miR-1290 and its potential targets are associated with characteristics of estrogen receptor α-positive breast cancer

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Abstract

Recent analyses have identified heterogeneity in estrogen receptor α (ERα)-positive breast cancer. Subtypes called luminal A and luminal B have been identified, and the tumor characteristics, such as response to endocrine therapy and prognosis, are different in these subtypes. However, little is known about how the biological characteristics of ER-positive breast cancer are determined. In this study, expression profiles of microRNAs (miRNAs) and mRNAs in ER-positive breast cancer tissue were compared between ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors by miRNA and mRNA microarrays. Unsupervised hierarchical clustering analyses revealed distinct expression patterns of miRNAs and mRNAs in these groups. We identified a downregulation of miR-1290 in ER^{high} $Ki67^{low}$ tumors. Among 11 miRNAs that were upregulated in ER^{high} $Ki67^{low}$ tumors, quantitative RT-PCR detection analysis using 64 samples of frozen breast cancer tissue identified six miRNAs (let-7a, miR-15a, miR-26a, miR-34a, miR-193b, and miR-342-3p). We picked up 11 genes that were potential target genes of the selected miRNAs and that were differentially expressed in ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors. Protein expression patterns of the selected target genes were analyzed in 256 ER-positive breast cancer samples by immunohistochemistry: miR-1290 and its putative targets, *BCL2, FOXA1, MAPT,* and *NAT1*, were identified. Transfection experiments revealed that introduction of miR-1290 into ER-positive breast cancer cells decreased expression of NAT1 and FOXA1. Our results suggest that miR-1290 and its potential targets might be associated with characteristics of ER-positive breast cancer.

Key Words
- breast cancer
- microRNA
- estrogen receptor
- miR-1290

Endocrine-Related Cancer (2013) **20**, 91–102

Introduction

There are large-scale molecular differences between estrogen receptor α (ERα)-positive and ER-negative breast cancers (Sorlie *et al.* 2003). ER is essential for estrogen-dependent growth, and its level of expression is a crucial determinant of response to endocrine therapy and prognosis in ER-positive breast cancer (Harvey *et al.* 1999, Yamashita *et al.* 2006, Dowsett *et al.* 2008). Recent analyses have identified heterogeneity in ER-positive

http://erc.endocrinology-journals.org
DOI: 10.1530/ERC-12-0207

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breast cancer. Subtypes, named luminal A and luminal B, have been defined according to expression levels of Ki67, and the characteristics of these two subtypes are different (Goldhirsch *et al.* 2011). There is no doubt that higher concentrations of ER in the tumor cells are associated with a greater likelihood of a favorable response to endocrine therapy. However, little is known about how the expression of ER in breast cancer cells is regulated and how the biological characteristics of ER-positive breast cancer are determined. We recently analyzed expressions of microRNAs (miRNAs) that directly target ER in breast cancer. We found that miR-206 and miR-18a were downregulated in ER-positive breast cancer compared with ER-negative tumors and that low miR-18b expression was significantly associated with improved survival in HER2-negative breast cancer, although miR-18b expression was not correlated with ER protein expression (Kondo *et al.* 2008, Yoshimoto *et al.* 2011).

miRNAs are small (~21 nucleotides) noncoding RNAs that negatively regulate target genes by predominantly binding to the 3′-untranslated region (3′-UTR) of target mRNA, resulting in either mRNA degradation or translational repression (Krol *et al.* 2010). Recent studies have shown that miRNA mutations or dysregulated expression were associated with various human cancers and indicated that miRNAs can function as tumor suppressor genes and oncogenes (Esquela-Kerscher & Slack 2006). Expression profiling also revealed that miRNAs are differently expressed among molecular subtypes of breast cancer (Iorio *et al.* 2005). Significant associations were found between miRNA expression profiles and clinicopathological factors such as ER status and tumor grade (Blenkiron *et al.* 2007). Furthermore, recent studies have demonstrated that loss- or gain-of-function of specific miRNAs contributes to breast epithelial cellular transformation, tumorigenesis, and epithelial–mesenchymal transition and metastasis (Zhang & Ma 2012).

In this study, expression profiles of miRNAs and mRNAs in ER-positive breast cancer tissue were compared between ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors by miRNA and mRNA microarrays. Unsupervised hierarchical clustering analyses revealed distinct expression patterns of miRNAs and mRNAs in these two groups. We demonstrated that miR-1290 was downregulated and that six miRNAs were upregulated in ER^{high} $Ki67^{low}$ tumors. Protein expression patterns of the predicted target genes and the genes that were identified by mRNA expression profiling were analyzed in ER-positive breast cancer samples by immunohistochemistry (IHC). We identified miR-1290 and its potential target genes, forkhead box A1 (*FOXA1*) and *N*-acetyltransferase-1 (*NAT1*), being associated with characteristics of ER-positive breast cancer.

Materials and methods

Patients and breast cancer tissue

Breast tumor specimens from female patients with invasive breast carcinoma who were treated at Nagoya City University Hospital between 1995 and 2010 were included in the study (Table 1). The study protocol was approved by the institutional review board and conformed to the guidelines of the 1996 Declaration of Helsinki. Written informed consent for the use of surgically resected tumor tissues was provided by all patients before treatments. The samples were chosen from a continuous series of invasive carcinoma. All patients except those with stage IV disease underwent surgical treatment (mastectomy or lumpectomy). Tumor samples of patients with stage IV disease were taken by core needle biopsy. Patients received adequate endocrine or chemotherapy for adjuvant or metastatic diseases.

Microarray profiling of miRNA and mRNA expression

Total RNA was extracted from eight frozen samples of breast cancer tissue (Table 1). Extracted total RNA was labeled with Hy5 using the miRCURY LNA Array miR labeling kit (Exiqon, Vedbaek, Denmark). Labeled RNAs were hybridized onto 3D-Gene Human miRNA Oligo chips containing 1011 antisense probes printed in duplicate spots (Toray, Kamakura, Japan). The annotation and oligonucleotide sequences of the probes were conformed to the miRBase miRNA data base (http://microrna.sanger.ac.uk/sequences/). After stringent washes, fluorescent signals were scanned with the ScanArray Express Scanner (PerkinElmer, Waltham, MA, USA) and analyzed using GenePix Pro version 5.0 (Molecular Devices, Sunnyvale, CA, USA). These raw data of each spot were normalized by substitution with the mean intensity of the background signal determined by all blank spots' signal intensities at 95% confidence intervals. Measurements of both duplicate spots with signal intensities >2 S.D. of the background signal intensity were considered to be valid. A relative expression level of a given miRNA was calculated by comparing the signal intensities of the averaged valid spots with their mean value throughout the microarray experiments after normalization by their median values adjusted equivalently. miRNAs differentially expressed

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DOI: 10.1530/ERC-12-0207

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among the ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors were statistically identified using the Student's *t*-test and unsupervised hierarchical clustering analyses. Hierarchical clustering was performed with average linkage and Pearson's correlation. Differential expression was assessed by a nonparametric Wilcoxon's rank sum test for comparison between two groups. A heat-map was constructed by hierarchical clustering analysis using Cluster 2.0 Software (Tokyo, Japan) and the results were displayed with the TreeView program (http://rana.lbl.gov/eisen/). miRNA expression data are available from the National Center for Biotechnology Gene Expression Omnibus (GEO) at accession number (GEO:GSE38280).

mRNA expression profiles were examined using the same frozen breast cancer tissue samples as those used in miRNA analyses. Extracted total RNA was labeled with Cy5 using the Amino Allyl MessageAMP II aRNA Amplification kit (Applied Biosystems). Labeled RNAs were hybridized onto 3D-Gene Human mRNA Oligo chips 25k (Toray) was used (25 370 distinct genes). Hybridization signals were scanned and detected by the same method as that used in miRNA analyses. The gene expression data are available from GEO at accession number (GEO:GSE38280).

Quantitative RT-PCR detection of miRNAs

Total RNA was extracted from ~500 mg frozen breast cancer tissue using TRIzol reagent (Life Technologies, Inc.) as described previously (Kondo *et al.* 2008). cDNA was reverse transcribed from total RNA samples using specific miRNA primers from the TaqMan MicroRNA Assays and reagents from the TaqMan MicroRNA RT Kit (Applied Biosystems). The resulting cDNA was amplified by PCR using TaqMan MicroRNA Assay primers with the TaqMan Universal PCR Master Mix and analyzed with a 7300 ABI PRISM Sequence Detector System according to the manufacturer's instructions (Applied Biosystems). The relative levels of miRNA expression were calculated from the relevant signals by normalization with the signal for *U6B* miRNA expression. The assay names for each miRNA were as follows: hsa-let-7a for let-7a, hsa-miR-10a for miR-10a, hsa-miR-10b for miR-10b, hsa-miR-15a for 15a, hsa-miR-18a for miR-18a, hsa-miR-26a for miR-26a, hsa-miR-29c for miR-29c, hsa-miR-34a for miR-34a, hsa-miR-129 for miR-129, hsa-miR-146a for miR-146a, hsa-miR-193b for miR-193b, hsa-miR-342-3p for miR-342-3p, hsa-miR-1290 for miR-1290, and RNU6B for U6B miRNA (Applied Biosystems).

