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Novel classification based on immunohistochemistry combined with hierarchical clustering analysis in non-functioning neuroendocrine tumor patients

Shinya Iida,[1] Yasuhiro Miki,[1] Katsuhiko Ono,[1] Jun-ichi Akahira,[1] Takashi Suzuki,[1] Kazuyuki Ishida,[2] Mika Watanabe[2] and Hironobu Sasano[1,2,3]

[1]Department of Pathology, Tohoku University Graduate School of Medicine, Sendai, Miyagi; [2]Department of Pathology, Tohoku University Hospital, Sendai, Miyagi, Japan

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Somatostatin analogues ameliorated many symptoms caused by neuroendocrine tumors (NET), but their antitumor activities are limited especially in non-functioning cases. An overactivation of signaling pathways under receptor tyrosine-kinase (RTK) has been recently demonstrated in some NET patients, but its details have remained largely unknown. Therefore, in this study, we immunolocalized therapeutic factors and evaluated the data to study the clinical significance of the molecules in non-functioning Japanese gastrointestinal NET. Fifty-two NET cases were available for examination in this study and expression of somatostatin receptor (sstr) 1, 2A, 2B, 3 and 5, activated form of mammalian target of rapamycin (mTOR), eukaryotic initiation factor 4-binding protein 1 (4EBP1), ribosomal protein s6 (S6), extracellular signal-regulated kinase (ERK) and insulin-like growth factor 1 receptor (IGF-1R) was evaluated using immunohistochemistry. We then studied the correlation among the immunohistochemical results of the individual cases using hierarchical clustering analysis. Results of clustering analysis demonstrated that NET cases were basically classified into Cluster I and II. Cluster I was associated with higher expression of sstr1, 2B and 3 and Cluster II was characterized by an activation of the PI3K/Akt pathway and IGF-1R and higher proliferative status. Cluster II was further classified into Cluster IIa and IIb. Cluster IIa was associated with higher expression of sstr1 and 5 and higher proliferative status and Cluster IIb was characterized by ERK activation. Hierarchical clustering analysis of immunoreactivity of the therapeutic factors can classify NET cases into three distinctive groups and the medical treatment may be determined according to this novel classification method for non-functioning NET patients. (*Cancer Sci* 2010; 101: 2278–2285)

Gastroenteropancreatic endocrine tumors or neuroendocrine tumors (NET) are generally classified into two groups in terms of their localization: gastrointestinal (GI) NET or endocrine tumor and pancreatic NET.[1] Both of these tumors are considered to arise from neuroendocrine cells and are histologically characterized by positive reactions to various neuroendocrine markers, including chromogranin A, neuron specific enolase (NSE), synaptophysin, and so on. In Japan, the number of GI NET patients is far more than that of pancreatic NET[2] and the great majority of these cases are clinically non-functioning NET. A GI NET is often slow-growing and indolent, and may not become clinically apparent until the manifestation of metastatic spread,[3,4] especially those occurring in the foregut and hindgut, the most prevalent GI NET in Japanese population.[2] Therefore, it has become clinically important to manage these non-functioning advanced NET cases using medical therapy.

We previously reported that somatostatin receptor (sstr) subtypes have been recently demonstrated in the great majority of GI NET.[5] In addition, somatostatin analogues (SSA) such as octreotide that interact with sstr subtypes are the most widely used therapeutic option for NET. They are generally well tolerated and highly effective in reducing various hormone-related symptoms caused by NET, but are not necessarily so in controlling cell proliferation of tumor cells, especially in clinically non-functioning NET cases.[6] Therefore, it has become important to examine the mechanisms related to cell proliferation, especially those related to intracellular signaling pathways, other than the somatostatin pathway in individual NET patients, in order to further explore effective antitumor agents.

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase working as a central regulator of many biological processes that are essential for cell proliferation, translation and cell metabolism.[7–9] It is regulated by upstream, phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR signaling pathway.[10] An activation of this pathway has been commonly detected in various tumor types,[11] and may be caused by an overactivation of insulin-like growth factor 1 receptor (IGF-1R) in NET cells.[12] In addition, a previous study demonstrated an overexpression of activated (phosphorylated) extracellular signal-regulated kinase (ERK) in NET cases.[13] However, the correlation among these pathways and of those with sstr-mediated pathways has not been reported at all in NET cases.

Therefore, in our present study, we first evaluated sstr subtypes, those involved in the signaling pathway under receptor tyrosine-kinase (RTK) (PI3K/Akt/mTOR and MEK/ERK pathways) and a major therapeutic targeted RTK (IGF-1R) in 52 clinically non-functioning Japanese NET cases using immunohistochemical methods. We then examined the correlation among these factors above and that with the clinicopathological factors of individual patients using hierarchical clustering analysis, an established effective method for analyzing high-volume immunohistochemical data, which was recently applied to an analysis of leiomyosarcoma and breast carcinoma cases.[14,15] We then added the *in vitro* experiments in order to further evaluate the antitumor activities of rapamycin, one of the mTOR inhibitors.

Materials and Methods

Tumor tissues. Fifty-two Japanese cases of GI NET or endocrine tumors were retrieved from the surgical pathology files at the Department of Pathology, Tohoku University Hospital and Sendai Red Cross Hospital (both in Sendai, Miyagi, Japan). Review of the charts of individual patients showed that none of the cases examined had demonstrated any clinical evidence of

[3]To whom correspondence should be addressed.
E-mail: hsasano@patholo2.med.tohoku.ac.jp

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endocrine abnormalities prior to surgery and all tumors were diagnosed as non-functional GI NET. The specimens had all been fixed in 10% formalin and embedded in paraffin. The clinicopathological features are summarized in Table 1. We classified the status of age (years) into <60 or ≥60 based on the reported average year of NET patients in Japan.[2] Epidemiological studies in Japanese patients of GI NET demonstrated that the frequency of *MEN1* genetic abnormalities was only 1%.[2,16] However, further investigation is required for clarification. Research protocols for this study were approved by the Ethics Committee at the Tohoku University School of Medicine (2008-122) and Sendai Red Cross Hospital (No. 32).

Immunohistochemistry. Tissue specimens were immunostained using a biotin-streptavidin method with a Histofine kit (Nichirei Co. Ltd, Tokyo, Japan: phospho-ribosomal protein [p-S6], phospho-eukaryotic initiation factor 4-binding protein 1 [p-4EBP1], p-ERK, sstr subtypes and Ki-67) and an EnVision method (Dako, Kyoto, Japan: p-mTOR and p-IGF-1R). In the present study, we did not evaluate the immunoreactivity of sstr4, because sstr4 was rarely reported in NET cells.[17,18] The characteristics of antibodies used in our immunostaining are summarized in Table 2. Antigen retrieval for p-mTOR, p-S6, p-4EBP1, and p-ERK analyses was performed by heating the slides in a microwave at 500 W for 15 min in citric acid buffer (2 mM citric acid and 9 mM trisodium citrate dehydrate, pH 6.0). Antigen retrieval for Ki-67 and sstrs was performed by heating the slides in an autoclave at 121°C for 5 min in citrate acid buffer. No treatment for antigen retrieval was performed in immunostaining for p-IGF-1R. These slides were further incubated with primary antibodies for 36–48 h in a moist chamber at 4°C. The antigen-antibody complex was then visualized with 3,3′-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6 and 0.006% H_2O_2) and counterstained with hematoxylin. Immunoreactivity for p-mTOR, p-4EBP1, p-S6 and p-ERK was detected in the cytoplasm of tumor cells, and classified into three groups as follows: score 0, negative; score 1, weakly positive; score 2, strongly positive (Table 3). Immunoreactivity for sstr subtypes was detected in the membrane or cytoplasm of tumor cells. An evaluation of immunoreactivity of sstrs was performed as previously reported (Table 3).[5]

Table 1. Summary of clinicopathological findings in 52 non-functioning neuroendocrine tumor cases examined in the present study

	No. cases (n = 52)
Age (years)	
<60	35 (67.3%)
≥60	17 (32.7%)
Mean ± SD	51.3 ± 15.4
Gender	
Male	32 (61.5%)
Female	20 (38.5%)
Localization†	
Foregut	15 (28.9%)
Midgut	2 (3.8%)
Hindgut	35 (67.3%)
Lymph metastasis	
Presence	3 (5.8%)
Absence	49 (94.2%)
Vascular invasion	
Presence	14 (26.9%)
Absence	38 (73.1%)

†Details of localization are as follows: foregut, 15 (lung, 5; stomach, 4; bronchus, 2; duodenum, 2; liver, 1; middle ear, 1); midgut, 2 (appendix, 2); hindgut 36 (rectum, 35; sigmoid colon, 1).

Immunoreactivity for p-IGF-1R was classified into two groups as follows: score 0, negative; score 1, positive. Representative illustration of immunohistochemistry is shown in Figure 1. Ki-67 immunoreactivity was evaluated in more than 1000 cells and the percentage of immunoreactivity (i.e. labeling index [LI]) was subsequently obtained. We scored the Ki-67 LI followed by the histopathological grade in NET recently defined by the European Neuroendocrine Tumor Society (ENETS) as follows: score 0, <2%; score 1, 2–20%; score 2, >20% (Table 3).[19] Two of the authors (S.I. and Y.M.) independently evaluated immunoreactivity and the cases of interobserver differences of more than 5% were re-evaluated together using double-headed light microscopy. Intraobserver differences were <5%.

Cell culture and reagents. NCI-H727 (H727), a human bronchial NET cell line, was purchased from the American Type Culture Collection (Manassas, VA, USA). COLO320-DM (COLO), a human colon NET cell line, was purchased from The Health Science Research Resources Bank (Osaka, Japan). These cell lines were maintained in RPMI-1640 medium (Sigma Aldrich Co., St Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS; Nichirei Co. Ltd, Tokyo, Japan). Cells were maintained at 37°C, 95% relative humidity and 5% CO_2. Rapamycin was purchased from Wako (Osaka, Japan).

