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14. ABSTRACT
While the significance of the protein kinase AKT expression /activity in human breast cancer has become increasingly evident, consistent alterationsof a specific isoform have not been well documented. A specific isoform of AKT may bepreferentially activated or activated proteins may have different substrate preferences,providing a therapeutic opportunity to target a particular isoform. Previously, we havedemonstrated a cross-talk between the growth factor and estrogen transduction pathways,converging in the ErbB2/PI 3-K/AKT1 signaling. Moreover, in the presence of constitutivelyactive Akt but low ErbB2 levels, ER-dependent breast cancer cells become resistant towardstamoxifen. In this proposal we have analyzed 44 tumors (mostly high grade, invasive ductalcarcinomas) and their surrounding normal tissues for ErbB receptors (EGFR, ErbB2, ErbB3,and ErbB4), Akt isoforms (AKT1, AKT2, AKT3), pGSK3, and MAPK expression and activity usingboth immunohistochemistry and western blot analysis. Akt expression and activity waspositive in 93% and 84%, respectively and was higher in the cytoplasm than in the nucleus.In 70% of the cases all three Akt isoforms were present, suggesting that they all maycontribute to Akt activity. Akt activity correlated well with ErbB2 and ErbB3 expression,Tyr 1248 phosphorylation of ErbB2, GSK3 and MAPK activity, suggesting that the cross-talkbetween growth factor and estrogen signaling may also occur in aggressive cancers.

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INTRODUCTION

The significance of AKT expression and/activity in human cancer has become increasingly evident. However, consistent alterations in overexpression and/or activity of a specific AKT isoform in human breast tumors have not been well documented. A specific isoform of AKT may be preferentially activated (1-5) or activated proteins may have different substrate preferences, providing a therapeutic opportunity to target a particular isoform. Our *in vitro* data show that the growth factors epidermal growth factor (EGF), insulin-like growth factor-I (IGF-I), and heregulin β1 (HRG-β1), as well as estradiol, can activate the phosphatidylinositol 3-kinase/serine-threonine protein kinase PI 3-K/Akt pathway in estrogen receptor (ER)-positive breast cancer cells (6-10). The effects of estradiol and HRG-β1 are mediated by membrane ER-α and by the ErbB2 but not by the EGFR signaling pathway (8,9). Moreover, estradiol and growth factors can induce anchorage-dependent and independent proliferation and membrane ruffling that can be inhibited by antiestrogens, selective ErbB2 inhibitors and by either PI 3-K inhibitors or dominant negative Akt1 mutants (K179M-Akt1 or an Akt1 mutant with a mutation in the pleckstrin homology domain (PH), R25C-Akt1). In contrast, Akt1 exerts estrogen-like effects on cell growth, membrane ruffling, and ER-α regulation and tamoxifen cannot fully abolish its effect (10). Taken together, these data suggest that estradiol, EGF, and HRG-β1 interact with membrane ER-α and a heterodimer with ErbB2, leading to tyrosine phosphorylation. This results in activation of PI 3-K and Akt1. Akt1, in turn, may interact with nuclear ER-α, altering its expression and activity, as well as cell proliferation and response to tamoxifen. Additionally, we have validated that the ErbB2/PI 3-K/Akt1 pathway is also active in animals. Inoculation of parental or empty-vector-transfected MCF-7 cells into ovariectomized female nude mice led to appearance of tumors four weeks after estradiol supplementation and tamoxifen and an ErbB2 selective inhibitor, AG825 blocked the estradiol effect (11, 12). Moreover, tumor volumes after estradiol supplementation were substantially inhibited upon inoculation of MCF-7 cells stably transfected with either dominant negative Akt1 or R25C-Akt1 (11, 12).

Therefore, this proposal was aimed to determine ErbB, Akt, and ER-α expression and activity in 42 breast tumors, their surrounding normal tissue, and stroma with the end-point to compare responsiveness to tamoxifen in ER-positive, ErbB2 normal tumors with high Akt1 activity versus no Akt1 activity.