Immunohistochemistry

Tissue microarrays were constructed using paraffin-embedded, formalin-fixed tissue from 256 ER-positive breast cancer samples, including 64 samples from patients whose frozen samples were used in miRNA expression analysis. Tissue array sections were immunostained with 15 commercially available antibodies using the Bond-Max Autostainer (Leica Microsystems, Newcastle, UK) and the associated Bond Refine Polymer Detection Kit (Yamashita *et al.* 2006). Details of primary antibodies and scoring manners are described in Supplementary Table 1, see section on supplementary data given at the end of this article. HER2-positive tumors were excluded from this study.

Cell culture and transfections

MCF-7 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS), 2 mmol/l L-glutamine and penicillin–streptomycin (50 IU/ml and 50 mg/ml respectively), and 0.1% human insulin at 37 °C with 5% CO_2. T47D cells (ATCC) were grown in RPMI 1640 medium containing 10% FBS and 2 mmol/l L-glutamine and penicillin–streptomycin (50 IU/ml and 50 mg/ml respectively) at 37 °C with 5% CO_2. Transfections of pre-miR-1290 precursor (hsa-miR-1290; Ambion, Inc., Austin, TX, USA) were performed with Cell Line Nucleofector kits (Amaxa Biosystems, Cologne, Germany) using a Nucleofector device (Amaxa Biosystems) according to the manufacturer's instructions (Kondo *et al.* 2008). A nonspecific control miRNA (Pre-miR miRNA Inhibitors-Negative Control #1; Ambion, Inc.) was used as a negative control.

Quantitative RT-PCR detection of miR-1290 and mRNAs

Total RNA was extracted from 2×10^6 cells with miRNeasy Mini Kit (Qiagen) using a QIAcube (Qiagen) according to the manufacturer's instructions. cDNA was reverse transcribed using specific miRNA primers and the relative levels of miR-1290 expression were measured as described earlier. Total RNA (1 μg) was also subjected to RT with random primers in a 20 μl reaction volume using High-Capacity cDNA RT Kit (Applied Biosystems). mRNA expression was measured by quantitative RT-PCR with the TaqMan Universal PCR Master Mix using a 7500 ABI PRISM Sequence Detector System according to the manufacturer's instructions (Applied Biosystems; Kondo *et al.* 2008). The relative levels of mRNA expression were

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DOI: 10.1530/ERC-12-0207

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Table 1 Clinicopathological characteristics of patients and breast tumors with ER-positive, HER2-negative breast cancer.

	Samples for miRNA and mRNA microarray analyses		Samples for miRNA quantitative RT-PCR analysis	Samples for immunohistochemistry
	ER^{high} $Ki67^{low}$	ER^{low} $Ki67^{high}$	Total	Total
No. of patients	4	4	64	256
Age (years)				
Mean±S.D.	71.8±20.9	57.5±12.1	60.0±12.0	58.0±13.0
Range	44–91	42–69	32–88	28–91
Tumor size (cm)				
Mean±S.D.	1.5±0.4	1.6±0.7		
≤2.0			20 (31%)	148 (57.9%)
2.1–5.0			38 (59%)	102 (39.8%)
>5.0			6 (10%)	6 (2.3%)
No. of positive lymph nodes				
0	4 (100%)	4 (100%)	34 (53%)	135 (52.7%)
1–3	0 (0%)	0 (0%)	16 (25%)	72 (28.1%)
4–9	0 (0%)	0 (0%)	6 (10%)	11 (4.3%)
≥10	0 (0%)	0 (0%)	4 (6%)	7 (2.7%)
Unknown	0 (0%)	0 (0%)	4 (6%)	31 (12.2%)
Tumor grade				
1	4 (100%)	0 (0%)	16 (25%)	95 (37.1%)
2	0 (0%)	0 (0%)	36 (56%)	69 (27.0%)
3	0 (0%)	4 (100%)	12 (19%)	92 (35.9%)
ER (Allred score)				
Mean±S.D.	7.8±0.5	3.5±0.6		
0–2 (negative)			0 (0%)	0 (0%)
3–8 (positive)			64 (100%)	256 (100%)
PgR (Allred score)				
Mean±S.D.	7.8±0.5	2.5±0.6		
0–2 (negative)			10 (16%)	34 (13.3%)
3–8 (positive)			54 (84%)	222 (86.7%)
HER2 status				
Negative	4 (100%)	4 (100%)	64 (100%)	256 (100%)
Positive	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Ki67 (labeling index, %)				
Mean±S.D.	6.1±2.7	50.8±11.8		
Adjuvant therapy				
None			8	27
Endocrine therapy			32	127
Chemotherapy			3	4
Combined			21	98

calculated from the relevant signals by normalization with the signal for β-actin mRNA expression. The assay numbers for BCL2, FOXA1, microtubule-associated protein tau (MAPT), NAT1, and β-actin were as follows: Hs00608023_m1 for BCL2, Hs00270129_m1 for FOXA1, Hs00902314_m1 for MAPT, Hs00265080_m1 for NAT1, and 4333762T for β-actin (Applied Biosystems).

Western blotting

Cells were pelleted by centrifugation and solubilized in lysis buffer containing protease inhibitor and phosphatase inhibitor cocktails (Thermo Scientific, Yokohama, Japan). Equal amounts of total protein (30 μg) from whole cell lysates were prepared and electrophoresed on 12% (w/v) SDS–polyacrylamide gels (NuPAGE Bis–Tris Gel, Invitrogen) transferred to polyvinylidene difluoride membranes (Invitrogen) and immunoblotted using specific antibodies (Supplementary Table 1; Yamashita *et al.* 2003). Anti-mouse or anti-rabbit IgG, HRP-linked Whole Antibodies (GE Healthcare Japan, Tokyo, Japan) were used as secondary antibodies at 1:10 000 dilution. Antibody binding was visualized with ECL Western Blotting Detection System (GE Healthcare Japan) using Light-Capture AE-6981 (ATTO, Tokyo, Japan) according to the manufacturer's instructions. Image J Software from the National Institutes of Health (Bethesda, MD, USA) was used to quantify band intensities.

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DOI: 10.1530/ERC-12-0207

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Statistical analysis

Spearman's rank correlation test was used to study relationships between expression levels of miRNAs and clinicopathological factors, expression levels of proteins and clinicopathological factors, expression levels of miRNAs and proteins, and expression levels of miRNAs and mRNAs. $P<0.05$ is considered significant in Spearman's rank correlation test.

Results

Differentially expressed miRNAs in ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors in breast cancer tissue

Expression profiles of miRNAs and mRNAs in ER-positive breast cancer tissue were compared between ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors by miRNA and mRNA microarrays using eight frozen samples of breast cancer tissue (four tumors in each group; Table 1). Unsupervised hierarchical clustering analyses revealed 67 miRNAs in 1011 miRNAs and 657 mRNAs in 25 370 mRNAs that were differentially expressed in ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors ($P<0.01$; Supplementary Figure 1, see section on supplementary data given at the end of this article and Supplementary Table 2, see section on supplementary data given at the end of this article, and $P<0.01$; Supplementary Figure 2, see section on supplementary data given at the end of this article and Supplementary Tables 3 and 4, see section on supplementary data given at the end of this article respectively). We selected 12 miRNAs (let-7a, miR-10a, miR-10b, miR-15a, miR-26a, miR-29c, miR-34a, miR-129, miR-146a, miR-193b, miR-342-3p, and miR-1290) that were differentially expressed in these two groups. Among differentially expressed 67 miRNAs, the above 12 miRNAs, especially let-7a, miR-10a, miR-10b, miR-15a, miR-26a, miR-29c, miR-34a, miR-146a, and miR-342-3p, have been reported to be related to breast cancer development and carcinogenesis (Mattie *et al.* 2006, Blenkiron *et al.* 2007, O'Day & Lal 2010). miR-193b has been reported to be related to ERα (Yoshimoto *et al.* 2011). Moreover, we referred to the reported mRNA microarray analyses to classify luminal A and luminal B subtypes in order to select key genes (Sorlie *et al.* 2003, Parker *et al.* 2009), including *FOXA1*, *NAT1*, *MAPT*, *XBP1*, and *BCL2*, which have target sequences in the 3′-UTR regions of 67 differentially expressed miRNAs according to *in silico* analysis using TargetScan, PicTar, and MiRanda, and selected miR-146a and miR-1290, which were downregulated in ER^{high} $Ki67^{low}$ tumors. Quantitative RT-PCR detection analysis using 64 frozen breast cancer tissue samples (Table 2 and Supplementary Table 5, see section on supplementary data given at the end of this article) identified six miRNAs (let-7a, miR-15a, miR-26a, miR-34a, miR-193b, and miR-342-3p) that were upregulated in ER^{high} tumors ($P=0.0002$, $P=0.0006$, $P=0.0082$, $P<0.0001$, $P=0.0142$, and $P=0.0002$ respectively; Table 3). miR-1290 was also included in further analyses because it was the only miRNA among the selected miRNAs that was downregulated in ER^{high} $Ki67^{low}$ tumors and its expression levels were strongly correlated with tumor grade ($P<0.0001$; Table 3).