Cell proliferation assay. The status of cell proliferation of H727 and COLO cells was determined using WST-8 (2-[2-methoxy-4-nitrophenyl]-3-[4-nitrophenyl]-5-[2, 4-disulfophenyl]-2H-tetrazolium monosodium salt) method (Cell Counting kit-8; Dojindo Inc., Kumamoto, Japan). Cells were seeded in 96-well plates at a density of 5000 cells/well. After 24 h of incubation, different concentrations of rapamycin were added into the medium. Compound was added with the exchange of medium every 3 days and measured for 3, 6 and 9 days (H727) or 1, 2 and 3 days (COLO). The medium including reagents was changed every 3 days. A volume of 10 μL of 5 mM WST-8 was added and the plates were then incubated for 1–4 h at 37°C in 95% relative humidity and 5% CO_2. The resulting optical densities (OD; 450 nm) were obtained using a Model 680 microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). The status of cell proliferation (%) was calculated according to the following equation: (cell OD value after treatment with test materials/vehicle control cell OD value) ×100.

Statistical analysis. We used hierarchical clustering analysis to sort the results of the immunohistochemistry and to further evaluate the correlation of immunohistochemical data with clinicopathological findings of individual NET cases. Hierarchical clustering analysis attempts to identify homogeneous subgroups of the cases examined are as reported by Eisen *et al.*[20] The correlation between individual cases and cell signaling factors is depicted graphically as a dendrogram in which branch length is determined by the distance between the results of the immunohistochemistry. Data were subjected to hierarchical clustering analysis and visualization using Cluster and TreeView, respectively (downloaded from the Eisen Lab, Barkley, CA, USA). Chi-squared tests were used to determine which markers contributed to the formation of individual clusters. The statistical analysis on the results of cell proliferation was analyzed with Sheffe test (StatView ver. 5.0, SAS institute, Cary, NC, USA). A P-value < 0.05 indicated the statistical significance in this study.

Results

Immunoreactivity in cell-signaling molecules in NET cases. Results of immunohistochemical staining of sstr subtypes, activated (phosphorylated) forms of intracellular signaling factors (mTOR, 4EBP1, S6 and ERK) and IGF-1R examined are summarized in Table 4 and Figures 2 and 3. Immunoreactivity for

Table 2. Antibodies and their conditions of immunostaining

Primary antibody	Dilution	Source	Antigen retrieval	Positive control
p-mTOR	1/50	Cell Signaling Technology	MW	Rectum
p-4EBP1	1/50	(Beverly, MA, USA)		
p-S6	1/100			
p-p44/42 MAPK (p-ERK)	1/100			
sstr1, 2A, 2B, 3, 5	1/1000	Gramsh Laboratories (Schwabhausen, Germany)	MW	Pancreas
p-IGF-1R	1/100	Abnova (Taipei, Taiwan)	No treatment	Breast carcinoma
Ki-67	1/100	Dako Cytomation (Glostrup, Denmark)	AC	Tonsil

AC, autoclave treatment; 4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; MAPK, mitogen-activated protein kinase; mTOR, mammalian target of rapamycin; MW, microwave treatment; S6, ribosomal protein s6; sstr, somatostatin receptor.

Table 3. Summary of scoring of immunoreactivity used in the present study

Primary antibody	Score 0	Score 1	Score 2
p-mTOR	Negative	Weakly positive	Strongly positive
p-4EBP1			
p-S6			
p-ERK			
Ki-67 LI	<2%	2–20%	>20%
sstr1, 2A, 2B, 3, 5	Negative	Positive	
p-IGF-1R			

4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

p-mTOR, p-4EBP1, p-S6 and p-ERK was detected in the cytoplasm of tumor cells in 33 (63.5%), 43 (82.7%), 27 (51.9%) and 18 (34.6%) of 52 NET cases examined, respectively. Immunoreactivity for sstr1, 2A, 2B, 3, 5 and p-IGF-1R was detected in the membrane or cytoplasm of tumor cells in 27 (51.9%), 48 (92.3%), 20 (38.5%), 29 (55.8%), 39 (75.0%) and 38 (73.1%) of 52 cases examined, respectively.

Hierarchical clustering analysis of immunohistochemical results in individual clusters. Hierarchical clustering analysis was applied to results of the immunohistochemistry in NET cases and the correlation was subsequently displayed graphically using the computer program, Cluster and TreeView (downloaded from Eisen Lab; Fig. 4). The patterns of each sstr subtypes obtained were nearly identical in terms of staining patterns in the great majority of tumors, that is, co-expressing all or none of these markers, especially in those of sstr2A and 5. In addition, there was an almost identical scoring pattern among p-IGF-1R, p-mTOR and p-4EBP1 and also between p-ERK and p-S6, respectively, which indicated that activation of S6 was correlated more with the MEK/ERK pathway rather than the PI3K/Akt pathway in GI NET cases.

The results showed that the 52 NET cases examined were basically classified into two clusters, Cluster I (18 cases) and Cluster II (34 cases), and Cluster II was further sub-classified into Cluster IIa (15 cases) and Cluster IIb (17 cases) according to the branch length, which represents the correlation of the scoring data (Fig. 4). Two cases belonging to Cluster II were eliminated because of the branch length.

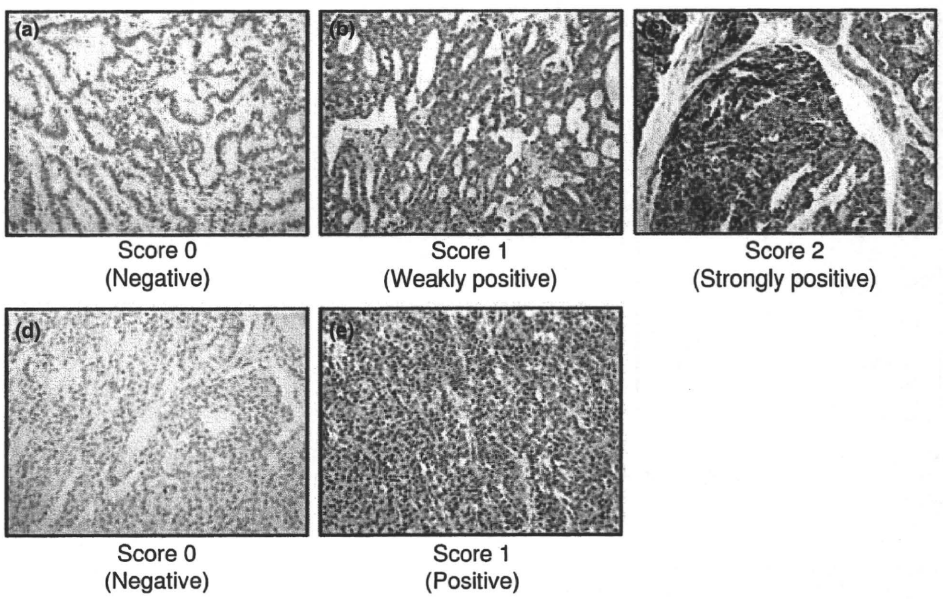

Fig. 1. Representative illustrations of immunohistochemistry. Results of immunohistochemistry were evaluated according to Table 3. (a) p-4EBP1 (score 0, negative); (b) p-4EBP1 (score 1, weakly positive); (c) p-4EBP1 (score 2, strongly positive); (d) somatostatin receptor 1 (sstr1) (score 0, negative); (e) sstr1 (score 1, positive). (a–e) Original magnification, ×100.

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Table 4. Summary of scoring of immunohistochemistry in the present study of neuroendocrine tumor cases

Total (n = 52)	Score 0	Score 1	Score 2	Total (n = 52)	Score 0	Score 1
p-mTOR	19 (36.5%)	29 (55.8%)	4 (7.7%)	sstr1	25 (48.1%)	27 (51.9%)
p-4EBP1	9 (17.3%)	33 (63.5%)	10 (19.2%)	sstr2A	4 (7.7%)	48 (92.3%)
p-S6	25 (48.1%)	24 (46.2%)	3 (5.8%)	sstr2B	32 (61.5%)	20 (38.5%)
p-ERK	34 (65.4%)	18 (34.6%)	0 (0.0%)	sstr3	23 (44.2%)	29 (55.8%)
Ki-67	39 (75.0%)	12 (23.1%)	1 (1.9%)	sstr5	13 (25.0%)	39 (75.0%)
				p-IGF-1R	14 (26.9%)	38 (73.1%)

4EBP1, eukaryotic initiation factor 4-binding protein 1; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

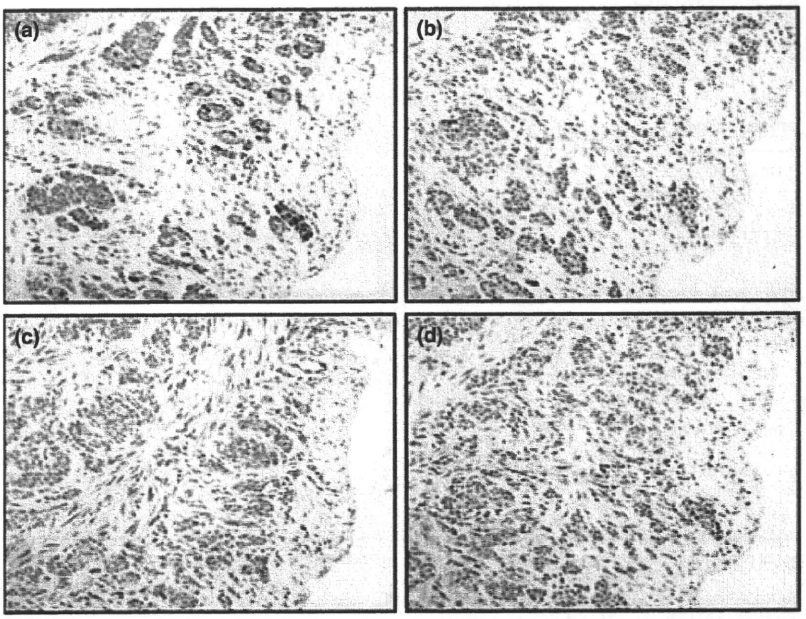

Fig. 2. Representative illustrations of immunohistochemistry of (a) p-mTOR, (b) p-4EBP1, (c) p-S6 and (d) p-ERK. Immunoreactivity of all signaling factors was detected in cytoplasmic of tumor cells. (a–d) Original magnification, ×100.

