of *HER2* amplification in the cell lines. The sequence information used in this quantitative genomic PCR is listed in Table S1.

Quantitative RT-PCR

The mRNA expression levels of genes such as *HER2* in the cell lines were assessed by SYBR green based quantitative RT-PCR (SYBR Green PCR Master Mix, Applied Biosystems, Carlsbad, CA). The RT-PCR data were normalized against the *GAPDH* expression in the cells. The sequence information used in this quantitative RT PCR was also listed in Table S1. The expression levels of individual miRNAs were determined by an ABI 7300 Sequence Detector™ (Applied Biosystems, Foster City, CA) with TaqMan MicroRNA Assay kits for hsa-miR-26a and 30b (Applied Biosystems). The miR-16 was used as an internal control to normalize the microRNA expression levels [11].

MicroRNA expression profiling

To identify trastuzumab-inducible microRNAs, we performed microRNA expression profiling using microRNA microarray technology. The RNA samples were extracted from two *HER2*-positive cell lines (SKBR3 andBT474) and two *HER2*-negative cell lines (MCF7 and T47D), that were cultured with and without trastuzumab (4 μg/ml) for 144 hours. The global microRNA expression profiles of the 8 RNA samples were obtained using a Toray's microRNA microarray platform based on miRBase version 12 (3D-Gene miRNA oligo chip, Toray Industries Inc., Tokyo, Japan), as previously described [12]. Briefly, for each patient, 500 ng of total RNA derived from both tumor and non-tumor samples were labeled using a miRCURY LNA™ microRNA Power Labeling Kit Hy5 (Exiqon, Vedbaek, Denmark). The labeled samples were individually hybridized onto the DNA chip surface, and were incubated at 42°C for 16 hours. The washed and dried DNA chip in an ozone-free environment was scanned using a ProScanArray™ microarray scanner (PerkinElmer Inc. Waltham, MA). The obtained microarray images were then analyzed using Genepix Pro™ 4.0 software (Molecular Device, Sunnyvale, CA). In this study, the median values of the foreground signal minus the local background were calculated as the feature intensities.

Transfection

The cells were plated at a density of 2×10^5 cells per well in a 6-well format, or 5×10^3 cells for a 96-well format 24 hours before the transfection. The microRNA precursor oligos, microRNA inhibitor, or negative control RNA (ncRNA) oligos (final concentration: 25 nM for mimic oligo, 40 nM for microRNA inhibitor) were transfected into cells using an X-tremeGENE siRNA Transfection Reagent (Roche). The medium was replaced eight hours after the transfection.

WST-1 assay

We utilized a WST-1 assay for assessing the sensitivity of the cells to trastuzumab and the effect of the microRNA on cell proliferation. Regarding the sensitivity of the cells to trastuzumab, 5000 breast cancer cells were plated per 96-well plate on Day 0. From Day 1 to Day 6, the cells were exposed to trastuzumab at different concentrations of 0.0625, 0.125, 0.25, 0.5, 1, 2, 4, 8, 16, and 32 μg/ml, and the culture media containing trastuzumab were replaced every 72 hours. On Day 6, 10 μl of WST-1 reagent was added into each well. After 1 hour of incubation, the absorbance at 450 nm was measured by a microplate reader (Biorad, Hercules, CA). To assess the effect the microRNA on cell proliferation, a WST-1 assay was performed in the 96-well format at 72 hours after the microRNA/ncRNA transfection.

Cell cycle assay

Flow cytometric analysis of the DNA content was performed to assess the effect of the microRNA on the cell cycle. Pn Day 0, miR-26a/30b precursor or ncRNA oligo (final concentration: 25 nM) was transfected into SKBR3 or BT474 cells in a 6-well

format. On Day 3, the cells were fixed in 70% ethanol at −20°C. After washing with PBS, the cells were treated with RNase A and stained with propidium iodide (PI) using a Cellular DNA Flow Cytometric Analysis kit (Roche Diagnostics, Basel, Switzerland). The DNA content was evaluated using a FACS Calibur flow cytometer (BD Biosciences, San Jose, CA) with Modfit LT software (Verity Software House) for histogram analysis. Each experiment was performed in triplicate.

Apoptosis assay

Annexin-V assays were performed for the detection of apoptotic cells. After the transfection of the microRNA precursors on Day 0, the cells were harvested on Day 3 and washed with PBS. The cells were then stained using an Annexin V-FITC Apoptosis Detection Kit I (BD Biosciences). The untreated cells served as a negative control for the double staining. The cells were analyzed immediately after staining using FACS Calibur flow cytometer and Cell Quest Pro software.