Table 2 Expression levels of 12 selected miRNAs and the control miRNA (U6B) in 64 ER-positive breast cancer tissues by quantitative RT-PCR analysis.

	Mean±S.E.M.
let-7a	22.079±0.173
miR-10a	26.585±0.278
miR-10b	27.636±0.247
miR-15a	27.100±0.285
miR-26a	22.711±0.201
miR-29c	24.295±0.390
miR-34a	26.339±0.240
mR-129	34.759±0.185
miR-146a	26.160±0.221
miR-193b	21.112±0.219
miR-342-3p	24.456±0.322
miR-1290	27.612±0.445
U6B	27.091±0.154

The potential target genes for seven selected miRNAs (let-7a, miR-15a, miR-26a, miR-34a, miR-193b, miR-342-3p, and miR-1290) were predicted according to *in silico* analysis using TargetScan, PicTar, and MiRanda. In addition, 657 mRNAs that were differentially expressed in ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors in microarray analysis were considered to select putative target genes. Finally, we picked up 11 proteins (ANKRD30, BCL2, cyclin D1, FOXA1, GATA3, LIN28, MAPT, NAT1, RB1, P53 (TP53), and XBP1) that were products of potential target genes for seven selected miRNAs and that were considered to be differentially expressed in ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors (Table 4). *ANKRD30* was the most differentially expressed gene between ER^{high} $Ki67^{low}$ tumors and ER^{low} $Ki67^{high}$ tumors. BCL2, cyclin D1, LIN28, and RB1 are potential targets of the selected miRNAs as shown in Table 4. FOXA1, GATA3, NAT1, and XBP1 were strongly downregulated in ER^{low} $Ki67^{high}$ tumors, putative targets of the selected miRNAs, and reported as to be related with ER-positive breast

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DOI: 10.1530/ERC-12-0207

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Table 3 Correlation between expression levels of miRNAs and clinicopathological factors ($n=64$).

	ER	PgR	Tumor grade	Ki67	Tumor size	No. of positive lymph nodes
let-7a	+0.533[a] 0.0002*,[b]	+0.349 0.0087*	−0.033 0.2536	−0.115 0.3717	−0.068 0.5854	+0.123 0.7959
miR-10a	+0.286 0.1114	+0.219 0.1113	+0.005 0.4012	−0.113 0.3757	−0.326 0.0098*	+0.132 0.7399
miR-10b	+0.268 0.1646	+0.130 0.3894	+0.074 0.8411	−0.114 0.375	−0.185 0.1439	+0.171 0.5025
miR-15a	+0.499 0.0006*	+0.081 0.6396	+0.215 0.3036	+0.055 0.6729	−0.129 0.3084	+0.062 0.7917
miR-26a	+0.414 0.0082*	+0.165 0.2585	+0.065 0.7953	−0.056 0.6674	−0.003 0.9712	+0.060 0.8038
miR-29c	+0.206 0.3839	−0.030 0.6671	+0.115 0.8782	+0.018 0.8917	−0.084 0.5117	+0.121 0.7546
miR-34a	+0.785 <0.0001*	+0.164 0.2558	+0.061 0.7535	+0.034 0.7941	−0.168 0.1851	+0.039 0.6458
mR-129	+0.334 0.0528	+0.043 0.8722	+0.334 0.0384*	−0.056 0.6711	−0.006 0.9746	+0.049 0.677
miR-146a	+0.101 0.9032	−0.149 0.1819	+0.425 0.0073*	+0.007 0.9586	−0.052 0.6966	−0.009 0.5031
miR-193b	+0.387 0.0142*	+0.203 0.1483	+0.223 0.2666	+0.078 0.5493	+0.046 0.7298	+0.214 0.2889
miR-342-3p	+0.539 0.0002*	+0.131 0.3975	+0.131 0.8024	−0.039 0.7657	−0.016 0.8932	+0.107 0.9081
miR-1290	+0.014 0.3987	−0.211 0.0581	+0.585 <0.0001*	+0.228 0.0748	+0.029 0.8267	+0.280 0.1109

*$P<0.05$ is considered significant.
[a]Spearman's correlation coefficient.
[b]P, Spearman's rank correlation test.

cancer. MAPT is also reported to be related with ER-positive breast cancer and a potential target of miR-1290. P53 was selected as a target of let-7a.

Expression of the potential target genes in ER-positive, HER2-negative breast cancer

We examined protein expression of 11 selected target genes in ER-positive, HER2-negative breast cancer by IHC (Supplementary Table 6, see section on supplementary data given at the end of this article). Expression levels of BCL2, FOXA1, GATA3, LIN28, MAPT, and NAT1 were positively correlated with expression levels of ER ($P<0.0001$, $P<0.0001$, $P<0.0001$, $P=0.0008$, $P<0.0001$, and $P=0.0005$ respectively; Table 4). Expression levels of ANKRD30, BCL2, FOXA1, GATA3, LIN28, MAPT, and NAT1 were positively correlated with expression levels of progesterone receptor (PgR; $P=0.0246$, $P=0.0059$, $P=0.0005$, $P<0.0001$, $P=0.017$, $P<0.0001$, and $P<0.0001$ respectively). Expression levels of ANKRD30, BCL2, and TP53 were positively correlated with tumor grade ($P=0.0012$, $P=0.0109$, and $P=0.0108$ respectively), whereas expression levels of CCND1, FOXA1, GATA3, LIN28, MAPT, NAT1, and XBP1 were negatively correlated with tumor grade ($P=0.0101$, $P<0.0001$, $P<0.0001$, $P=0.0099$, $P<0.0001$, $P<0.0001$, and $P=0.0018$ respectively). Expression levels of LIN28 and TP53 were positively correlated with expression levels of Ki67 ($P=0.0446$ and $P=0.002$ respectively), while expression levels of MAPT and NAT1 were negatively correlated with expression levels of Ki67 ($P=0.0419$ and $P=0.0095$ respectively). Expression levels of ANKRD30, FOXA1, GATA3, LIN28, MAPT, NAT1, TP53, and XBP1 were negatively correlated with tumor size ($P<0.0001$, $P=0.0009$, $P=0.0001$, $P<0.0001$, $P=0.0093$, $P=0.0004$, $P=0.0336$, and $P=0.0203$ respectively). There was no association between expression of 11 selected proteins and lymph node status (Table 4).

We then compared expression levels of seven selected miRNAs (let-7a, miR-15a, miR-26a, miR-34a, miR-193b, miR-342-3p, and miR-1290) and their potential target genes (*ANKRD30*, *BCL2*, cyclin D1, *FOXA1*, *GATA3*, *LIN28*, *MAPT*, *NAT1*, *RB1*, *P53*, and *XBP1*) using 64 samples of breast cancer tissue, simultaneously analyzing miRNA expression by quantitative RT-PCR and protein expression by IHC. Interestingly, expression levels of miR-1290 were

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DOI: 10.1530/ERC-12-0207

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Table 4 Correlation between expression levels of potential target proteins and clinicopathological factors ($n=256$).