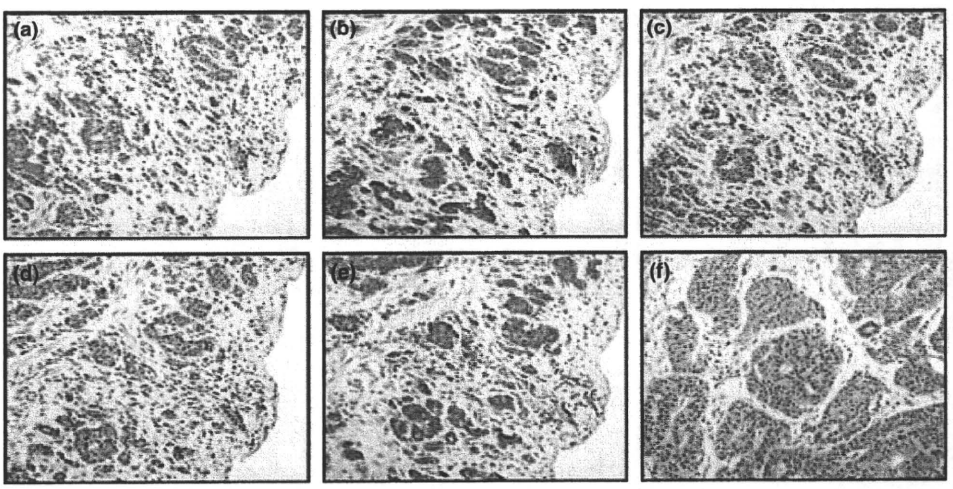

Fig. 3. Representative illustrations of immunohistochemistry of somatostatin receptor (sstr) subtypes and p-IGF-1R. Immunoreactivity of all sstr subtypes was detected in the membrane or cytoplasmic of the tumor cells. (a) sstr1; (b) sstr2A; (c) sstr2B; (d) sstr3; (e) sstr5; (f) p-IGF-1R. (a–f) Original magnification, ×100.

Multivariate analysis of clusters of immunoreactivity and clinicopathological characteristics of NET cases examined. We performed chi-squared tests in order to define the features of each clusters regarding the patterns of immunoreactivity of the factors examined. We first defined the features between Cluster I and Cluster II. Results of this analysis demonstrated that immunoreactivity of p-ERK, sstr2A and sstr5 did not show any significant differences among the three groups above, but that of p-mTOR (P = 0.038), p-4EBP1 (P = 0.026), p-S6 (P = 0.0066), sstr1 (P = 0.0066), sstr2B (P < 0.001), sstr5 (P = 0.0036),

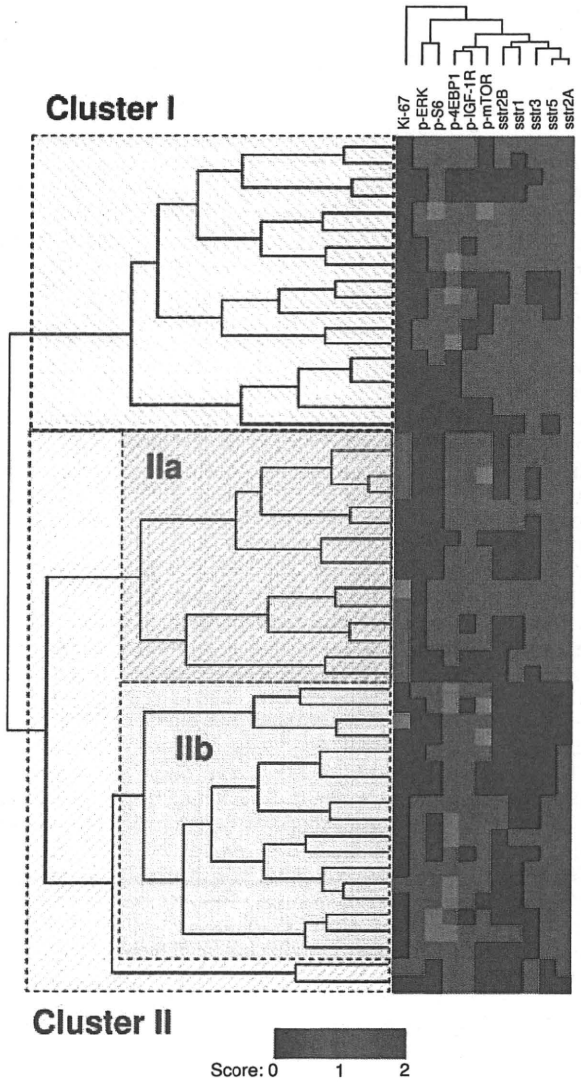

Fig. 4. Summary of hierarchical clustering analysis of the immunohistochemical data of 52 neuroendocrine tumor cases. The branch length represents the similarity between results obtained in this system. Neuroendocrine tumor cases in the present study were classified into the following three different groups according to the results: Cluster I, 18 cases; Cluster IIa, 15 cases; Cluster IIb, 17 cases. Two cases belonging to Cluster II were excluded because of the branch length. 4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor.

p-IGF-1R ($P = 0.016$) and Ki-67 ($P = 0.018$) did show significant differences among the three clusters above (Table 5). These results indicated that the Cluster I cases ($n = 18$) were associated with expression of the sstr subtypes rather than the proteins in the intracellular signaling pathways. In contrast, the Cluster II cases ($n = 34$) were associated with relative abundance of p-mTOR, p-4EBP1 and p-S6, compared with the sstr subtypes above and higher proliferative activities. We then studied the correlation between clinicopathological features of individual cases and the clusters above using chi-squared tests, but there were no significant differences between the clusters of the patients examined (data not shown).

We subsequently performed chi-squared tests between Cluster IIa and Cluster IIb. Results showed that the Cluster IIa cases

Table 5. Summary of scoring of immunohistochemistry between Cluster I and II

	Cluster I ($n = 18$)	Cluster II ($n = 34$)	*P*-value
p-mTOR (Score 0 *vs* 1, 2)	8	25	**0.038**
p-4EBP1 (Score 0 *vs* 1, 2)	12	31	**0.026**
p-S6 (Score 0 *vs* 1, 2)	14	13	**0.0066**
p-ERK (Score 0 *vs* 1, 2)	7	11	0.64
sstr1, positive	14	13	**0.0066**
sstr2A, positive	18	30	0.13
sstr2B, positive	13	7	**<0.001**
sstr3, positive	15	14	**0.0036**
sstr5, positive	15	24	0.31
p-IGF-1R, positive	10	28	**0.016**
Ki-67 LI			
<2%	17	22	**0.018**
≥2%	1	12	

4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor. The bold values indicate the statistical significance.

Table 6. Summary of scoring of immunohistochemistry between Cluster IIa and IIb

	Total ($n = 32$)	Cluster IIa ($n = 15$)	Cluster IIb ($n = 17$)	*P*-value
p-mTOR (Score 0 *vs* 1, 2)	25 (78.1%)	12	13	0.81
p-4EBP1 (Score 0 *vs* 1, 2)	30 (93.8%)	13	17	0.12
p-S6 (Score 0 *vs* 1, 2)	14 (43.8%)	4	10	0.067
p-ERK (Score 0 *vs* 1, 2)	11 (34.3%)	0	11	**<0.001**
sstr1, positive	13 (40.6%)	10	3	**0.0048**
sstr2A, positive	29 (90.6%)	15	14	0.087
sstr2B, positive	7 (21.9%)	4	3	0.54
sstr3, positive	12 (37.5%)	8	4	0.082
sstr5, positive	25 (78.1%)	15	10	**0.0049**
p-IGF-1R, positive	26 (81.3%)	12	14	0.84
Ki-67 LI				
<2%	20 (62.5%)	5	15	**0.0014**
≥2%	12 (37.5%)	10	2	

4EBP1, eukaryotic initiation factor 4-binding protein 1; ERK, extracellular signal-regulated kinase; IGF-1R, insulin-like growth factor 1 receptor; LI, labeling index; mTOR, mammalian target of rapamycin; S6, ribosomal protein s6; sstr, somatostatin receptor. The bold values indicate the statistical significance.

were associated with higher expression of sstr1 and 5 and higher proliferative status evaluated by Ki-67 immunohistochemistry (Table 6; $P = 0.0048$, 0.0049 and 0.0014, respectively). However, the Cluster IIb cases were associated with ERK activation ($P < 0.001$). Therefore, we then evaluated the correlation of the results with the clinicopathological features above and the results indicated that the status of age and their localization was significantly different between these clusters (Table 7; $P = 0.0078$ and 0.0043, respectively).

Effects of mTOR inhibitors on the cell proliferation in NET cell lines. Because the Cluster II cases were associated with the expression of p-mTOR and higher proliferative activities, we examined the effects of mTOR inhibitor, rapamycin, on cell proliferation using two NET cell lines, H727 and COLO. We performed a cell proliferation assay at a range of 10^{-9} to 10^{-7} M for 9 days (H727) or 3 days (COLO), and the results showed that there was a significant decrease in the cell number for 9 days in H727 and 3 days in COLO treated with rapamycin in a concentration-dependent manner (Fig. 5).

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Table 7. Characteristics of the clinicopathological findings of individual patients in Cluster IIa and IIb

	Total (n = 32)	Cluster IIa (n = 15)	Cluster IIb (n = 17)	P-value
Age (years)				
<60	23 (71.9%)	7	16	**0.0078**
≥60	9 (28.1%)	8	1	
Mean ± SD	52.7 ± 14.2	56.4 ± 17.4	49.4 ± 10.0	
Gender				
Male	22 (68.8%)	8	14	0.077
Female	10 (31.3%)	7	3	
Localization				
Foregut	9 (28.1%)	8	1	**0.0043**
Midgut	1 (3.1%)	1	0	
Hindgut	22 (68.8%)	6	16	
Lymph metastasis				
Presence	3 (9.4%)	3	0	0.053
Absence	29 (90.6%)	12	17	
Vascular invasion				
Presence	9 (28.1%)	6	3	0.16
Absence	23 (71.9%)	9	14	

The bold values indicate the statistical significance.