BODY

Task 1: Selection of frozen tumors (including adjacent control tissue) and paraffin sections)

Since the Tissue Shared Resource of the Lombardi Cancer Center could not provide us with more than 4 frozen tumor tissues, we contacted the National Disease Research Interchange (NDRI) and we almost completed the selection of frozen tumors (including adjacent control tissue) and paraffin sections for gathering the 7 samples in each of the following 6 groups of tumors based on ER-α and ErbB2 levels: Group 1 (ER+/ErbB2high), Group 2 (ER+/ErbB2low), Group 3 (ER+/ErbB2-), Group 4 (ER-/ErbB2high), Group 5 (ER-/ErbB2low), and Group 6 (ER-/ErbB2-)(Table 1). Instead of

being a retrospective study as it was designed, it will now be a prospective study. We are requesting follow-up information (tamoxifen responsiveness) and we have gathered all

Table 1. Tumor Classification

# OD#\Group1	Group2	Group 3	Group 4	Group5	Group6
ER+/ErbB2>>	ER+/ErbB2low	ER+/ErbB2-	ER-/ErbB2>>	ER-/ErbB2low	ER-/ErbB2-
1. 13485-10	15299	13365-12	16426-05	12748-02	17453-11
2. 12959-06	13425-02	13835-03	17223-14	12867-14	12746-04
3.	13819-01	15685-05	17520-12	15378-03	12870-02
4.	14495-01	18205-01	18205-01	14658-13	15371-07
5.	13781-02	13285-02	18470-01	12593-09	15091-05
6.	17905-01	15428-05	18473-01	15699-01	13721-07
7.	18333-04	14851-05		18175-06	15806-03
8.		15823-09		18472-01	
9.		17974-04			
10.		16765-16			
11.		17016-06			
12.		17659-01			
13.		17467-03			
% 5	16	30	14	19	16

the traditional clinical and pathological markers (tumor type, stage, nodal status, invasiveness, patient age, ER, PR, ErbB2 status, etc) (we are still waiting for ER, PR, and ErbB2 status for the tumor with OD# 17174-17) (Table 2):

Table 2. Tumors from NDRI

#	OD#	Tumor Type	L.Nodes	Age	ER	PR	ErbB2	Other
1.	12593-09	invasive ductal (comedo) carcinoma, gr.3	0/12	88	-	-	-/1	
2.	12746-04	ductal carc., poorly differ., gr. 3	0/15	51	-	-	-	
3.	12748-02	ductal carcinoma, poorly differ., gr.2-3	0/6	61	-	-	1	
4.	12867-14	bilateral infiltrating ductal carcinoma, gr.3	1 met	52	-	-	2	
5.	12870-02	invasive ductal carcinoma, gr.3 (nucl gr. 2)	-	50	-	-	-	
6.	13365-12	mucinous adenocarcinoma, inv. gr.2	0/5	84	+	+	-	
7.	13425-02	invasive ductal carcinoma, gr.2 (nucl gr. 3)	4/7	38	+	+	2	
8.	13485-10	infiltrating ductal carc., gr.3, poorly diff.	ND	87	+	-	3	
9.	13546-03	infiltrating ductal carcinoma, gr.3	5/9	57	ND	ND	-/1	
10.	13721-07	inv. carc, mod. diff.,metapl, squamous,gr.2	0/5	69	-	-	-	K_i67high
11.	13819-01	ductal carc, poorly diff., dormal lymph, inv, rec	ND	72	+	+	2	
12.	13835-03	infiltr duct carc, lympha and pagetoid, gr.2	-	73	+	+	-	
13.	12959-06	in situ ductal carcinoma, gr.3,2foci microinv	0/5	39	+	+	3	
14.	14495-01	invasive ductal carcinoma, gr.2	0/9	78	+	+	-	
15.	13285-02	residual intraductal carcinoma	2/8	62	+	-	-	
16.	13781-02	invasive duct carc in fibromusc tissue, gr.2-3	ND	69	+	-	2	
17.	14658-13	ductal carcinoma, invasive T4N2M1	1/12	68	-	-	1	
18.	14851-05	invasive lobular carcinoma, gr.2	1/2	77	+	+	-	K_i67high