	ER	PgR	Tumor grade	Ki67	Tumor size	No. of positive lymph nodes	miRNAs
ANKRD30	+0.260[a] 0.2265[b]	+0.250 0.0246*	+0.002 0.0012*	+0.142 0.5865	−0.145 <0.0001*	+0.176 0.2769	miR-193b
BCL2	+0.467 <0.0001*	+0.320 0.0059*	+0.102 0.0109*	+0.132 0.4585	+0.078 0.0968	+0.278 0.8608	let-7a, miR-10a, miR-15a, miR-26a, miR-29c, miR-34a, miR-1290
CCND1	+0.177 0.5364	+0.083 0.6216	−0.190 0.0101*	+0.078 0.5415	−0.085 0.4981	+0.046 0.6898	miR-15a, miR-34a, miR-193b
FOXA1	+0.407 <0.0001*	+0.234 0.0005*	−0.235 <0.0001*	−0.082 0.1939	−0.210 0.0009*	+0.009 0.103	miR-129, miR-1290
GATA3	+0.448 <0.0001*	+0.286 <0.0001*	−0.224 <0.0001*	−0.004 0.9441	−0.242 0.0001*	−0.005 0.0655	miR-10a, miR-10b, miR-34a
LIN28	+0.289 0.0008*	+0.173 0.017*	−0.081 0.0099*	+0.138 0.0446*	−0.238 <0.0001*	+0.068 0.3681	let-7a, miR-26a, miR-34a, miR-129, miR-342-3p
MAPT	+0.356 <0.0001*	0.494 <0.0001*	−0.254 <0.0001*	−0.144 0.0419*	−0.149 0.0093*	+0.030 0.1314	miR-34a, miR-1290
NAT1	+0.316 0.0005*	+0.394 <0.0001*	−0.274 <0.0001*	−0.122 0.0095*	−0.180 0.0004*	+0.105 0.4956	miR-1290
RB1	+0.248 0.0751	+0.261 0.8369	+0.327 0.4651	+0.290 0.2424	+0.263 0.6956	+0.374 0.6748	let-7a, miR-26a, miR-34a, miR-129, miR-1290
TP53	−0.016 0.0743	−0.010 0.6815	+0.211 0.0108*	+0.197 0.002*	−0.133 0.0336*	+0.074 0.5783	let-7a
XBP1	+0.183 0.5653	−0.042 0.5318	−0.236 0.0018*	+0.079 0.5906	−0.278 0.0203*	−0.040 0.2069	miR-34a

*$P<0.05$ is considered significant.
[a]Spearman's correlation coefficient.
[b]P, Spearman's rank correlation test.

inversely correlated with expression levels of *BCL2*, *FOXA1*, *MAPT*, and *NAT1*, all of which are predictive targets of miR-1290 according to *in silico* analysis ($P=0.020$, $P=0.044$, $P=0.040$, and $P=0.0098$ respectively; Fig. 1A, B, C and D), suggesting that miR-1290 might downregulate these four genes in ER-positive breast cancer. Moreover, let-7a expression was inversely correlated with P53 expression ($P=0.038$; Fig. 1E). No association was found between other miRNA expressions and their putative target gene expressions.

miR-1290 downregulates FOXA1 and NAT1 in ER-positive breast cancer cells

We extended our analysis to clarify whether miR-1290 downregulates BCL2, FOXA1, MAPT, and NAT1 in ER-positive breast cancer cells. Pre-miR-1290 precursor was introduced into T47D and MCF-7 cells. Cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at various concentrations (10–300 nmol/l) and incubated for 24 h in T47D cells and for 36 h in MCF-7 cells. Expression levels of miR-1290 and mRNA expression levels of *BCL2*, *FOXA1*, *MAPT*, and *NAT1* were quantitatively measured using parallel samples. Transfection with pre-miR-1290 produced a dose-dependent increase in miR-1290 expression levels (Fig. 2A, left), whereas expression levels of miR-1290 were inversely correlated with expression levels of *FOXA1* ($P=0.0003$; Fig. 2A, top right) and *NAT1* ($P<0.0001$; Fig. 2A, bottom right) mRNAs, but not with *BCL2* or *MAPT* mRNA, in T47D cells (Fig. 2A). Moreover, expression levels of miR-1290 were inversely correlated with expression levels of *NAT1* mRNA ($P=0.037$; Fig. 2B, bottom right), but not with *BCL2*, *FOXA1*, or *MAPT* mRNA, in MCF-7 cells (Fig. 2B).

The effects of miR-1290 on protein expression of BCL2, FOXA1, MAPT, and NAT1 were examined in T47D and MCF-7 cells by western blot analysis. When T47D cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at various concentrations (30–1000 nmol/l) and incubated for 48 h, miR-1290 induced a dose-dependent decrease in protein expression of NAT1, reducing it ~60%, but not BCL2, FOXA1, or MAPT (Fig. 2C). Effects of miR-1290 on protein expression of BCL2, FOXA1, MAPT, and NAT1 were not clear in MCF-7 cells (Fig. 2D). From these analyses, we conclude that miR-1290 might downregulate FOXA1 and NAT1 in ER-positive breast cancer cells.

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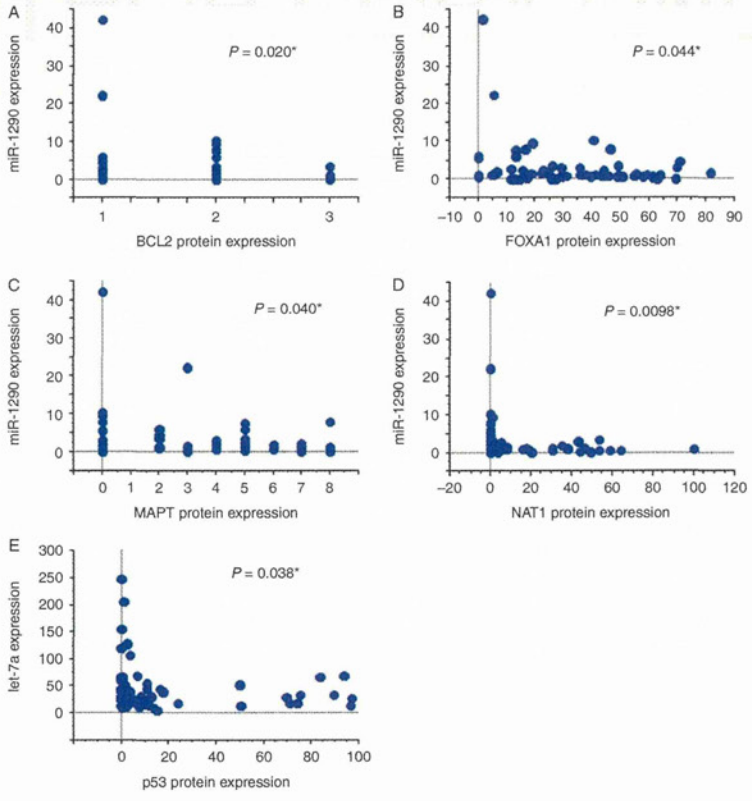

Figure 1

miR-1290 expression is inversely correlated with expressions of BCL2, FOXA1, MAPT, and NAT1. Scatter plots show inverse correlations between miR-1290 and BCL2 (A), FOXA1 (B), MAPT (C), and NAT1 (D) protein expression in breast cancer tissue ($P=0.020$, $P=0.044$, $P=0.040$, and $P=0.0098$ respectively). (E) let-7 expression is inversely correlated with P53 protein expression in breast cancer tissue ($P=0.038$).

Discussion

In this study, we have shown distinct expression patterns of miRNAs and mRNAs in luminal A and luminal B subtypes in ER-positive breast cancer. We demonstrated that miR-1290 and its potential target genes, *FOXA1* and *NAT1*, might be associated with characteristics of ER-positive disease. miR-1290 expression was strongly downregulated in ER^{high} $Ki67^{low}$ tumors and was positively correlated with tumor grade. Although the role of miR-1290 has not been analyzed as yet, it was reported that 36 miRNAs, including miR-1290, were circulating at increased levels in patients with renal cell carcinoma and were overexpressed in corresponding renal cell carcinoma tissue (Wulfken *et al.* 2011). It was also reported that six miRNAs, including miR-1290, were upregulated in drug-sensitive cells following Y-Box protein 1 inhibition, but no differences in miRNA expression could be detected in multidrug-resistant gastric carcinoma cells (Belian *et al.* 2010).

FOXA1, a forkhead family transcription factor, has been reported to be expressed predominantly in luminal A breast cancer with favorable prognosis (Badve *et al.* 2007, Mehta *et al.* 2012). Hurtado *et al.* recently reported that FOXA1 creates an open conformation at ER-binding sites and that ER can bind and activate target gene expression in the presence of estrogen. Thus, FOXA1 is a key determinant of ER function and endocrine response in breast cancer (Hurtado *et al.* 2011). They also reported that the differential ER-binding program observed in tumors from patients with poor outcome is due to the FOXA1-mediated reprogramming of ER binding (Ross-Innes *et al.* 2012). We demonstrated that FOXA1 expression is much higher in ER^{high} $Ki67^{low}$ tumors than in ER^{low} $Ki67^{high}$ tumors and that expression levels of FOXA1 were strongly and positively correlated with expression levels of ER and