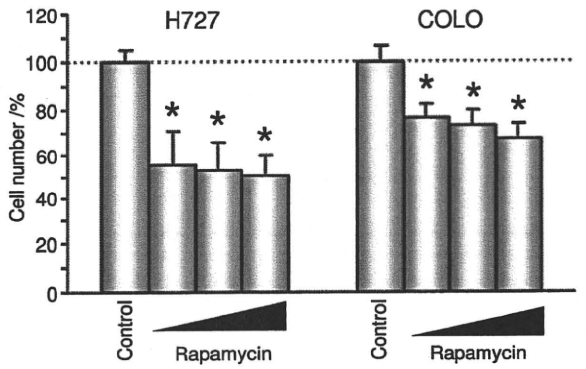

Fig. 5. Antitumor effects of rapamycin in neuroendocrine tumor cell lines in a concentration-dependent manner. Rapamycin, 10^{-9}, 10^{-8}, 10^{-7} M; H727, NCI-H727; COLO, COLO320-DM. All data are shown as mean (n = 6) ±SD. *P < 0.001 (*vs* Control).

Discussion

It is true that the main therapy of NET is surgical excision. Neuroendocrine tumor patients are generally considered resistant to traditional cytotoxic agents when they are in an advanced clinical stage.[21] In particular, the majority of NET cases arising in the foregut and hindgut, which were the predominant NET cases in Japan,[2] do not manifest clinically detectable endocrine manifestations and may be first detected at advanced clinical stages.[22] Somatostatin receptor subtypes have been demonstrated in the great majority of NET cases, including those arising in the foregut and hindgut, even at advanced clinical stages.[5] Octreotide is well known to inhibit the release of hormones and subsequently control symptoms in NET patients. Recently, a newly developed SOM230 (pasireotide; Novartis, St Louis, MO, USA), which could react with wider sstr subtypes, has been reported to be more effective in controlling cell proliferation and symptoms in preclinical studies.[23] In addition, some groups, including our laboratories, showed the antitumor effects of SSA in preclinical and clinical study.[5,24,25] However, its clinically effective antitumor activity has not necessarily been detected with octreotide alone, because the tumor response rate for octreotide represents <10%,[4,26] and thus, the antitumor activities of SSA have been controversial. Therefore, other modes of medical therapy have been in demand clinically, particularly for controlling tumor cell proliferation of non-functioning NET cases including those arising in the foregut and hindgut.

Other modes of intracellular signaling pathways have been reported to be involved in NET cases and among these pathways, in particular, mTOR activities have been shown to increase in NET cells, as a result of mutations of the tumor suppressor genes in the PI3K/Akt/mTOR pathway, rather than the genes encoding mTOR. For instance, the loss of heterozygosity of the *NF1* gene led to constitutive mTOR activation.[27] Neurofibromatosis type 1 (NF-1) is an autosomal dominant disorder clinically characterized by the presence of cutaneous and subcutaneous neurofibromas, café-au-lait spots and Lisch nodules. Neurofibromatosis type 1 appears to play a role as a tumor suppressor gene to function the *Ras* pathway.[28] Tumors associated with NF-1 include not only neurogenic neoplasms such as neurofibromas and neurofibrosarcomas, but also pheochromocytomas and NET, suggesting a broader role for *NF-1* as a tumor suppressor gene. However, the GI NET harboring *NF-1* genetic abnormalities often occurs in duodenal, ampullary NET and somatostatinomas. In addition, the presence of *NF-1* mutations in NET was reported in only 1–2% of cases.[16,29] However, it also awaits further investigations to clarify the possible involvement of *NF-1* genetic abnormalities in patients with NET. The overactivation of IGF-1R is also reported to be correlated with activation of the PI3K/Akt/mTOR pathway in NET cells.[13,30] von Wichert *et al.*[12] demonstrated that low-grade NET co-expressed IGF-1 and IGF-1R, and BON, a human pancreatic NET cell line, expressed functionally active IGF-1R and secreted IGF-1, which all suggest an autocrine action of this growth factor in NET. In addition, a Phase II clinical trial in which the IGF-1R monoclonal antibody is used for NET patients is in progress.[31] However, the immunohistochemical study of p-IGF-1R in human NET cases has not been previously reported. In addition, correlation of the sstr subtypes with the IGF-1R signaling pathway has also not been reported.

Therefore, in this study, we evaluated sstr subtypes, key factors in major signaling pathways under RTK and a potential therapeutic targeted RTK in NET cases using immunohistochemistry combined with hierarchical clustering analysis. Neuroendocrine tumors have been reported to be associated with specific patterns of sstr expression and sstr2 and sstr5 were predominant subtypes reported in Japanese NET patients.[5] Somatostatin receptor 1 and sstr3 are expressed less frequently and sstr4 is rarely expressed in NET as described above.[17,18] Results of our present immunohistochemical study were also consistent with those reported previously, and in particular, sstr2A and sstr5 were the most frequently detected sstr subtypes in these GI NET.[2,5] In addition, results of our present study also showed that the NET cases were basically classified into two different groups, Cluster I and II, and Cluster II was then further sub-classified into Cluster IIa and IIb. Between Cluster I and II, Cluster I was associated with a higher expression of the sstr subtypes, but there were no significant differences between these two clusters in the expression of sstr2A and sstr5. In addition, all Cluster IIa cases expressed sstr2A, but not Cluster IIb. Therefore, the status of the proliferative activity and lymph node metastasis was indeed associated with that of sstr2A and sstr5 expressions regardless of the status of p-IGF-1R immunoreactivity in the cases examined.

Shah *et al.*[14] also demonstrated a relative high abundance of p-endothelial growth factor receptor (p-EGFR) and p-ERK in NET cases using immunohistochemistry. In our present study, phosphorylated factors in the PI3K/Akt/mTOR pathway were also detected in many of the NET cases examined, but the cases associated with activated ERK were relatively low in number.

Possible reasons for the discrepancy between the report of Shah *et al.* and our present study might be due to differences of the sensitivities of the primary antibodies, or the majority of the localization (midgut *vs* hindgut) of the cases examined. In addition, results of our present study demonstrated that the cases belonging to Cluster IIb were associated with PI3K/Akt/mTOR and MEK/ERK pathways related to IGF-1R. These cases were associated with a relatively low proliferative status but may be treated with mTOR inhibitors/IGF-1R antagonists combined with MEK inhibitors, but further investigation is required for clarification.

Mammalian target of rapamycin inhibitors are macrolide antibiotics with potent immunosuppressive and antitumor activities. These agents bind immunophilin FK506-binding protein 12 (FKBP12), and this complex subsequently binds to mTOR, which inhibits downstream signaling pathways.[32,33] Recently, the antitumor activities of mTOR inhibitors have been extensively studied, and treatment of the mTOR inhibitors such as temsirolimus (CCI-779; Wyeth, Philadelphia, PA, USA) and everolimus (RAD001; Novartis, Basel, Switzerland) for advanced renal cell carcinoma after vascular endothelial growth factor receptor (VEGFR)-targeted therapy have been approved in Europe, the USA and Japan.[34,35] In NET patients, the effects of mTOR inhibitors have also been evaluated in both preclinical and clinical studies.[36,37] In the present study, we performed an *in vitro* study using NET cell lines in order to evaluate whether this classification has any relationship with sensitivity to the molecular target therapy. We examined the antitumor effects of rapamycin in NET cell lines, in which the PI3K/Akt/mTOR pathway was shown to be activated,[38,39] which suggests that the cases associated with overexpression of p-mTOR may be treated with mTOR inhibitors.

We subjected the results of the immunohistochemistry into hierarchical clustering analysis. This analysis is one of the multivariative statistical methods that identifies groups of samples that behave similarly or show similar characteristics.[20] Therefore, hierarchical clustering analysis following immunohistochemistry of different molecules may contribute to a potential new classification method according to biological features. Results of the present study revealed that NET cases were basically classified into the "sstr subtypes expressing predominant" group (Cluster I) and the "activating signaling pathways predominant" group (Cluster II), and the latter group was further sub-classified into the "sstr expression with higher proliferative status predominant" group, or Cluster IIa, and the "activating ERK cascade predominant" group, or Cluster IIb.

In conclusion, we are first to demonstrate the application of a novel classification method for non-functioning NET patients using hierarchical clustering analysis based on the immunohistochemical data of sstr subtypes, factors of a major signaling pathway under RTK and major RTK and clinicopathological factors of individual patients. It will be important to evaluate which group the cases with non-functioning NET belong to, and to determine the treatment of adequate drugs for individual NET patients.

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SPECIAL REVIEW SERIES: Breast carcinoma and steroid hormones

**Takashi Suzuki · Yasuhiro Miki · Kiyoshi Takagi
Hisashi Hirakawa · Takuya Moriya · Noriaki Ohuchi
Hironobu Sasano**

Androgens in human breast carcinoma

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Abstract Sex steroids play important roles in the development of human breast carcinoma. Androgen receptor (AR) is expressed in a majority of breast carcinoma tissues. However, the significance of androgen actions remains largely unclear in breast carcinoma, differing from estrogen actions. Therefore, in this review, we summarized recent studies on androgens in breast carcinoma. Concentration of a potent androgen, 5α-dihydrotestosterone (DHT), was significantly higher in breast carcinoma tissue than in plasma, and DHT is considered to be locally produced from circulating androstenedione by 17β-hydroxysteroid dehydrogenase type 5 and 5α-reductase. On the other hand, aromatase was recently reported as a negative regulator for intratumoral DHT production by possibly reducing the precursor testosterone. Androgens predominantly show antiproliferative effects in breast carcinoma cells, but association between AR status and the clinical outcome of the patient remains controversial, perhaps partly because AR status does not necessarily reflect androgenic action in breast carcinoma. Recently, molecular apocrine breast carcinoma was identified by microarray analysis. Molecular apocrine carcinoma was characterized by being estrogen receptor (ER) negative and AR positive and by being associated with increased androgen signaling and apocrine features. Therefore, androgenic actions may also be involved in apocrine features in breast carcinoma.