19. 15091-05	invasive ductal carcinoma, gr.3	ND	88	- - -
20. 15299	invasive ductal carcinoma, gr.2	0/17	74	+ + 2 K_i67high
21. 15371-07	spindle-cell sarcoma, intermed.-high gr.	1/11	69	- - -
22. 15378-03	invasive duct., infiltrating carcinoma, gr.3 (3)	0/8	50	- - 2
23. 15428-05	infiltrating ductal carcinoma, gr.2	0/3	58	+ + -K_i67bord
24. 15685-05	invasive ductal carcinoma, gr.3	0/6	80	+ + -
25. 15806-03	inv. duct. carc. with medullary features, gr.3	0/4	81	- -- K_i67high
26. 15823-09	Infiltrating ductal invasive	17/17	80	+ +- K_i67high
27. 15699-01	inv. ductal carc., ZL, gr.3, 4cm	ND	30	- - + CK7+
28. 16426-05	invasive ductal carcinoma, gr.3, fibrocistic	12/12	46	- - 3+
29. 16765-14	inv. duct. adenocarc., gr.2, 4x4 cm	3/19	81	+ +- K_i67high
29. 16765-16	metastasis of 16765-14	3/19	81	+ +- K_i67high
30. 17016-06	infiltrating duct. carc., inv., colloid, gr.3	0/12	93	+ + - K_i67low
31. 17223-14	poorly differ. ductal carcinoma, gr.3,invasive	5/13	75	- -3+ K_i67high
32. 17174-17	invasive duct carc, gr.2, lobular feat, nipple	13/29	63	
33. 17453-11	invasive duct carc, gr.3	5/24	37	- - - K_i67high
34. 17467-03	invasive lobular carcinoma, T3N2MX		85	+ + -
34. 17467-05	metastasis of 17467-03		85	ND
35. 17520-12	invas. duct. carc., gr.3, extens.angiolymph, L	9/9	52	- - 3+
36. 17659-01	invas. lobular carc, gr.2-3, 6cm	0/5	62	+ + - K_i67low
37. 17905-01	in situ (25%) and invas. duct.carc, gr.3 (nucl2)	ND	50	+ + 2+
38. 17974-04	inv. duct. carc. and high gr. DCIS, gr3	ND	36	+ + - K_i67high
30. 18205-01	inv. duct. infiltr. adencarc, gr.3, L, 4.5x3.5x2.4	16/22	68	- - 3+ K_i67bord
40. 18175-06	inv duct carc, poorly diff 3.5x2.6x2.5, gr 3, inv	1/2	83	- - 2+ MIB-1
41. 18333-04	inv duct carc w DCIS, 9cm, gr 3 (3), inv	20/28	72	+ + 2+
41. 18333-24	met of 18333-04 inflammat		72	ND
42. 18470-01	duct adenocarc, gr 3(nucl2), fibrocys backgr, L	5/9	80	- - 3+
43. 18472-01	infiltr duct w medull feat, gr 2(nucl3), w necrosis, R	0/9	55	- - 1+
44. 18473-01	inv duct carc, gr 3(3), w necrosis, fibrocys backgr, R	1/10	45	- - 3+

ND- not determined

Although we have received more than the 42 expected tumors, as you can see from Table 1, we still need 5 tumors for Group 1 and 1 tumor for Group 4 in order to have 7 samples in each group for our final end point (according to our power analysis). This is not unexpected since tumors that are ER+ and ErbB2 high are very rare.