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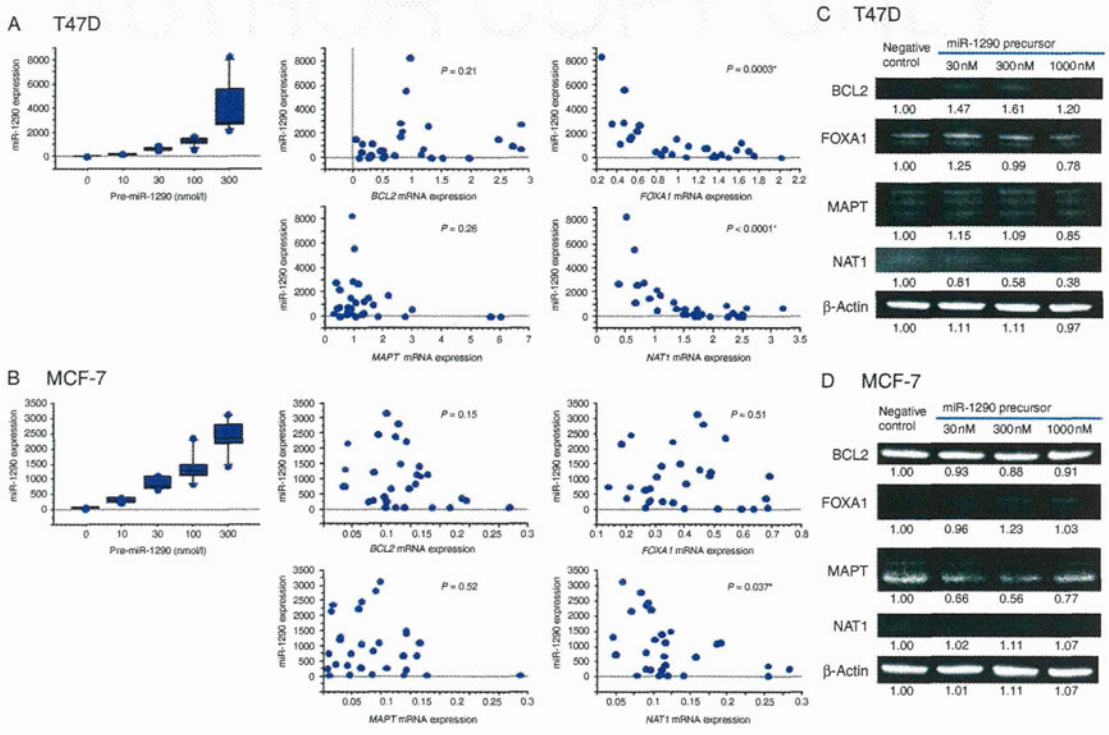

Figure 2

Gene expressions of miR-1290 putative targets in T47D and MCF-7 cells transfected with miR-1290. (A) T47D cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at 10–300 nmol/l and incubated for 24 h. Expression levels of miR-1290 and mRNA levels of BCL2, FOXA1, MAPT, and NAT1 were measured by quantitative RT-PCR. Scatter plots show inverse correlation between miR-1290 expression and *FOXA1* and *NAT1* mRNA expression ($P=0.0003$ and $P<0.0001$ respectively). (B) MCF-7 cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at 10–300 nmol/l and incubated for 36 h. Expression levels of miR-1290 and mRNA levels of BCL2, FOXA1, MAPT, and NAT1 were measured by quantitative RT-PCR. Scatter plots show inverse correlation between miR-1290 expression and *NAT1* mRNA expression ($P=0.037$). (C) T47D cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at 30–1000 nmol/l and incubated for 48 h. Protein expression of BCL2, FOXA1, MAPT, and NAT1 was assayed by western blot analysis. The number below the band represents the mean value from densitometry reading, relative to the negative control, which was set at 1.00. Representative results from one of the three experiments are shown. (D) MCF-7 cells were transfected with either control miRNA (300 nmol/l) or pre-miR-1290 precursor at 30–1000 nmol/l and incubated for 48 h. Protein expression of BCL2, FOXA1, MAPT, and NAT1 was assayed by western blot analysis. The number below the band represents the mean value from densitometry reading, relative to the negative control, which was set at 1.00. Representative results from one of the three experiments are shown.

PgR and negatively associated with tumor grade in ER-positive breast cancer. Moreover, introduction of miR-1290 into estrogen-dependent breast cancer cells reduced FOXA1 expression. Because FOXA1 is a putative target of miR-1290 according to *in silico* analysis, we suggest that miR-1290 is a key factor for regulating FOXA1, which is associated with characteristics of ER-positive breast cancer.

Arylamine NATs, known as drug- and carcinogen-metabolizing enzymes, transfer an acetyl group from acetyl coenzyme A to arylamines (Sim *et al.* 2008). Several studies have shown higher mRNA and protein expression of NAT1 in ER-positive breast cancer compared with the expression in ER-negative disease (Perou *et al.* 2000, Adam *et al.* 2003, Tozlu *et al.* 2006, Wakefield *et al.* 2008). Moreover, it was reported that high expression of NAT1 was correlated with better outcome in ER-positive breast cancer (Bieche *et al.* 2004, Dolled-Filhart *et al.* 2006). Our results demonstrated that *NAT1* mRNA expression was much higher in ER^{high} $Ki67^{low}$ tumors than in ER^{low} $Ki67^{high}$ tumors by microarray analyses and that NAT1 protein expression by IHC showed positive correlation with expression levels of ER and PgR and negative correlation with expression levels of Ki67, tumor grade, and tumor size. In addition, introduction of miR-1290 into estrogen-dependent breast cancer cells strongly

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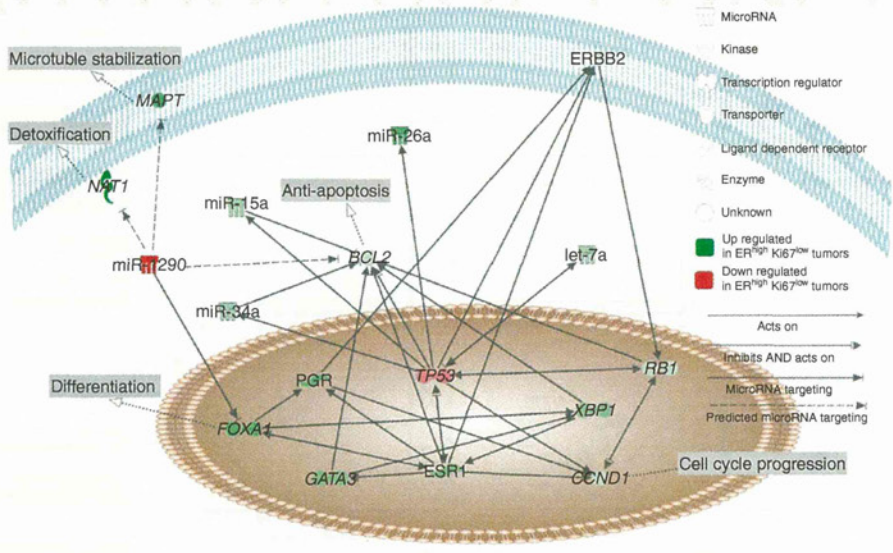

Figure 3

Interaction between miRNAs and putative target proteins that might be associated with characteristics of ER-positive breast cancer. Pathway analyses show five miRNAs (let-7a, miR-15a, miR-26a, miR-34a, and miR-1290) and nine target genes (*BCL2*, *CCND1*, *FOXA1*, *GATA3*, *MAPT*, *NAT1*, *RB1*, *TP53*, and *XBP1*) that were picked up in our present analyses. These proteins and their pathways have diverse cellular functions, such as differentiation, detoxification, anti-apoptosis, cell cycle progression, and microtubule stabilization.

reduced NAT1 expression. Because NAT1, as well as FOXA1, is a putative target of miR-1290 according to *in silico* analysis, it is possible that miR-1290 also regulates NAT1, which will be associated with characteristics of ER-positive breast cancer.

BCL2 and MAPT are also potential targets of miR-1290 according to *in silico* analysis. BCL2 is an anti-apoptotic protein that has an anti-proliferative effect influencing cell cycle entry (Zinkel *et al.* 2006). *BCL2* is an ER-induced gene, and its protein expression assessed by IHC has been shown to be a favorable prognostic marker in breast cancer (Callagy *et al.* 2006, Dawson *et al.* 2010). Our results also showed that expression levels of BCL2 were strongly and positively correlated with expression levels of ER and PgR in ER-positive breast cancer. It was recently reported that miR-195, miR-24-2, and miR-365-2 act as negative regulators of BCL2 through direct binding to their respective binding sites in the 3′-UTR of human *BCL2* gene (Singh & Saini 2012).

MAPT binds to both the outer and the inner surfaces of microtubules, leading to tubulin assembly and microtubule stabilization. As taxanes also bind to the inner surface of microtubules, MAPT might be considered to obstruct the function of these drugs. Most of the studies reported that MAPT expression has prognostic value, with high expression associated with favorable patient outcome. However, at the present time, there are few studies indicating that MAPT is a predictive marker for taxane-based chemotherapy (Baquero *et al.* 2011, Smoter *et al.* 2011). We demonstrated that expression levels of MAPT showed positive correlation with expression levels of ER and PgR and negative correlation with expression levels of Ki67, tumor grade, and tumor size in ER-positive breast cancer. Because miR-1290 did not decrease BCL2 or MAPT protein expression in ER-positive breast cancer cells in our analysis, BCL2 and MAPT might be regulated by other mechanisms.