Key words Androgen · Androgen receptor · Aromatase · Breast cancer · Estrogen receptor · 5α-Reductase

T. Suzuki (✉) · K. Takagi
Department of Pathology and Histotechnology, Tohoku University Graduate School of Medicine, 2-1 Seiryo-machi, Aoba-ku, Sendai 980-8575, Japan
Tel. +81-22-717-7947; Fax +81-22-717-7947
e-mail: t-suzuki@patholo2.med.tohoku.ac.jp

Y. Miki · H. Sasano
Department of Anatomic Pathology, Tohoku Kosai Hospital, Sendai, Japan

H. Hirakawa
Department of Surgery, Tohoku Kosai Hospital, Sendai, Japan

T. Moriya
Department of Pathology 2, Kawasaki Medical School, Kurashiki, Japan

N. Ohuchi
Department of Surgical Oncology, Tohoku University Graduate School of Medicine, Sendai, Japan

Introduction

It is well known that sex steroids play important roles in the development of hormone-dependent human breast carcinoma. Among these sex steroids, estrogens immensely contribute to growth of breast carcinoma through binding with estrogen receptor (ER). A majority of breast carcinoma tissues express ER, and estrogen deprivation is an effective treatment for breast carcinoma as an endocrine therapy. Therefore, antiestrogens such as tamoxifen, aromatase inhibitors, or luteinizing hormone-releasing hormone (LH-RH) agonists are currently used in breast carcinoma patients to block intratumoral estrogen action. Androgen receptor (AR) is also expressed in a majority of human breast carcinoma tissues,[1-6] suggesting important roles of androgens in human breast carcinomas. However, the clinical and/or biological significance of androgen actions in breast carcinomas remains largely unclear, in contrast to estrogen actions. Therefore, in this review, we summarized results of recent studies on androgens in breast carcinoma tissues.

In situ production of androgens in breast carcinoma

Among the androgens, 5α-dihydrotestosterone (DHT) binds with the highest affinity to AR, and together with testosterone promotes AR transcriptional activity.[7] Plasma concentrations of DHT are very low in normal woman and in breast cancer patients.[8] However, DHT concentrations were significantly (threefold) higher in breast carcinoma tissues than in plasma,[9] and the tissue concentration of DHT was threefold higher in ductal carcinoma in situ of the

breast (DCIS) than in the nonneoplastic breast,[10] suggesting possible local production of DHT in breast carcinomas. Figure 1 summarizes a representative pathway of in situ production of DHT in breast carcinoma tissue that is currently postulated. A high concentration of circulating inactive steroid androstenedione is converted to DHT by androgen-producing enzymes, such as 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5: conversion from androstenedione to testosterone) and 5α-reductase (5α-Red: reduction of testosterone to DHT). Therefore, it is very important to examine these enzymes in breast carcinoma tissues to obtain a better understanding of the significance of androgens in breast carcinomas.

17β-HSD5

17β-HSDs are key enzymes that catalyze reversible interconversions between biologically active and inactive sex steroids. The removal of the hydrogen at position 17 of steroid skeletons by oxidative 17β-HSDs inactivates the steroids, whereas hydrogenation by reductive 17β-HSDs results in activation of androgens and estrogens.[11] To date, 14 isozymes of 17β-HSD have been cloned, and 17β-reduction (17β-HSD1, -3, -5, -7, etc.) or oxidation (17β-HSD2, -4, -6 etc) of estrogens and/or androgens is catalyzed by different 17β-HSD isozymes.[12] Among these isozymes, 17β-HSD3 biosynthesizes testosterone from androstenedione and is expressed in testicular Leydig cells. However, testicular Leydig cells provide approximately 50% of the total amount in men, and the rest of the amount is converted from circulating androstenedione in peripheral tissues.[8] This enzymatic reaction is catalyzed by different enzymes, namely 17β-HSD5.[13] 17β-HSD5 is a member of the aldo-keto reductase (AKR) superfamily and is formally termed AKR1C3.[14]

mRNA expression of 17β-HSD5 was detected in 65%–83% of breast carcinoma tissues.[15–17] Vihko et al.[17] reported that 17β-HSD5 mRNA expression was significantly higher in breast tumor specimens than in normal tissues, and they also demonstrated that the group of patients with overexpression of 17β-HSD5 mRNA had a worse prognosis than other patients. 17β-HSD5 immunoreactivity was positive in 53%–56% of invasive breast carcinomas[18,19] and 71% of DCIS.[10] Immunoreactivity of 17β-HSD5 was significantly associated with that of 5α-Red type 1 (5α-Red1),[18] but it was not significantly associated with other clinicopathological factors.[19,20]

17β-HSD5 also possesses 3α-HSD and 20α-HSD activities.[21] The 3α-HSD and 20α-HSD activities are involved in the inactivation of progesterone.[21–23] However, the significance of 17β-HSD5 for these activities in breast carcinoma remains unclear.

5α-Red

5α-Red catalyzes the conversion of testosterone to a potent androgen, DHT, and is considered as an important regulator of local actions of androgens. 5α-Red activity was elevated four- to eightfold in breast carcinoma tissues compared to nontumorous breast tissues.[22] Two isoforms of 5α-Red (e.g., 5α-Red1 and 5α-Red2) have been cloned and characterized in mammals. Immunoreactivity for 5α-Red1 was detected in 58% of breast carcinomas, whereas that of 5α-Red2 was detected only in 15% of breast carcinomas,[18] suggesting that 5α-Red1 may mainly determine 5α-Red activity in the breast carcinoma. 5α-Red1 immunoreactivity was significantly correlated with AR and inversely associated with histological grade or tumor size in breast carcinoma tissues.[18] 5α-Red1 immunoreactivity was also detected in 63% of DCIS and, interestingly, 5α-Red1 immunoreactivity was positively associated with the Van Nuys classification, Ki-67, and increased risk of recurrence of DCIS patients.[10]

5α-Red metabolizes progesterone to 5α-dihydroprogesterone (5α-DHP), suggesting that this enzyme is also involved in local regulation of progesterone actions. Wiebe et al.[22,24] reported that 5α-DHP stimulated proliferation and detachment of breast cell lines in vitro, which was blocked by the 5α-Red inhibitor dutasteride.

Aromatase as a negative regulator of in situ DHT production in breast carcinoma

Aromatase catalyzes the aromatization of androgens (androstenedione or testosterone) to estrogens (estrone or estradiol) (Fig. 1). Aromatase is a key enzyme in the estrogen biosynthesis, and aromatase inhibitors (e.g., anastrozole, letrozole, and exemestane) are currently used in postmenopausal patients with breast carcinoma as an estrogen-

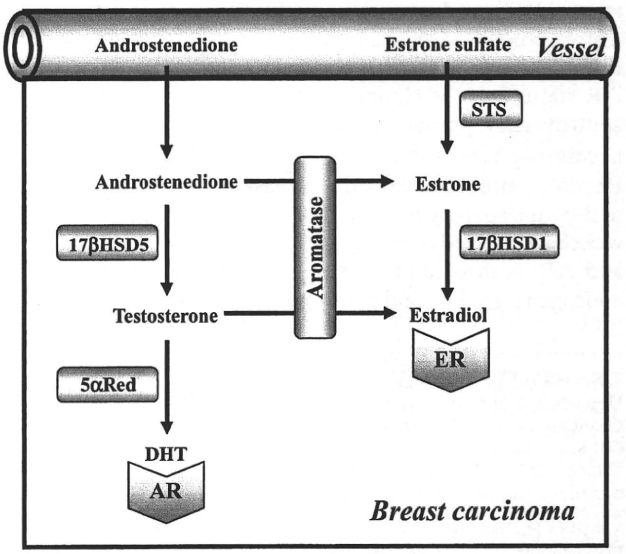

Fig. 1. Scheme representing in situ production of 5α-dihydrotestosterone (DHT) in breast carcinoma tissue. Biologically active DHT is locally produced from circulating androstenedione by 17β-hydroxysteroid dehydrogenase type 5 (17β-HSD5) and 5α-reductase (5α-Red) and acts on the breast carcinoma cells through androgen receptor (AR). In contrast, estradiol is synthesized by aromatase, steroid sulfatase (STS), and 17β-HSD1, then acts on the breast carcinoma cells through estrogen receptor (ER). Androstenedione and testosterone are not only precursors of DHT production but also precursors of estradiol synthesis. (Adapted from Suzuki et al.[31] with permission)

deprivation therapy. Approximately 70% of breast carcinoma specimens had aromatase activity comparable with or greater than that found in other tissues, and aromatase mRNA levels were significantly increased in breast carcinomas compared to those in nonmalignant tissues.[20]

The substrates of aromatase, androstenedione, and testosterone are not only precursors of estradiol synthesis but also precursors of DHT production (see Fig. 1). DHT itself is nonaromatizable. Intratumoral concentration of DHT was significantly associated with that of testosterone in the breast carcinoma tissue,[9,25] suggesting that DHT level in breast carcinoma is greatly influenced by amounts of precursor. Previously, Spinola et al.[26] showed that treatment with an aromatase inhibitor markedly elevated intratumoral testosterone concentrations in dimethylbenz(*a*) anthracene (DMBA)-induced rat mammary tumors, and Sonne-Hansen and Lykkesfeldt[27] reported that aromatase preferred testosterone as a substrate in MCF-7 breast carcinoma cells. Recently, we[28] have demonstrated that aromatase expression was inversely associated with intratumoral DHT concentration in breast carcinomas, and that aromatase suppressed DHT production from androstenedione in coculture experiments of MCF-7 cells and intratumoral stromal cells isolated from breast carcinoma. Therefore, aromatase is suggested a negative regulator for intratumoral DHT production in breast carcinoma by possibly reducing concentrations of the precursor testosterone.