Luciferase reporter assay for the association between the 3′UTR of target gene candidates and miR-30b

First, an EcoRI site was introduced into the XbaI site of the luciferase reporter vector pGL4.13 (Proamega, Madison, WI, USA) by ligation with the oligonucleotides 5′-CTAGACT-GAATTC-3′ and 5′-CTAGGAATTCAGT-3′, yielding the pGL4.13EcoRI vector [13]. Second, the 3′-untranslated regions (UTRs) of the *CCNE2*, *cyclin A1* (*CCNA1*), and *cell division cycle 7* (*CDC7*) genes were amplified fromBT474 cells using the PCR primers listed in Table S2, and cloned into a pCR4-TOPO vector (Invitrogen). The cloned EcoRI fragments containing the putative miR-30b binding sites were then inserted into the EcoRI site of pGL4.13EcoRI, and were designated CCNE2-wt, CCNA1-wt, and CDC7-wt, respectively. Three derivative constructs o CCNE2-wt with mutations in the putative miR-30b-binding sites were generated using a Gene-Taylor Mutagenesis kit (Invitrogen) and the primers listed in Table S2, and were designated CCNE2-mut1, -mut2, and –mut1+2. All of the constructs were verified by direct sequencing. MicroRNA oligos or ncRNA were co-transfected with 200 ng each of the constructed reporter vector constructs and an internal control vector (pGL4.73, Promega) into HEK293 cells (5×10^4 cells) in a 24-well format. Twenty-four hours later, the luciferase activity was measured using a dual-luciferase reporter assay system (Promega) and a Lumat LB9507 luminometer (Berthold Technologies, Germany). The firefly luciferase activities of the reporter constructs were normalized against the renilla luciferase activities of the internal control vector, The reduction ratio of the luciferase activity from the ncRNA-transfected samples was used as an index of the effect of the microRNAs on the post-transcriptional regulation of these 3 genes.

Statistical analysis

The unpaired student-t test was used for evaluating whether a difference between two mean values was statistically significant. Matlab 2011a (Mathworks, MA, USA) or Microsoft Excel (Microsoft, Redmond, WA) software was used for these analyses, and a P-value of less than 0.05 was considered statistically significant.

Results

HER2 status and trastuzumab sensitivity in breast cancer cell lines

To select HER2-positive and negative breast cancer cell lines, we determined the *HER2* status of the breast cancer cells in terms of genomic amplification and the mRNA expression of *HER2*. Figure 1A and 1B show the genome copy number in the *HER2* locus and the mRNA expression levels of *HER2* gene assessed by quantitative PCR, respectively. Among the 11 breast cancer cells, SKBR3 andBT474 exhibited marked genomic amplification and the overexpression of *HER2*, and MDA-MB-453 had a moderate level of *HER2* overexpression. Thus, for further study, we chose SKBR3 and BT474 as *HER2*-positive cells and MCF7 and MDA-MB-231 as *HER2*-negative cells.

The WST-1 assays showed that even very low concentrations of trastuzumab significantly reduced cell proliferation in SKBR3 andBT474 cells by 40–60%, whereas the proliferation of MCF7 and MDA-MB-231 cells was not affected by trastuzumab exposure (Fig. 1C). Thus, trastuzumab exposure directly reduced the growth of *HER2*-positive cells.

Identification of trastuzumab-responsive microRNAs

In this study, we hypothesized that some of the trastuzumab-inducible/reducible microRNAs would play roles in the molecular mechanisms responsible for the therapeutic effect of trastuzumab. To identify these microRNAs, we performed microRNA expression profiling analysis. First, the two *HER2*-positive and two *HER2*-negative breast cancer cell lines were exposed to trastuzumab at a concentration of 4 μg/mL for six days. The control treatment consisted of PBS. Thus, a total of 8 RNA samples were extracted from these cells, and were subjected to microRNA profiling analysis. Second, the obtained microRNA profiling data were normalized by a quantile normalization method, and filtered using the criterion that the microRNA signals before or after trastuzumab exposure for each of the four cells, SKBR3, BT474, MCF7, and MDA-MB-231 cells, should be more than 6 in log2 transformed value. After this filtration, 94 microRNAs were subjected to further screening. All normalized and raw data from the microarray is available in Minimum Information about Microarray Gene Experiment (MIAME)-compliant format via the Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo). The accession numbers (GSM-numbers) are currently in the registration process. Third, the expression of trastuzumab-responsive microRNAs should not be changed by trastuzumab exposure in *HER2*-negative breast cancer cell lines MCF7 and MDA-MB-231. We eliminated those microRNAs, which had a more than 1.5-fold up/down-regulation in the MCF7 or MDA-MB-231 cells. Thus, 71 microRNAs remained. Fourth, the relative fold-change (fold change of microRNAs – average fold change of the microRNAs in MCF7 and MBA-MD-231 cells) of the remaining microRNAs, and microRNAs with more than a 1.5-fold up/down-regulation, were listed in Table 1.