Interaction between miRNAs and putative target proteins that might be associated with characteristics of ER-positive breast cancer is shown in Fig. 3, which was created by Ingenuity systems Pathway Analysis (http://www.ingenuity.com/index.html) and referring to previous reports (Gomez *et al.* 2007, Badve & Nakshatri 2009, Clarke *et al.* 2009, O'Day & Lal 2010).

Finally, our results indicated that let-7a was strongly upregulated in ER^{high} $Ki67^{low}$ tumors and that expression levels of p53, one of the let-7a targets, was inversely correlated with let-7a expression in ER-positive breast cancer. The let-7 miRNA family is a group of tumor suppressing miRNAs that can inhibit both tumorigenesis

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and metastasis (Zhang *et al.* 2010). It was recently reported that let-7 family miRNAs, especially let-7a, let-7b, and let-7i, were downregulated in breast cancer tissue compared with normal tissue and that let-7 miRNAs induced apoptosis in MCF-7 cells (Zhao *et al.* 2011). Thus, let-7 might have a role in ER-positive breast cancer.

In conclusion, this study indicates for the first time that miR-1290 and its potential targets, NAT1 and FOXA1, are strongly downregulated in ER^{high} $Ki67^{low}$ tumors and are associated with characteristics of ER-positive breast cancer. miR-1290 could be a novel therapeutic target in ER-positive breast cancer.

Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/ERC-12-0207.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported in part by grant-in-aid for scientific research from Japan Society for the Promotion of Science.

Author contribution statement

Y Endo designed the study, executed miRNA and mRNA expression profiling, target prediction and target validation, carried out immunostaining and western blotting, and drafted the manuscript. T Toyama, N Yoshimoto, M Iwasa, and T Asano provided tissue samples. S Takahashi assessed the immunostaining and western blotting. Y Fujii participated in its design and coordination. H Yamashita conceived of the study and participated in its design, coordination, and manuscript writing. All authors read and approved the final manuscript.

Acknowledgements

The authors wish to thank Prof. Edith Sim and Dr Hilary Long (University of Oxford, UK) for kindly providing anti-NAT1 antibodies.

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Received in final form 29 October 2012
Accepted 23 November 2012
Made available online as an Accepted Preprint
26 November 2012

Published by Bioscientifica Ltd.

Qual Life Res
DOI 10.1007/s11136-013-0387-8

Using the EORTC-QLQ-C30 in clinical practice for patient management: identifying scores requiring a clinician's attention

Claire F. Snyder · Amanda L. Blackford · Toru Okuyama · Tatsuo Akechi · Hiroko Yamashita · Tatsuya Toyama · Michael A. Carducci · Albert W. Wu

Accepted: 4 March 2013

Abstract
Purpose Patient-reported outcomes (PROs) are used increasingly for individual patient management. Identifying which PRO scores require a clinician's attention is an ongoing challenge. Previous research used a needs assessment to identify EORTC-QLQ-C30 cutoff scores representing unmet needs. This analysis attempted to replicate the previous findings in a new and larger sample.
Methods This analysis used data from 408 Japanese ambulatory breast cancer patients who completed the QLQ-C30 and Supportive Care Needs Survey-Short Form-34 (SCNS-SF34). Applying the methods used previously, SCNS-SF34 item/domain scores were dichotomized as no versus some unmet need. We calculated area under the receiver operating characteristic curve (AUC) to evaluate QLQ-C30 scores' ability to discriminate between patients with no versus some unmet need based on SCNS-SF34 items/domains. For QLQ-C30 domains with AUC $\geq$ 0.70, we calculated the sensitivity, specificity, and predictive value of various cutoffs for identifying unmet needs. We hypothesized that compared to our original analysis, (1) the same six QLQ-C30 domains would have AUC $\geq$ 0.70, (2) the same SCNS-SF34 items would be best discriminated by QLQ-C30 scores, and (3) the sensitivity and specificity of our original cutoff scores would be supported.
Results The findings from our original analysis were supported. The same six domains with AUC $\geq$ 0.70 in the original analysis had AUC $\geq$ 0.70 in this new sample, and the same SCNS-SF34 item was best discriminated by QLQ-C30 scores. Cutoff scores were identified with sensitivity $\geq$0.84 and specificity $\geq$0.54.
Conclusion Given these findings' concordance with our previous analysis, these QLQ-C30 cutoffs could be implemented in clinical practice and their usefulness evaluated.

Keywords EORTC-QLQ-C30 · Patient-reported outcomes · Clinical practice · Cancer

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Published online: 27 March 2013

Abbreviations

AUC	Area under the curve
ECOG	Eastern Cooperative Oncology Group
EORTC-QLQ-C30	European Organization for the Research and Treatment of Cancer Quality of Life Questionnaire Core 30
NPV	Negative predictive value
PPV	Positive predictive value
PRO	Patient-reported outcome
ROC	Receiver operating characteristic
SCNS-SF34	Supportive Care Needs Survey-Short Form-34

Introduction

The use of patient-reported outcome (PRO) measures in clinical practice for individual patient management involves having a patient complete a questionnaire about his/her functioning and well-being and providing that patient's scores to his/her clinician to inform care and management [1, 2]. The procedure is analogous to laboratory tests that inform the clinician about the patient's health—the difference being that PROs are based on scores from patient-reported questionnaires rather than values from chemical or microscopic analyses. The use of PROs for individual patient management has been consistently shown to improve clinician–patient communication [3–6]. It has also been shown to improve detection of problems [6–9], affect management [5], and improve patient outcomes, such as symptom control, health-related quality-of-life, and functioning [3, 10, 11].

Although we have demonstrated that PROs can effectively identify the issues that are bothering patients the most [12], an ongoing challenge to the use of PROs in clinical practice is determining which scores require a clinician's attention. That is, after patients complete the PRO questionnaire, their responses are scored and a score report is generated. However, for clinicians reviewing the scores, it is not intuitive which scores represent a problem that should motivate action. Various methods have been applied to assist with score interpretation, including providing the mean score for the general population for comparison [3] or highlighting scores using the lowest quartile from the general population as a cutoff [13]. However, these methods do not actually reflect whether a score represents an unmet need from the perspective of the patient, which would require a clinician's attention.

To address this issue, in a previous study, we used the Supportive Care Needs Survey-Short Form (SCNS-SF34) to determine cutoff scores on the European Organization for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire-Core 30 (QLQ-C30) that identify unmet needs [14]. We demonstrated that QLQ-C30 scores can discriminate between patients with and without unmet needs; however, the study was conducted in a limited sample ($n = 117$) of breast, prostate, and lung cancer patients from a single institution. The present analysis was undertaken to attempt to replicate the findings using a new and larger sample.

Patients and methods

Research design and data source

The objective of this study was to test the replicability of the QLQ-C30 cutoff scores from our previous study. To address this objective, we conducted a secondary analysis of data originally collected in the validation study of the Japanese version of the Supportive Care Needs Survey-Short Form (SCNS-SF34-J). The methods of this Japanese study have been reported previously [15]. Briefly, ambulatory breast cancer patients were recruited from the Oncology, Immunology and Surgery outpatient clinic of Nagoya City University Hospital. Inclusion criteria included diagnosis of breast cancer, age at least 20 years, awareness of cancer diagnosis, and Eastern Cooperative Oncology Group (ECOG) performance status of 0–3. Exclusion criteria were severe mental or cognitive disorders or inability to understand Japanese. Participants were selected at random using a list of visits and a random number table to limit the number of patients enrolled each day.

After providing written consent, subjects completed a paper survey that included the SCNS-SF34-J (validated in the parent study [15]) and the Japanese version of the EORTC-QLQ-C30 (described below). In addition to these PRO questionnaires, the survey included basic sociodemographic questions. Patients were instructed to return the completed survey to the clinic the following day, and follow-up by telephone was used to clarify inadequate answers. The attending physician provided ECOG performance status, and information on cancer stage and treatments was abstracted from the patients' medical records.

The SCNS-SF34 was originally developed by investigators in Australia to identify unmet needs cancer patients have in five domains: physical and daily living, psychological, patient care and support, health system and information, and sexual [16, 17]. The 34-item questionnaire uses five response options: 1 = not applicable, 2 = satisfied, 3 = low unmet need, 4 = moderate unmet need, and 5 = high unmet need and a recall period of the "last month." To calculate domain scores, we averaged the

scores of the items within the domain; thus, domain scores >2.0 reflected some level of unmet need.

The QLQ-C30 [18] is a cancer health-related quality-of-life questionnaire that has been widely used in clinical trials and investigations using PROs for individual patient management [3, 6, 11, 19]. It includes five function domains (physical, emotional, social, role, and cognitive), eight symptoms (fatigue, pain, nausea/vomiting, constipation, diarrhea, insomnia, dyspnea, and appetite loss), as well as global health/quality-of-life and financial impact. Subjects respond on a four-point scale from "not at all" to "very much" for most items. Most items use a "past week" recall period. Raw scores are linearly converted to a 0–100 scale with higher scores reflecting higher levels of function and higher levels of symptom burden. The Japanese version of the QLQ-C30 has been validated previously [20].