Results of large multicenter trials demonstrated the superior efficacy of aromatase inhibitors compared to antiestrogen tamoxifen. Although this result might be caused by the agonistic effects of tamoxifen in an estrogen-deprived environment,[29] it may be possible to speculate that aromatase inhibitor therapy caused increased androgen actions with estrogen deprivation. However, intratumoral concentration of androgens has not been reported in breast carcinomas treated with aromatase inhibitor, and further examinations are required to clarify the clinical importance of androgenic actions in association with a response to aromatase inhibitors in breast cancer patients.

When we compared aromatase mRNA expression and intratumoral DHT concentration levels between invasive ductal carcinoma (IDC) and DCIS, the expression levels of aromatase mRNA in both carcinoma cells and intratumoral stromal cell components were significantly higher in IDC than those in DCIS (17 fold in the carcinoma cell component and 100 fold in the stromal cell component), whereas the intratumoral concentration of DHT was significantly lower (0.5 fold) in IDC than in DCIS.[10] Subsequent coculture experiments demonstrated that aromatase activity was significantly increased under coculture with MCF-7 cells and intratumoral stromal cells isolated from breast carcinoma tissue compared to that found in each single culture.[30] Previous in vitro studies demonstrated that breast carcinoma cells secrete various factors that induce aromatase expression in adipose fibroblasts, including prostaglandin E_2, interleukin (IL)-1, IL-6, IL-11, and tumor necrosis factor-α.[31] On the other hand, it has been also reported that exogenous growth factors such as epidermal growth factor, transforming growth factor, and keratinocyte growth factor stimulated aromatase activity in MCF-7 cells.[31] Therefore, aromatase expression may be, at least in part, regulated by tumor–stromal interactions in breast carcinoma, which may be promoted by invasion of the carcinoma cells into the stroma.

Androgen action in breast carcinoma cells

Various studies have demonstrated that androgens predominantly exerted antiproliferative effects on the mitogenic effects of estrogens in breast carcinoma cell lines.[32,33] These antiproliferative effects were partly independent on the presence of estrogens and were associated with an increase in a proportion of cells in G_0/G_1 phase in MCF-7 cells.[34] DHT also caused accumulation of cyclin-dependent kinase inhibitor p27 in CAMA-1 cells,[35] and DHT treatment resulted in a rapid fall in tumor volume of ZR75–1 cells injected into athymic mice. Proapoptotic effects of DHT were also reported in breast carcinoma cells, and expression of antiapoptotic protein bcl-2 was strongly inhibited by DHT through AR.[36,37] However, it is also true that some divergent findings have been reported. For instance, Birrell et al.[38] showed that both DHT and the synthetic nonmetabolizable androgen mibolerone increased cell proliferation of MCF-7 and MDA-MB-453 cells. In addition, Zhang et al.[39] reported that DHT-bezonate (DHT-B) induced growth of mouse mammary ductal cells, although its effect is much weaker than that of estradiol, and that treatment with both estradiol and DHT-B caused more pronounced hyperplasia of mammary ducts and alveoli compared to treatment with each hormone alone. Androgen-responsive genes are not characterized well in breast carcinomas in contrast to estrogen-responsive genes, and detailed mechanisms of androgenic actions in breast carcinomas remain largely unclear.

AR is expressed in 70%–90% of breast carcinomas, and the frequency is comparable to, or higher than, that of ER.[3,40] Ogawa et al.[6] examined AR immunoreactivity in 227 Japanese breast carcinomas and showed that the AR positive rate was significantly higher in smaller carcinomas, tumors with negative lymph node metastasis, scirrhous-type tumors, tumors of low histological grade, and p53-negative tumors. Several groups have examined the correlation between AR status and clinical outcome of breast carcinoma patients, but the results were not necessarily consistent. Previously, Bryan et al.[41] found a significant association between AR status evaluated by AR assays and overall survival of the patients, and Peters et al.[42] demonstrated that AR immunohistochemical status was significantly associated with better prognosis in ER-positive breast carcinoma, when AR immunoreactivity was categorized into two groups according to the median value. On the other hand, Soreide et al.[1] did not detect any significant correlation between AR status and relapse-free survival. In our study, effects of DHT primarily exist in breast carcinoma tissues positive for both AR and 5α-Red1, and AR status alone does not necessarily reflect androgenic actions.[28] Therefore,

inconsistent results regarding the correlation between AR status and prognosis in previous studies may partly be caused by the different ratios of breast carcinomas positive for both AR and 5α-Red1 examined.

Possible interaction of AR and ER functions was proposed by several groups. For instance, Panet-Raymond et al.[43] reported that coexpression of ER with AR decreased AR transactivation by 35%, and demonstrated that both AR and ER can interact directly using yeast and mammalian two-hybrid systems. In addition, Lanzino et al.[44] showed that an AR-specific coactivator ARA70 also increased ER transcriptional activity and modulated functional ER–AR interplay in MCF-7 cells. Recently, Peters et al.[42] showed that AR potently inhibited ER-α transactivation activity and estradiol-stimulated growth of breast carcinoma cells through binding of the AR to an estrogen-responsive element. On the other hand, when we examined interaction of AR and ER functions in T-47D breast carcinoma cells, DHT-mediated expression of prostate-specific antigen (PSA) mRNA was dose dependently suppressed by estradiol (Fig. 2). Therefore, androgen actions may be, at least in part, suppressed in breast carcinoma by predominant estrogen actions, even if the carcinoma cells expressed AR and intratumoral DHT reached a significant level. Results of studies regarding effects of androgens on breast carcinoma cells are not necessarily consistent, which may be the result of different experimental conditions including the specific cell line used, the androgen used and its dose, and estrogen status.

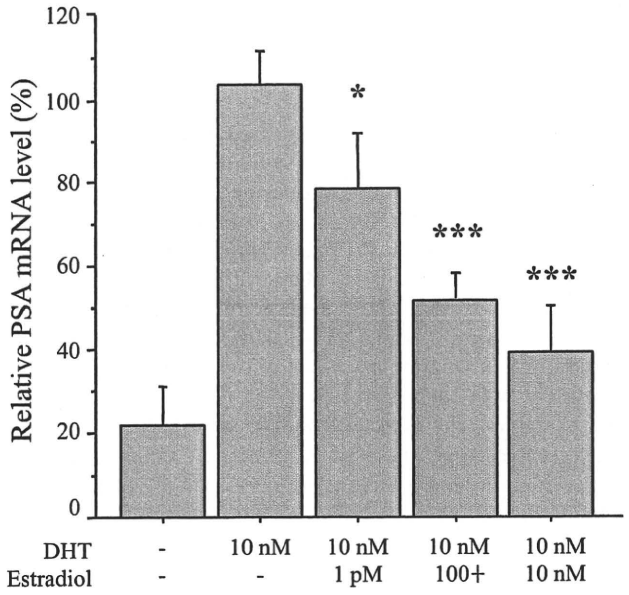

Fig. 2. Effects of estradiol on DHT-mediated prostate-specific antigen (PSA) mRNA expression by real-time polymerase chain reaction (PCR) analysis. T-47D breast carcinoma cells were treated with DHT (10 nM) and indicated concentrations of estradiol for 24 h. Relative PSA mRNA level was summarized a ratio (%) compared with the ribosomal protein L13A mRNA level. Data are presented as mean ± SD ($n = 3$), respectively; $^{*}P < 0.05$, $^{***}P < 0.001$ vs. treatment with DHT alone (*second column*)

ER status in DCIS was inversely associated with the histological differentiation or nuclear grade.[45–47] However, AR status was not correlated with ER status in DCIS,[48] and a significant number of poorly differentiated DCIS was reported to be ER negative but AR positive.[5] Recently, we reported that 5α-Red1 immunoreactivity was significantly associated with Ki-67 and the Van Nuys classification in DCIS cases, and it was associated with an increased risk of recurrence in DCIS patients.[10] Therefore, DHT might be involved in the development of DCIS. However, no information is currently available on the effects of androgens in DCIS, and further examination is required to clarify the significance of androgens in DCIS.

Androgens in ER-negative breast carcinoma

Although AR is frequently coexpressed in a majority of ER-positive breast carcinoma, it is also detected in approximately 50% of breast carcinomas negative for ER.[6,42,49] AR immunoreactivity was shown to be associated with a good prognosis in ER-negative carcinomas,[3] and loss of AR was associated with a poor prognosis in lymph node-positive ER/HER2-negative breast cancers.[50] These findings are consistent with cell-based assays as already described and suggest that AR also initiates a growth inhibitory signal in ER-negative breast carcinoma.[51]

Breast carcinoma is a heterogeneous group of diseases that includes a wide range of histological types. Recent DNA microarray profiling studies on breast carcinoma have identified five distinct subtypes of breast carcinomas that were associated with different clinical outcomes,[52–54] and subsequent investigations revealed that these subtypes substantially overlapped with the immunohistochemical features summarized in Table 1 in clinical specimens.[55–57] In

Table 1. Association between intrinsic subtypes based on gene expression profiling and their immunohistochemical definitions

Microarray-based intrinsic subtypes	Immunohistochemical definitions
ER-positive groups	
Luminal A	ER and/or PR: +; HER2: –
Luminal B	ER and/or PR: +; HER2: +
ER-negative groups	
HER2	ER: –; PR: –; HER2: +
Basal-like	ER: –; PR: –; HER2: –; CK5/6 and/or EGFR: +
Normal-like	Negative for all markers

PR, progesterone receptor; CK, cytokeratin; EGFR, epidermal growth factor receptor

addition, Farmer et al.[58] identified a discrete subset of breast carcinomas by microarray analysis, characterized by ER– and AR+, and termed "molecular apocrine." Molecular apocrine carcinomas encompass tumors that share ER-negative groups and were found to represent 8%–14% of the breast carcinomas.[58] Apocrine cells are generally ER negative and AR positive in the breast tissue, and AR is suggested to be implicated in apocrine morphogenesis, rather than the progression of the apocrine lesion.[59] Previously, Miller et al.[60] reported that breast carcinoma in which apocrine characteristics significantly increased conversion to DHT from testosterone. Also, Farmer et al.[58] demonstrated that molecular apocrine breast carcinoma was characterized by increased androgen signaling and associated with apocrine features. Although these molecular apocrine carcinomas were not necessarily classified into classical apocrine carcinoma, which corresponds to 0.5%–3% of all invasive breast carcinomas,[61] these data suggest that androgen actions may be involved in the apocrine features in ER-negative and AR-positive breast carcinomas, in addition to the growth inhibition.