Trastuzumab exposure upregulated 16 and 9 microRNAs in SKBR3 and BT474 cells, whereas it down-regulated 0 and 10 microRNAs, respectively. As shown in a clustergram of 94 prefiltered microRNAs (Figure 2A), all pairs of the same cells with versus without trastuzumab treatment were clustered most closely, which indicated that the trastuzumab treatment changed microRNA profile slightly. The first branch of the clustergram divided *HER2*-positive and HER2-negative cells. This clustergram shows that the microRNA profile was reflected by the HER2-characteristics ofSKBR3, BT474, MCF7, and MDA-MB-231 cells, indicating that this profiling analysis worked well. The height of the last branch in the clustergram for the *HER2*-positive cells was higher than that of the *HER2*-negative cells, indicating that the *HER2*-positive cells had more altered microRNA expression than the *HER2*-negative cells. A heatmap and the clustergram in Figure 2B illustrated the fold-change pattern of the four cells following trastuzumab treatment.

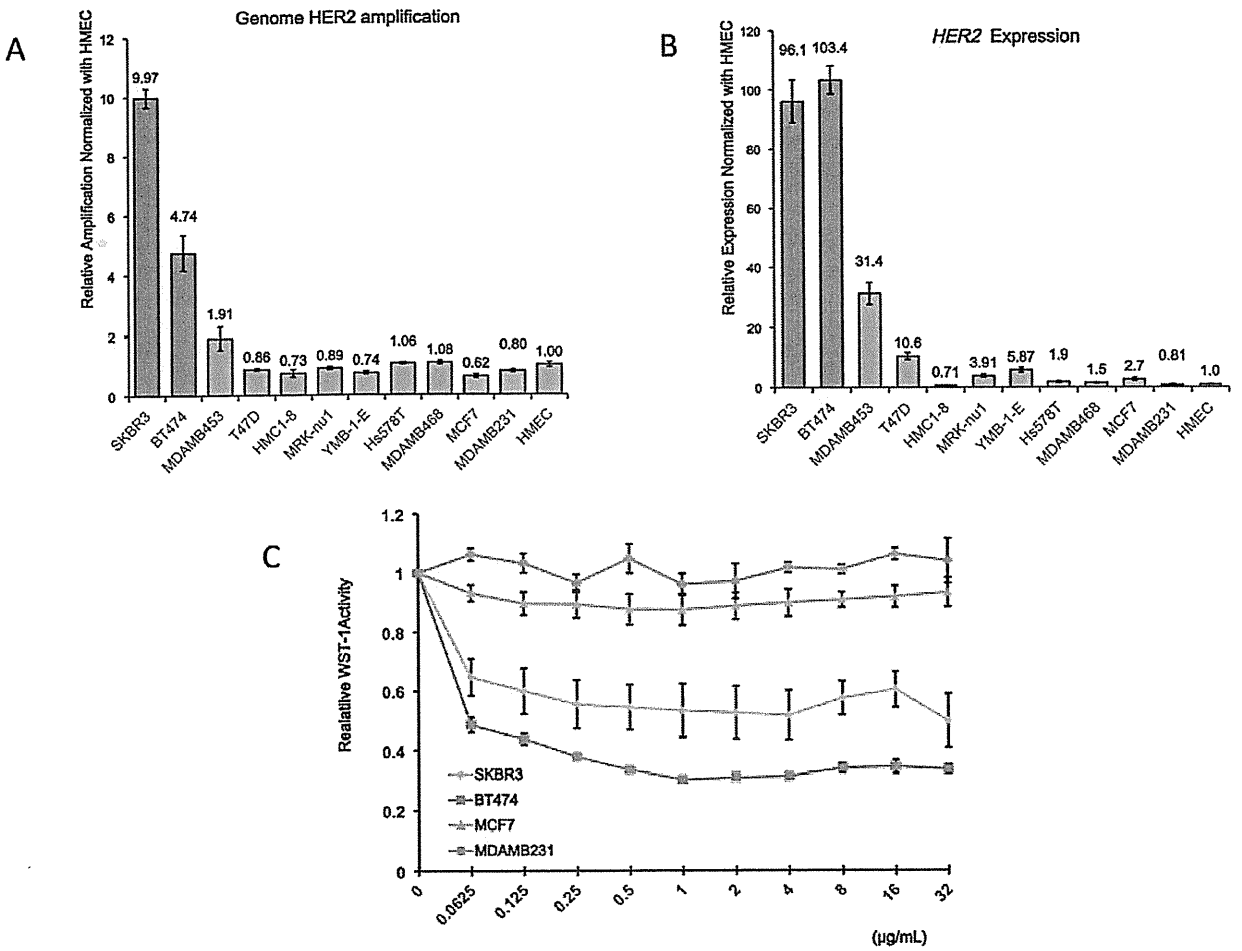

Figure 1. HER2-status of breast cancer cell lines. The genomic amplification (1A) and mRNA expression level (1B) of HER2 in 11 human breast cancer cell lines and normal human mammary epithelial cells (HMEC) were assessed using quantitative PCR and quantitative RT-PCR (n = 3). The mRNA abundance was normalized by the *GAPDH* expression levels. 1C: The trastuzumab sensitivity of SKBR3, BT474, MCF7, and MDA-MB-231 cells was determined using the WST-1 assay. The cells were incubated in trastuzumab-containing media at different concentrations for 144 hours, and then the absorbance at 450 nm was measured after a 2-hour incubation with WST-1 reagent. The ratio of the absorbance to that of the non-treated cells represented the trastuzumab sensitivity of cells.