Task 2: Measurement of expression of ErbB receptors

a) Lysates from all 44 frozen tumors and adjacent normal tissue were prepared

b) Protein expression/activity for EGFR, ErbB2, ErbB3, and ErbB4 were analyzed for most of the lysates (Figure 1). We used the following primary antibodies:

Table 3. Primary Antibodies Used for Western Blot Analysis

# Antibody		Dilution	Source
1. ErbB2	Polyclonal	1:1,000	Cell Signaling Technology
2. EGFR	Polyclonal	1:1,000	Cell Signaling Technology
3. ErbB3	Monoclonal	1:1,000	Upstate Biotechnology

4. ErbB4	Polyclonal	1:1,000	Upstate Biotechnology
5. Phospho-EGFR(Tyr845)	Polyclonal	1:1,000	Cell Signaling Technology
6. Phospho-EGFR(Tyr1068)	Polyclonal	1:1,000	Cell Signaling Technology
7. Phospho-EGFR(Tyr992)	Polyclonal	1:1,000	Cell Signaling Technology
8. Phospho-EGFR(Tyr1045)	Polyclonal	1:1,000	Cell Signaling Technology
9. Phospho-ErbB2(Tyr1248)	Polyclonal	1:1,000	Cell Signaling Technology
10. Phospho-ErbB2(Tyr847)	Polyclonal	1:1,000	Cell Signaling Technology
11. Phospho-ErbB2(Tyr1221)	Polyclonal	1:1,000	Cell Signaling Technology
12. Phospho-ErbB3(Tyr1289)	Monoclonal	1:1,000	Cell Signaling Technology
13. Actin	Monoclonal	1:500	Santa Cruz Biotechnology
14. Akt	Polyclonal	1:1,000	Cell Signaling Technology
15. Phospho-Akt(1/2/3)(Ser473)	Polyclonal	1:1,000	Cell Signaling Technology
16. Akt1	Polyclonal	1:1,000	Upstate Biotechnology
17. Akt2	Polyclonal	1:1,000	Upstate Biotechnology
18. Akt3	Polyclonal	1:1,000	Upstate Biotechnology
19. Phospho-GSK3(Ser21/9)	Polyclonal	1:1,000	Cell Signaling Technology
20. MAPK	Polyclonal	1:1,000	Cell Signaling Technology
21. Phospho-MAPK(Thr202/204)	Polyclonal	1:1,000	Cell Signaling Technology

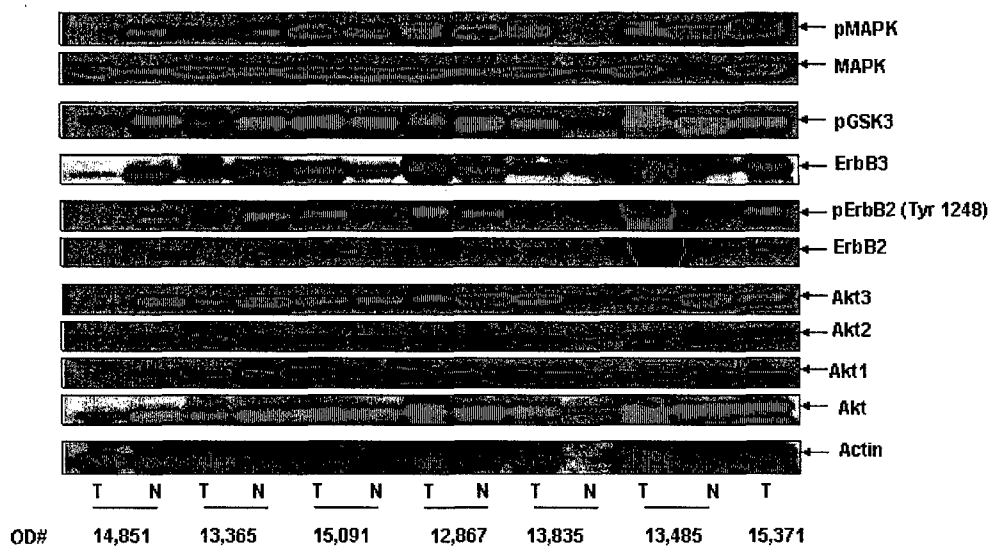

Figure 1. Expression/Activity of ErbB2, ErbB3, Akt Isoforms, GSK3, and MAPK in Human Breast Tumors (T) and Adjacent Normal Tissue (N). *Western blot analysis: lysates of seven T and six N were analyzed by immunoblotting using the primary antibodies from Table 3. Actin was used as an internal control.*