Invasive apocrine carcinoma is a histological variant of invasive breast carcinoma. Previous studies reported that invasive apocrine carcinoma had a similar prognosis to IDC not otherwise specified (IDC-NOS), but Japaze et al.[62] demonstrated that pure invasive apocrine carcinoma represented a distinct clinicopathological entity with a less aggressive behavior than IDC-NOS and might be regarded as an independent prognostic factor in early breast cancer.

Recent studies have demonstrated some specific apocrine biomarkers, including 15-hydroxyprostaglandin dehydrogenase, 3-hydroxymethylglutaryl coenzyme A reductase, and cyclooxygenase 2,[61,63] and some of these are known therapeutic targets with pharmacological agents already available. Therefore, it may be possible to speculate that ER-negative and AR-positive breast carcinoma might benefit from a different therapeutic regimen, and further studies are required.

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Down regulation of Heat Shock Protein 70 (HSP-70) correlated with responsiveness to neoadjuvant aromatase inhibitor (AI) therapy in breast cancer patients

CHRISTOPHER CP YIU[1,2,3,4], NIRAMOL CHANPLAKORN[1,6], MONICA SM CHAN[1,2,5], WINGS TY LOO[1,2,3], LOUIS WC CHOW[1,2,3,4], MASAKAZU TOI[7] and HIRONOBU SASANO[1]

[1]*Department of Pathology, Tohoku University School of Medicine, Japan;*
[2]*UNIMED Medical Institute, Comprehensive Centre for Breast Diseases, Hong Kong;*
[3]*Organization of Oncology Translational Research, Hong Kong;*
[4]*Department of Surgery, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong;*
[5]*Department of Radiology, Li Ka Shing Faculty of Medicine, The University of Hong Kong, Hong Kong;*
[6]*Department of Pathology, Faculty of Medicine, Ramathibodi Hospital, Mahidol University, Bangkok, Thailand;*
[7]*Department of Surgery and Oncology, Graduate School of Medicine, Kyoto University, Japan*

Abstract. *Background: Aromatase inhibitor (AI) has been established as an effective endocrine therapy in estrogen receptor (ER)-positive postmenopausal breast cancer patients. Our recent proteomic analysis demonstrated that ten proteins were significantly altered in their expression levels before and after the therapy in the patients receiving neoadjuvant AI. Among these newly identified proteins, heat shock protein 70 (HSP-70) was the most significantly correlated with both clinical and pathological responses. Therefore, in this study, we further evaluated the significance of this HSP-70 alteration using immunohistochemistry. Materials and Methods: A total of 32 patients treated with neoadjuvant exemestane or letrozole in whom pre- and post-treatment tumor tissues were available were included. Immunohistochemical evaluation of ER, progesterone receptor (PgR), Her-2, Ki-67 and HSP-70 was performed. Results obtained were compared to both clinical and biological responses of the patients. Results: The majority of the patients responded to treatment (16 patients with partial response, 14 with stable disease and 2 with progressive disease). The means of ER, Ki-67 and HSP-70 were significantly different between treatment responders and non-responders. Decrement of HSP-70 and Ki-67 after AI treatment and pretreatment Ki-67 LI of >10% tumor cells were significantly associated with clinical responsiveness to AI treatment ($p<0.0001$). There was a significant positive correlation between changes of HSP-70 and Ki-67 before and after the therapy. Conclusion: Decrement of HSP-70 in breast carcinoma cells plays important roles in therapeutic mechanisms of AIs through suppressing tumor cell proliferation in breast cancer patients.*

Correspondence to: Professor Hironobu Sasano, Department of Pathology, Tohoku University School of Medicine, 2-1 Seiryou-machi, Aoba-Ku, Sendai, Miyagi, Japan 980-8575. E-mail: hsasano@patholo2.med.tohoku.ac.jp

Key words: Heat shock protein 70, Ki-67, neoadjuvant, aromatase inhibitor, breast cancer.

Aromatase inhibitor (AI) has become a gold standard of endocrine therapy for estrogen receptor (ER)-positive post-menopausal women with breast cancer (1-5). Breast cancer patients have been in general presenting at earlier clinical stages due to a wide availability of screening programs and increased breast cancer awareness among the general population, but it is also true that there are patients who manifest with advanced clinical stages on their first visit to clinicians (6-7). Neoadjuvant therapy aiming for tumor shrinkage could allow the choice of breast conservative surgery for these advanced breast cancer cases (8). In these neoadjuvant settings, chemotherapy has been frequently used but adverse effects and complications are quite common among the patients. Therefore, the ideas of using endocrine therapy in these neoadjuvant settings have evolved, at least for ER-positive breast cancer patients. However, the determination of objective therapeutic effectiveness in neoadjuvant endocrine therapy has not been well established, with the possible exception of alterations of Ki-67 labeling index (LI) before and after the treatment (9). In addition, the alterations of carcinoma cell biology following the therapy have not been well studied compared to those of chemotherapy, with the exception of recent studies of

microarray analysis reported by Miller *et al.* (10) and Mackay *et al.* (11). We have recently demonstrated that ten proteins had different expression profiles after three months of neoadjuvant AI compared to those before the therapy using proteomic approach in ER-positive postmenopausal breast cancer patients (12). Among these proteins, heat-shock protein 70 (HSP-70) was most significantly correlated with both clinical and biological responses of the patients. Therefore, in this study, we further examined the potential role of HSP-70 in therapeutic effectiveness of neoadjuvant AI therapy using immunohistochemistry and correlated the findings with clinicopathological features and biological responses of these patients.

Materials and Methods

Patients. A neoadjuvant clinical, trial termed Celecoxib Anti-Aromatase Neoadjuvant trial (CAAN trial), was conducted on post-menopausal breast cancer patients and its details were previously reported (13). Briefly, all the patients were postmenopausal women with invasive ductal breast carcinoma and positive ER/PgR status determined by immunohistochemistry. These patients either suffered from local advanced breast cancer, in which the purpose of neoadjuvant treatment was to downstage the cancer for a better chance of subsequent surgical complete resection, or they were anticipated to have high operative risks due to advanced age or co-morbidities that prevented them from upfront surgical treatment. The treatment duration was three months of AI and all the patients were randomized into three different treatment groups: group A patients received combined treatment of exemestane 25 mg daily and celecoxib 400 mg twice daily; group B received exemestane 25 mg daily; and group C received letrozole 2.5 mg daily. As reported previously, there were no significant differences in term of clinical and pathological responses among these three different treatment groups [13]. Therefore, the responses toward AI were not influenced by the use of celecoxib.

Institutional Ethical Committee approval of this analysis was obtained from The University of Hong Kong and Queen Mary Hospital, Hong Kong. Informed consent of participation in the trial were obtained from all the patients before enrollment into the trial. During the treatment period, participating patients were monitored serially with both clinical and radiological assessments for the responses to treatments and potential adverse effects. After completion of 3-month treatment, standard surgical treatments were offered to the patients. The responses to AI treatment were measured according to RECIST scales in the outpatient clinics at the Queen Mary Hospital, The University of Hong Kong (14).

Immunohistochemistry. Mouse monoclonal antibodies for ER, PgR and HER2 were purchased from Roche Diagnostics, Switzerland. Mouse monoclonal antibody for Ki-67 was purchased from DAKO Cytomation (Glostrup, Denmark). Mouse monoclonal antibody for HSP-70 (HSPA2) was purchased from ABNOVA (Taipei, Taiwan). The dilutions of primary antibodies were as follows: ER, PgR and HER2 ready for use; Ki-67, 1:100; HSPA2, 1:200. ER, PgR and HER2 were stained by auto-immunohistochemical system BENCHMARK® XT (Roche Diagnostics). Ki-67 and HSPA2 were immunostained by a biotin-streptavidin method using Histofine kit

Table I. *Demographic data of the studied patients.*

Total no. of patients: 32	
Mean age (years)	71.0+9.3 (51–93)
Pre-treatment mean (range) tumour size (cm)	
Clinical assessment	4.1+1.2 (2.0–8.0)
USG assessment	3.0+0.9 (1.2–5.5)
Treatment arm	
A	12 (37.5%)
B	10 (31.3%)
C	10 (31.3%)
RECIST response	
CR	0 (0.0%)
PR	16 (50.0%)
SD	14 (43.8%)
PD	2 (6.3%)
Objective treatment response	
Responder (size reduction)	28 (87.5%)
Non-responder (size increment)	4 (12.5%)
Biological treatment response	
Group 1 (increase)	8 (25.0%)
Group 2 (no change)	5 (15.6%)
Group 3 (decrease)	19 (59.4%)

CR: complete response; PR: partial response; SD: stable disease; PD: progressive disease.

Table II. *Data on the biological markers of the studied patients.*

ER (mean Allred's score)	
Pre-treatment	7.0+1.6
Post-treatment	7.4+1.2
PgR (mean Allred's score)	
Pre-treatment	6.7+1.7
Post-treatment	5.2+2.3
Her-2 (IHC score, no. of patients)	
Pre-treatment (0–1+, 2+, 3+)	15, 11, 6
Post-treatment (0–1+, 2+, 3+)	18, 9, 5
Ki-67	
Pre-treatment (mean %)	17.6+14.0
Post-treatment (mean %)	10.0+9.3
High pre-treatment Ki-67 index* (no. of patients)	
Yes	22 (68.8%)
No	10 (31.2%)
HSP-70	
Pre-treatment (mean H score)	85.9+42.6
Post-treatment (mean H score)	56.1+30.4
Change of HSP-70 after AI treatment (no. of patients)	
Down-regulation	24 (75%)
Up-regulation	8 (25%)

*Pretreatment Ki-67>10% is considered as having high proliferative index. Results expressed in mean+SD. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70.