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Among the listed microRNAs, we selected seven microRNAs (miR-18a, miR-21, miR-26a, miR-26b, miR-30b, miR98 and miR-210) to validate the array-based expression data by Taqman quantitative RT-PCR (Figure 2C, 2D, S1). Most of the microRNAs showed consistent results with the array data. In particular, miR-26a and miR-30b in both cells were significantly upregulated in trastuzumab dose-dependent manner (Figure 2E). Interestingly, 3 out of 5 miR-30 family members (miR-30a~e) were upregulated in the BT474 cells following trastuzumab exposure. Therefore, in this study, we focused on miR-26a and miR-30b for a further functional study.

A list of microRNAs which expression were altered only in HER2-negative cells is shown in Table S3. These microRNAs. The changes in these microRNAs could help to identify non-specific side effects of trastuzumab.

Cell growth suppressive effects of miR26a and miR-30b

Using WST-1 assay, we examined whether miR-26a and miR-30b had growth suppressive effects. Six days after transfection, miR-26a significantly reduced the proliferation ofSKBR3 andBT474 cells by 56% and 24%, whereas miR-30b inhibited 37% and 26% of the cell growth, respectively (Figure 3, $p<0.05$).

Next, we checked whether the mechanisms responsible for this cell growth suppression by miR-26a and miR-30b included changes in the cell cycle and apoptosis. The proportion of cells in the G1 phase increased from 57% to 64% in theSKBR3 cells, and from 65% to 91% in the BT474 cells (Figure 4, $p<0.005$), and that in the S phase decreased from 37% to 31%, and from 29% to 6%, respectively. Thus, the trastuzumab treatment induced G1 arrest in both cell types. The transfection of miR-26a also showed a 22% ($p = 0.14$) and 11% ($p = 0.0002$) increment of the G1 proportion, and a 20% ($p = 0.005$) and 10% ($p = 0.0005$) decrement in the S phase in SKBR3 andBT474 cells, respectively. In contrast, the G2/M phase had no significant changes in both cell types. miR-30b also increased the G1 phase by 6% and 8%, and decreased the S phase by 5% and 7%, respectively, whereas the G2/M phase did not change. Thus, exogenous miR-26a and miR-30b induced G1 arrest in SKBR3 and BT474 cells.

Table 1. Trastuzumab responsive microRNAs.