The Japanese study was approved by the Institutional Review Board and Ethics Committee of Nagoya City University Graduate School of Medical Sciences [15]. A de-identified dataset was provided to the Johns Hopkins investigators for this analysis, which was exempted for review by the Johns Hopkins School of Medicine Institutional Review Board.

Analyses

The data were analyzed using the methods applied in the original study using the SCNS-SF34 to identify cutoff scores on the QLQ-C30 that represent unmet need [14]. First, we dichotomized the SCNS-SF34 item and domain scores into no unmet need (scores ≤ 2.0) versus some unmet need (scores > 2.0). We then tested the ability of QLQ-C30 domain scores to discriminate between patients with and without an unmet need using the SCNS-SF34 domains and items we tested in our previous analysis (see Table 1 for a summary of the SCNS-SF34 items/domains tested for each QLQ-C30 domain). Variables for the discriminant analysis were selected to correspond as closely as possible to the content of the QLQ-C30 domains. In some cases, the content was quite similar (e.g., pain on the QLQ-C30 and pain on the SCNS-SF34). For a few QLQ-C30 domains, there was no SCNS-SF34 item or domain with similar content. In these cases we used a generic SCNS-SF34 item such as "feeling unwell a lot of the time."

The discriminative ability of each QLQ-C30 domain score was summarized using the area under the receiver operating characteristic (ROC) curve (AUC). The AUC summarizes the ability of QLQ-C30 scores to discriminate between patients with and without a reported unmet need. Higher AUCs indicate better discriminative ability. For the domains with AUC ≥ 0.70, we then calculated the sensitivity and specificity, as well as the positive and negative predictive values, associated with various QLQ-C30 cutoff scores. We used a threshold of AUC ≥ 0.70 because Hosmer and Lemeshow suggest that values below 0.70 represent poor discrimination, between 0.70 and 0.80 represent acceptable discrimination, and above 0.80 represent excellent discrimination [21]. It was also the standard used for our previous analysis [14]. We hypothesized that compared to our original analysis, (1) the same QLQ-C30 domains would have AUC ≥ 0.70, (2) the same SCNS-SF34 items would be best discriminated by the QLQ-C30 and thus provide the highest AUC, and (3) the sensitivity and specificity of our original cutoff scores would be supported. Analyses were performed using statistical freeware R version 2.15.1.

Results

The sample has been described previously [15]. Briefly, from a pool of 420 potential participants, 12 were excluded due to declining participation ($n = 7$), cognitive deficits ($n = 2$), advanced disease ($n = 1$), and failure to respond after consenting ($n = 2$). The study sample included 408 subjects with a mean age of 56 years, 100 % female, 76 % married, and 45 % employed full- or part-time. The ECOG performance status was 0 for 90 % of the sample; the clinical stage was I or II for 71 %; 93 % had received surgery, 44 % chemotherapy, and 39 % radiation; and the median time from diagnosis was 701 days (range 11–17,915 days). Complete data were available for all 408 subjects, with the exception of one participant who was missing a single SCNS-SF34 item. That observation was excluded from analyses that required that item.

Table 1 shows which SCNS-SF34 items/domains were used to evaluate the discriminative ability for each QLQ-C30 domain, as well as the resulting AUCs both from our original analysis [14] and from this replication analysis. The AUCs were largely similar between studies. As hypothesized, the same six QLQ-C30 domains with AUCs ≥ 0.70 in the original analysis had AUCs ≥ 0.70 in the replication sample. Further, the SCNS-SF34 item that was best discriminated by the QLQ-C30 with the highest AUC in the original analysis also had the highest AUC in the replication sample. The following QLQ-C30 domain–SCNS-SF34 item pairings were used: physical function–work around the home (AUC = 0.74), role function–work around the home (AUC = 0.70), emotional function–feelings of sadness (AUC = 0.75), pain–pain (AUC = 0.74), fatigue–lack of energy/tiredness (AUC = 0.75), and global health/QOL–feeling unwell a lot of the time (AUC = 0.76).

Using these pairings, we evaluated the sensitivity, specificity, and predictive value of various cutoff scores on the QLQ-C30 (Table 2). Again, the results were largely similar between the original analysis and this replication

Table 1 Hypothesized relationship between QLQ-C30 and SCNS-SF34 domains and resulting areas under the curve (AUC): original and replication analysis

QLQ-C30 Domain	SCNS-SF34 Domain/Item(s)	AUC	
		Original Analysis [14]	Replication Analysis
Hypothesized AUC≥0.70			
Physical Function	Physical & daily living needs (overall score and individual items)	0.69–0.81	0.69–0.74
Role Function	Work around the home Not being able to do the things you used to	0.71–0.73	0.70–0.70
Emotional Function	Psychological needs (overall score and individual items)	0.56–0.74	0.61–0.75
Pain	Pain	0.78	0.74
Fatigue	Lack of energy/tiredness	0.74	0.75
Global Health /QOL	Feeling unwell a lot of the time	0.73	0.76
Hypothesized AUC <0.70			
Social Function	Not being able to do the things you used to	0.64	0.68
Sleep	Lack of energy/tiredness Feeling unwell a lot of the time Being given information about aspects of managing your illness and side effects at home	0.41–0.51	0.39–0.55
Cognitive Function	Feeling unwell a lot of the time Being given information about aspects of managing your illness and side effects at home	0.54–0.60	0.53–0.63
Nausea/vomiting		0.19–0.36	0.22–0.27
Dyspnea		0.37–0.48	0.32–0.48
Appetite Loss		0.47–0.49	0.32–0.49
Constipation		0.31–0.37	0.32–0.40
Diarrhea		0.34–0.34	0.18–0.21

sample. Examples of cutoff scores (sensitivity, specificity) from the replication sample are as follows: physical function <90 (0.85, 0.65); role function <90 (0.85, 0.62); emotional function <90 (0.84, 0.60); global health/QOL < 70 (0.86, 0.56); pain >10 (0.93, 0.54); and fatigue >30 (0.86, 0.62). Thus, each domain had at least one cutoff score with sensitivity ≥0.84 and specificity ≥0.54. This means that patients who reported unmet needs in a domain were identified correctly at least 84 % of the time and that patients who reported no unmet needs in a domain were identified correctly at least 54 % of the time using these cutoffs. In general, the negative predictive values (NPVs) associated with these cutoffs were higher than the positive predictive values (PPVs), with the NPVs ranging from 0.86 to 0.94 and PPVs ranging from 0.33 to 0.58. This means that if a patient was identified by the cutoff as not having an unmet need in a domain, 86–94 % of the time they did not report an unmet need and that if a patient was identified by the cutoff as having an unmet need, 33–58 % of the time they actually did report an unmet need. While we describe these cutoff scores for illustrative purposes, the specific cutoff scores used in a given application should be determined based on the relative importance of sensitivity and specificity.

Discussion

This analysis was undertaken to test the generalizability of the findings from our previous study which evaluated the ability of different cutoff scores on the QLQ-C30 to identify patients with an unmet need in a given domain. Such cutoff scores facilitate the interpretation of PROs used clinically for individual patient management by helping clinicians determine which scores deserve further attention. Currently, there are few guides available to help clinicians determine which PRO scores represent a problem. For example, in PatientViewpoint, the PRO webtool used at Johns Hopkins [13, 22], we highlight in yellow QLQ-C30 domain scores representing the lowest quartile based on published general population norms [23] as an indication to the clinician reviewing the report that the patient may be having a problem in this area. However, these cutoff scores using distributions of the data are not empirically based on whether the score is likely to represent a problem from the patient's perspective. For example, the results from this analysis suggest that domain scores <90 on role or emotional function likely represent a patient-reported unmet need. However, at our institution, we are currently using cutoff scores <66.7 for these two

Table 2 Sensitivity and specificity of example cutoff scores: original and replication analysis

QLQ-C30 Domain	SCNS-SF34 Item	Cutoff	Cohort	Sensitivity	Specificity	Positive Predictive Value	Negative Predictive Value
Physical Function	Work around the home	80	Original [14]	0.65	0.83	0.55	0.89
			Replication	0.40	0.92	0.63	0.82
		90	Original [14]	0.85	0.58	0.39	0.92
			Replication	0.85	0.65	0.45	0.93
Role Function	Work around the home	80	Original [14]	0.69	0.79	0.50	0.89
			Replication	0.69	0.79	0.52	0.88
		90	Original [14]	0.85	0.69	0.46	.94
			Replication	0.85	0.62	0.43	0.93
Emotional Function	Feelings of sadness	90	Original [14]	0.89	0.53	0.48	0.91
			Replication	0.84	0.60	0.58	0.86
		100	Original [14]	0.94	0.35	0.41	0.93
			Replication	0.92	0.42	0.51	0.89
Global Health/QOL	Feeling unwell a lot of the time	70	Original [14]	0.71	0.69	0.52	0.84
			Replication	0.86	0.56	0.33	0.94
		80	Original [14]	0.89	0.58	0.50	0.91
			Replication	0.89	0.45	0.29	0.94
Pain	Pain	20	Original [14]	0.66	0.84	0.64	0.85
			Replication	0.70	0.81	0.62	0.86
		10	Original [14]	0.91	0.66	0.54	0.95
			Replication	0.93	0.54	0.47	0.94
Fatigue	Lack of energy/ tiredness	30	Original [14]	0.77	0.71	0.73	0.75
			Replication	0.86	0.62	0.54	0.90
		20	Original [14]	0.91	0.55	0.68	0.86
			Replication	0.97	0.42	0.46	0.97

domains, based on the population distribution of scores. This means that our current cutoffs are missing patients with unmet needs with scores between 67 and 90. Based on the results of this analysis, we will explore changing the cutoffs to those presented here to highlight QLQ-C30 scores for the clinician's attention.