(Nichirei Co. Ltd, Tokyo, Japan). Antigen retrieval for Ki-67 analysis was performed by heating the slides in an autoclave at 121°C for 5 min in citric acid buffer (2 mmol/l citric acid and 9 mmol/l trisodium citrate dehydrate, pH 6.0). These slides were further incubated with the primary antibodies for 12-18 h in a moist chamber at 4°C. The antigen-antibody complex was then visualized with 3,3'-diaminobenzidine (DAB) solution (1 mM DAB, 50 mM Tris-HCl buffer, pH 7.6, and 0.006% H_2O_2), and counterstained with hematoxylin. Immunohistochemical H-score was calculated by adding the sum of 100× of 1+ (weak), 2+(moderate) and 3+(strong) of staining intensity. All IHC stained slides were evaluated independently by two authors (CY and NP).

Biological response as determined by Ki-67 alterations. There has been no consensus on the absolute value of the pretreatment Ki-67 level at which the definition of high proliferative index is set at this juncture (15-32). However, several investigators reported that setting the cut-off Ki-67 level of >10% as the definition of high proliferative index was associated significantly with poorer disease free survival (DFS) and overall survival (OS) regardless of the nodal status in breast cancer patients (16, 28-29). Therefore in our study, pretreatment tumor specimens with Ki-67 level of >10% were tentatively defined as the highly proliferative group. In addition to the RECIST criteria, the responses to AI treatment were also graded according to their change in proliferative index as the biological response. The changes of Ki-67 were also tentatively classified into three groups using the criteria reported by Ellis *et al.* (33), Miller *et al.* (34) and Chanplakorn *et al.* (35), in which the significant changes were defined as more than 40% of the original measurement: group 1, the increased group (Ki-67 increment more than 40%); group 2, the unchanged group (increment or reduction of Ki-67 less than 40%); and group 3, the decreased group (Ki-67 level reduction more than 40%).

Statistical analysis. The software SPSS 15.0 (Inc, Chicago, IL, USA) was used. Independent student *t*-test was used to test the correlation of parametric variables while Pearson Chi-square test was used to test the correlation between non-parametric variables.

Results

Clinicopathological features of the patients. Clinical and pathological findings of the patients are summarized in Table I. Patients were evenly distributed into three treatment arms described above. There was no complete response (CR) achieved in the study, and 2 patients were found to have progressive disease (PD). Together with the 2 patients who had stable disease (SD) associated with size increment during the course of treatment, in all, 4 patients had a size increment after this 3-month neoadjuvant treatment. Therefore, the proportion of objective responders was 87.5% (n=28), while that of non-responders was 12.5% (n=4).

Immunohistochemistry. The great majority of the patients (n=22) had high Ki-67 level before treatment (Ki-67>10%; Table II). There were 23 and 24 patients who demonstrated significant decrement in proliferative index and HSP-70 expression respectively after treatment. There were no significant differences in both clinical and biological

Table III. *Comparison of the mean differences of various factors among objective treatment responders (with size reduction) and non-responders (with size increment).*

	Responders	Non-responders	*p*-Value
Age	71.36 + 9.75	69.00+5.72	0.643
ER	0.185+1.11	2.000+2.71	0.019*
PgR	–1.864+1.83	0.250+4.11	0.097
Her-2	–3.214+0.86	0.500+1.00	0.090
Ki-67	–9.861+12.74	8.875+15.31	0.012*
% change of Ki-67	–42.20+61.24	272.55+392.35	<0.0001*
HSP-70	–36.961+48.91	20.025+61.82	0.043*

Data shown as mean+SD; independent sample *t*-test used for comparison of means between responders and non-responders. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70. *p-Value <0.05 is considered as statistically significant.

Table IV. *Correlation of treatment response with different factors.*

	Objective treatment response		
	Responders (no. of pts)	Non-responders (no. of pts)	*p*-Value
Pre-treatment Ki-67 level (high/low)*	21 / 7	1 / 3	<0.0001
Change in Ki-67 after treatment (decrease/ increase)	23 / 5	1 / 3	<0.0001
Change in HSP-70 after treatment (decrease/ increase)	23 / 5	1 / 3	<0.0001

Pearson Chi-square test used, p-value<0.05 considered as statistically significant. *Pretreatment Ki-67>10% is considered as high. Other factors with non-significant correlations are not shown.

Table V. *Comparison of the mean differences of various factors among biological responders and non-responders in terms of % change of Ki-67.*

	Biological responders (% decrease in Ki-67≥40%)	Biological non-responders (% increase in Ki-67≥40%)	*p*-Value
Age	70.58±9.86	74.60±8.41	0.414
ER	0.111±1.21	1.60±2.51	0.073
PgR	–2.31±2.15	0.40±2.88	0.034*
Her-2	–0.42±0.96	0.20±1.10	0.224
HSP-70	–46.56±44.19	16.22±73.10	0.022*

Data shown as mean+SD; independent sample *t*-test used for comparison of means between responders and non-responders. ER: Estrogen receptor; PgR: progesterone receptor; Her-2: human epidermal growth factor receptor type 2; Ki-67: Ki-67 protein; HSP-70: heat-shock protein 70. *p-Value <0.05 is considered as statistically significant.

responses among these three treatment groups (p=0.202 and 0.057 respectively in Pearson Chi-square test, results not shown in table).

Changes of ER, Ki-67 and HSP-70 expression were statistically significant among objective treatment responders and non-responders (p-value=0.019, 0.012 and 0.043 respectively) (Figure 1, Table III). The clinical treatment response was significantly correlated with the biological response (42.2% mean Ki-67 reduction in the responder group and more than 2-fold Ki-67 increment (272.55%) in the non-responder group, p<0.0001). Results of Allred's score of ER in tumor cells were similar before and after treatment in responders (0.185±1.11), while an increment of Allred's score of 2.000±2.71 was detected in non-responders.

Immunoreactivity of Ki-67 and HSP-70 demonstrated both significant and consistent reductions among treatment responders. Table IV summarized significant factors associated with treatment responses including the pretreatment high proliferative index determined by Ki-67 LI and post-treatment decrement of Ki-67 and HSP-70. The pretreatment Ki-67 was also significantly associated with decrement of HSP-70 (p<0.0001 on Pearson Chi square test, data not shown in table). Representative illustrations of immunoreactivity of HSP-70 before and after the treatment are demonstrated in Figure 2.

Factors associated with biological response of the patients. A total of 19 patients (59.4%) had significant decrement of Ki-67 level following the treatment, *i.e.* they were biological responders, and the biological non-responders were 5 patients (15.6%). The remaining 8 patients (25%) did not have significant alterations of Ki-67 LI after completion of treatments. Table V summarizes the changes of biological markers before and after the treatment among the three subgroups of patients. Using the changes of Ki-67 level as a marker for biological response, the down-regulation of HSP-70 still represented a significant predictor for AI response (p=0.022) (Figure 3). Change in PgR expression was also found to be a significant factor (p=0.034).

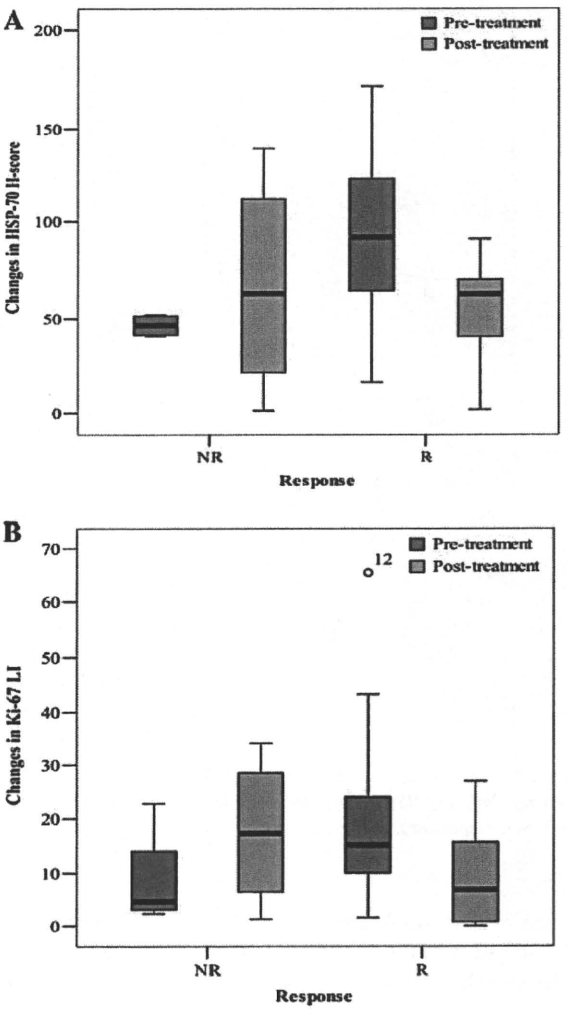

Figure 1. *Changes (mean difference) of HSP-70 (A) and Ki-67 (B) among the clinical objective responders (R) and non-responders (NR).*

Discussion

AI has been established an effective treatment for ER positive postmenopausal breast cancer but the problem of *de novo* resistance has remained the major clinical obstacle. It is also very important to evaluate the changes of carcinoma tissues following AI treatment in order for us to have a better understanding of the mechanisms of AI actions on breast carcinomas. Various alterations of histopathological features following AI treatment have been reported in the literature, which included decreased cellularity, increased interstitial fibrosis, decreased histological grading and others (34, 36) but it is also true that there have been no histological parameters following neoadjuvant AI therapy which are able to predict clinical outcome of the subsequent adjuvant AI treatment at this juncture.

Chen et al reported significant reduction of PgR and Ki-67 levels following the letrozole neoadjuvant trial (37). Decrement in Ki-67 after AI treatment was also reported by Ellis *et al.* (38). Among these factors, an alteration of Ki-67 has been probably the most consistent finding among different studies. Dowsett *et al.* reported the serial changes of Ki-67 level among 330 post-menopausal breast cancer women taking neoadjuvant anastrozole at the 2nd week and 12th week in the IMPACT trial (39). The change of Ki-67 level was more substantial in the anastrozole-treated group than in the tamoxifen-treated or combined groups and the degrees of such changes were also more pronounced at the 2nd week of treatment (93% of patients showed a certain