Rank	microRNA	RFC* in SKBR3	microRNA	RFC* in BT474	microRNA	Mean RFC*
Up-regulated microRNAs						
1	miR-663	2.6847	miR-1246	3.5825	miR-1246	2.9505
2	miR-1228-5p	2.5757	miR-26a	2.3305	miR-26a	2.1640
3	miR-1246	2.4300	miR-125a-5p	1.7146	miR-1228-5p	2.0419
4	miR-21	2.2558	miR-23a	1.6897	miR-663	1.9317
5	miR-26b	2.0749	miR-30c	1.6455	miR-125a-5p	1.6925
6	miR-98	2.0446	miR-1228-5p	1.6187	miR-1908	1.6487
7	miR-26a	2.0093	miR-30b	1.6076	miR-23a	1.6303
8	miR-195	1.9996	miR-100	1.5625	miR-28-5p	1.6176
9	miR-28-5p	1.8877	miR-30d	1.5330	miR-26b	1.5799
10	miR-1908	1.8548			miR-100	1.5768
11	miR-29b	1.7555			miR-21	1.5335
12	miR-1268a	1.6898			miR-29b	1.5211
13	miR-125a-5p	1.6707				
14	miR-100	1.5913				
15	miR-23a	1.5730				
16	miR-149*	1.5439				
Down-regulated microRNAs						
1	none		miR-296-5p	0.4668	miR-296-5p	0.6417
2			miR-1308	0.4889		
3			miR-1280(d)	0.5604		
4			miR-18a	0.5609		
5			miR-425	0.5631		
6			miR-210	0.5670		
7			miR-720	0.5995		
8			miR-125a-3p	0.6064		
9			miR-494	0.6352		
10			miR-187*	0.6501		

MicroRNAs with more than 1.5-fold change in HER2-positive cells but not in HER2-negative cells.
*: RFC, relative fold change = (Fold change of miR) – (average fold change of the miR in MCF7 and MBA-MD-231).
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Using the Annexin-V assay, we also examined whether apoptosis was involved in the cell growth suppression induced by miR-26a and miR-30b. The trastuzumab treatment significantly increased the portion of apoptotic cells from 8.1% to 14.7% (Figure 5, $p = 0.012$), and from 2.5% to 6.1% ($p = 0.003$) inSKBR3 and BT474 cells, respectively. The transfection of miR-26a induced apoptosis in both cell types, as compared with the non-targeting control microRNA (from 11.3% to 39%, $p = 0.012$ in SKBR3, and from 4.7% to 15.2%, $p = 0.012$ in BT474 cells), whereas miR-30b did not show any significant effect on apoptosis.

Identification of target genes against miR-30b

In the present study, we tried to identify the target mRNAs of miR-30b that were related to miR-30b-induced G1 arrest. First, we utilized three different algorithms for predicting the microRNA targets, TargetScan5.1 (http://www.targetscan.org/), miRanda (http://www.microrna.org), and PicTar (http://www.pictar.org/). Among the putative target genes listed by all of three prediction engines, we selected 3 cell cycle-related genes, *CCNE2*, *CCNA1*, and *CDC7*. We then examined whether these three genes were actually regulated by miR-30b or not, using luciferase reporter vectors containing the 3′UTR of these genes (Figure 6A). *CCNE2* and *CDC7* have two and one putative binding sites for miR-30b in the conserved regions of the 3′-UTR, respectively, whereas *CCNA1* possesses one miR-30b binding site in a poorly conserved region of the 3′-UTR.

Among the three reporter constructs with the wild-type 3′-UTR of these genes, miR-30b reduced the luciferase activity only of the CCNE2-wt construct (27% reduction, $p = 0.005$, Figure 6B). To confirm whether miR-30b was associated with the predicted binding sites, we generated three derivative constructs with mutations at the miR-30b binding sites (Figure 6A). These mutations abolished the post-transcriptional repressive effect of miR-30b (Figure 6C), which indicated that miR-30b interacts directly with both binding sites. However, transfecting excessive exogenous microRNA may lead an artificial effect. Thus, we tried to assess suppressive effect of microRNAs at the endogenous level. First, we used microRNA inhibitor for co-transfection (Figure S2), which did not show any significant effect. We speculated that other miR-30 family members with the same seed sequence could compensate the function of blocked miR-30b. Alternatively, we transfected reporter constructs without miR-30b mimic oligos into cells (Figure 6D). Endogenous microRNAs suppressed 54–59% of reporter actively by binding *CCNE2*. When mutated construct at