Our findings should be interpreted in the context of the study's strengths and limitations. First, the approach of using the SCNS-SF34 to identify QLQ-C30 cutoff scores only works well for the six QLQ-C30 domains where there is content overlap between the SCNS-SF34 and QLQ-C30. For the domains without a corresponding SCNS-SF34 item to use for comparison, we do not have indicators of appropriate cutoffs. Future research could address this issue by using items similar in format to the SCNS-SF34 but covering the content of the relevant QLQ-C30 domains for which no data are currently available. Also, the SCNS-SF34 uses a recall period of the "past month," whereas the QLQ-C30 generally uses a recall period of the "past week." Ideally, the comparison between scores would be made with questionnaires that use the same recall period. The study design used in both the current sample and the original analysis was cross-sectional, so while absolute cutoff scores can be identified, important changes in scores are not addressed. Research from longitudinal studies using both the QLQ-C30 and SCNS-SF34 could explore changes in scores representing an unmet need.

Notably, this validation sample used QLQ-C30 and SCNS-SF34 data collected using the Japanese versions of the questionnaires. That we found such similarity between our original analysis and the current sample, despite differences in language and culture, suggests that these findings are robust. While the Japanese study provided a new sample to test our original cutoffs, and almost four times as many patients, only breast cancer patients were enrolled in the Japanese study, whereas our original analysis included three different cancer types (breast, prostate, and lung). Also, the Japanese sample included women with a wide range of time since diagnosis (11–17,915 days). The symptom burden for women who had completed treatment years previously may be lower than for women in active treatment. Nevertheless, given the substantial concordance between this replication sample and our original sample, we believe there is adequate evidence to support implementing these cutoffs in Patient-Viewpoint and other applications of the QLQ-C30 being used in clinical practice.

The next important step will be to evaluate whether clinicians and patients find these cutoffs helpful. A key

consideration is which cutoff to use. We presented several example cutoff scores for illustrative purposes here, but the cutoff scores appropriate for a specific application depend on the relative importance between sensitivity and specificity. That is, the more likely a cutoff score is to identify patients with unmet needs (true positives), the more likely it will also identify patients without an unmet need (false positives). Thus, it is important to consider the implications of false positives versus false negatives.

In general, the use of PROs for individual patient management involves helping the clinician identify problems the patient may be experiencing and facilitating a focused discussion of PRO topics that might otherwise go unaddressed. This is essentially a screening function. We therefore expect follow-up of a "positive" score based on the cutoff to involve the clinician simply asking the patient about the issue and determining whether there is something that can and should be done to address any unmet needs. Given that this requires a minimal effort, it may be appropriate to favor high sensitivity over high specificity. However, it is also important to avoid alert fatigue, a phenomenon that leads to clinician inattention to potential problems and resistance to the tools in general. In addition, if the cutoff scores were to be applied by, for example, generating an automatic page to the clinician, then false positives would be much more problematic. Another issue is how to address PRO scores representing an unmet need. In previous research, we developed a range of suggestions for how to address issues identified by PRO questionnaires [24]. However, it is important to consider resource and reimbursement limitations for certain services (e.g., psychosocial services, home care), as well as their effectiveness, before implementing them as part of care pathways. Consideration of how these cutoff scores will be applied in practice will help determine the appropriate compromise between sensitivity and specificity.

In summary, this analysis was conducted to replicate our original analysis to determine whether specific cutoff scores effectively identify patients with unmet needs. For the QLQ-C30 domains with appropriate SCNS-SF34 content matches, our findings from the original analysis were largely supported. This suggests that these cutoff scores could be applied in practice, with an evaluation of their effectiveness from the clinician and patient perspectives. Specifically, it will be important to see how clinicians actually respond when presented with information from PROs using these (or other appropriate) cutoffs and whether the information helps increase clinicians' awareness of unmet needs. Further research is also needed to identify cutoff scores for QLQ-C30 domains without SCNS-SF34 content matches, as well as to identify changes in scores that represent unmet need. In the meantime, the results for these six domains provide critical guidance to clinicians interpreting PRO reports on which scores require their attention.

Acknowledgments This analysis was supported by the American Cancer Society (MRSG-08-011-01-CPPB). The original data collection was supported in part by Grants-in-Aid for Cancer Research and the Third Term Comprehensive 10-Year Strategy for Cancer Control from the Ministry of Health, Labour and Welfare, Japan. Drs. Snyder and Carducci are members of the Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins (P30CA006973). The funding sources had no role in study design, data collection, analysis, interpretation, writing, or decision to submit the manuscript for publication.

Conflict of interest The authors report no conflict of interest.

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革新的癌ワクチン，H/K-HELPの開発
――ショートペプチドからヘルパー/キラーロングペプチドへの移行
Helper/killer-hybrid epitope long peptide（H/K-HELP）

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◎本稿では，ヘルパーエピトープとキラーエピトープを化学的に結合させた人工癌抗原ロングペプチド（helper/killer hybrid epitope long peptide：H/K-HELP）癌ワクチン開発に至る癌免疫の基盤研究と，その成果の臨床研究への応用，そしてなぜ H/K-HELP ロングペプチドワクチンが，従来のショートペプチドに比べ有効であるかを解き明かす基盤研究の成果について概説したい．

Key word：癌ワクチン治療，H/K-HELP癌ワクチン，ヘルパー T細胞，Th1細胞，CTL

1991 年のテリー・ブーン博士らによる癌抗原の発見によって，癌に対する特異的免疫誘導が可能であることが示された[1]．アミノ酸 8～9 個からなるクラス Ⅰ 結合性癌抗原キラーペプチドを用いた癌ワクチン治療の臨床研究は，一時は無効とされたが，最近では制癌剤との併用により，あるいは数種のペプチドを混合したマルチペプチドを用いて，癌組織の縮小は認められない場合が多いものの，癌特異的 CTL が弱いながらも誘導され，癌患者の生存日数が大幅に延長されることが示されている[2]．さらに，最近ではクラス Ⅱ 結合性癌抗原ヘルパーペプチドの同定もなされ，ヘルパー T 細胞とキラー T 細胞の両者を活性化できる synthetic long peptide（SLP）の混合ワクチンがオランダの Melief らによって開発され，HPV で誘発されるヒト外陰部上皮異形成の治療効果があることが示された[3]．

著者らは 30 数年に及ぶ基盤的癌免疫研究から，より有効な癌免疫治療を開発するためには，①担癌生体の免疫抑制性癌エスケープ機構の解明と，②宿主免疫抑制を打破するためのヘルパー T 細胞，とくに癌特異的 Th1 細胞の活性化が重要であることを提唱してきた[4-6]．最近，①に関してはあらたな分子メカニズムや免疫抑制性細胞群が明らかにされ，さらには癌治療抵抗性を担う癌幹細胞の存在も明らかになってきている．また，②に関しては癌抗原ヘルパーエピトープの同定により癌特異的 Th1 細胞の誘導が可能となり，さらに，ヘルパーエピトープとキラーエピトープを化学的に結合させた人工癌抗原ロングペプチド（helper/killer hybrid epitope long peptide：H/K-HELP）も開発され，臨床研究において Th1 依存的免疫の誘導効果や癌消失効果も証明されている[7]．

担癌生体における免疫抑制・癌エスケープ機構

癌患者末梢血リンパ球は健常人のそれに比べ，異常に T 細胞応答が低下している．これは癌が増殖とともに宿主の免疫応答を抑制し，癌が増殖しやすい場を形成するためと考えられる．従来は，①癌細胞における MHC の消失，②癌細胞あるいは免疫担当細胞による免疫抑制因子（TGF-β や IL-10）の産生などが免疫逃避のおもなメカニズムとして報告されてきた．しかし最近は，担癌生体の癌局所において異常に集積する $CD4^+$ $Foxp3^+$ 制御生 T 細胞（Treg），$CD11b^+Gr\text{-}1^+$ 未