ErbB2 expression correlated well with ErbB2 activity (Figure 1). The tyrosine phosphorylated was Tyr1248. None of the other phospho-specific ErbB2 antibodies gave any signal in any tumors, suggesting that Tyr1248 and not Tyr 847 or Tyr1221 is phosphorylated in the analyzed tumors. When we compared our western blot results with the immunohistoscores obtained from NDRI, a good correlation was observed for most of the tissues. However, some of the reported negative ErbB2 scores were perceived as weak positive by our western blot analysis (e.g. OD# 15091, OD#15371). ErbB3 was similarly expressed to ErbB2,

but no ErbB3 activity was detected. ErbB3 correlated well with pAkt and pGSK3, a downstream target of Akt. This suggests that in these tumors ErbB2 heterodimerizes with ErbB3, phosphorylating Tyr1248 of ErbB2. ErbB3 may further interact with PI-3K, activating Akt and phosphorylating GSK3 that leads to its inactivation. No EGFR expression/activity or ErbB4 expression was detected in any of the tumors tested.

Comparison of tumor tissue (T or C) with normal adjacent tissue (N) generally shows higher expression/activity in T than in N (Figure 1) or even no expression/activity of ErbB2 and ErbB3 in N (Figure 2). In some cases, however, we could see weak expression/activity of the ErbB receptors in N. Moreover, in OD# 14851, no expression/activity of ErbB receptors was observed in T but a moderate expression/activity was seen in N (Figure 1).

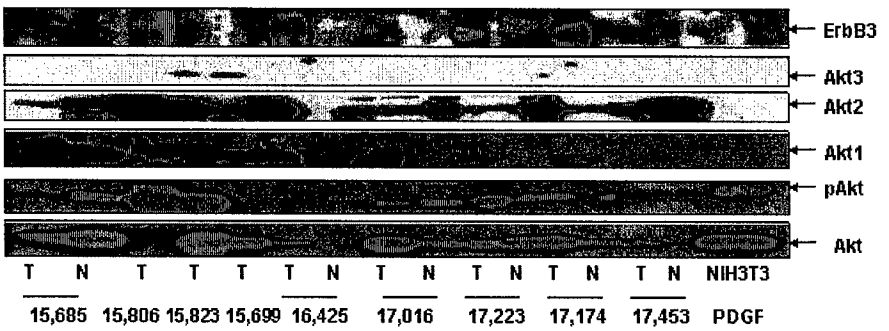

Figure 2. <u>Expression/Activity of ErbB3 and Akt Isoforms in Human Breast Tumors (T) and Adjacent Normal Tissue (N).</u> Western blot analysis: lysates of nine T and six N were analyzed by immunoblotting using the primary antibodies from Table 3. Lysate from PDGF-treated NIH3T3 cells was used as a positive control.

c) Paraffin sections from 8 tumors and 8 adjacent normal tissues were analyzed by immunohistochemistry for ErbB2 and PR (Figures 3, 4) and were compared to the values received from NDRI. A very good correlation was observed between the two scores this time.

Table 4. Primary Antibodies Used for Immunohistochemistry

# Antibody		Dilution	Source
1. ErbB2	Polyclonal	1:100	Cell Signaling Technology
2. PR	Polyclonal	1:100	Santa Cruz Biotechnology
3. Akt	Polyclonal	1:100	Cell Signaling Technology
4. Phospho-Akt(1/2/3)(Ser473)	Polyclonal	1:200	Cell Signaling Technology

<u>Tasks 3 and 4: Determination of the expression/activity of AKT1, AKT2, AKT3 in frozen tumor tissue</u>

a) We prepared total RNA from each of the 44 tumors and normal adjacent tissue

b) We designed primers and probes for each AKT isoform for real-time RT-PCR and tested them. Unfortunately, they did not work specifically for each isoform. Major cross-reaction was observed for each of the three isoforms. Therefore, we don't have any data yet for AKT1mRNA, AKT2mRNA, and AKT3mRNA. Our final decision is to use semi-quantitative RT-PCR for each Akt isoform and use actin as an internal control. We will

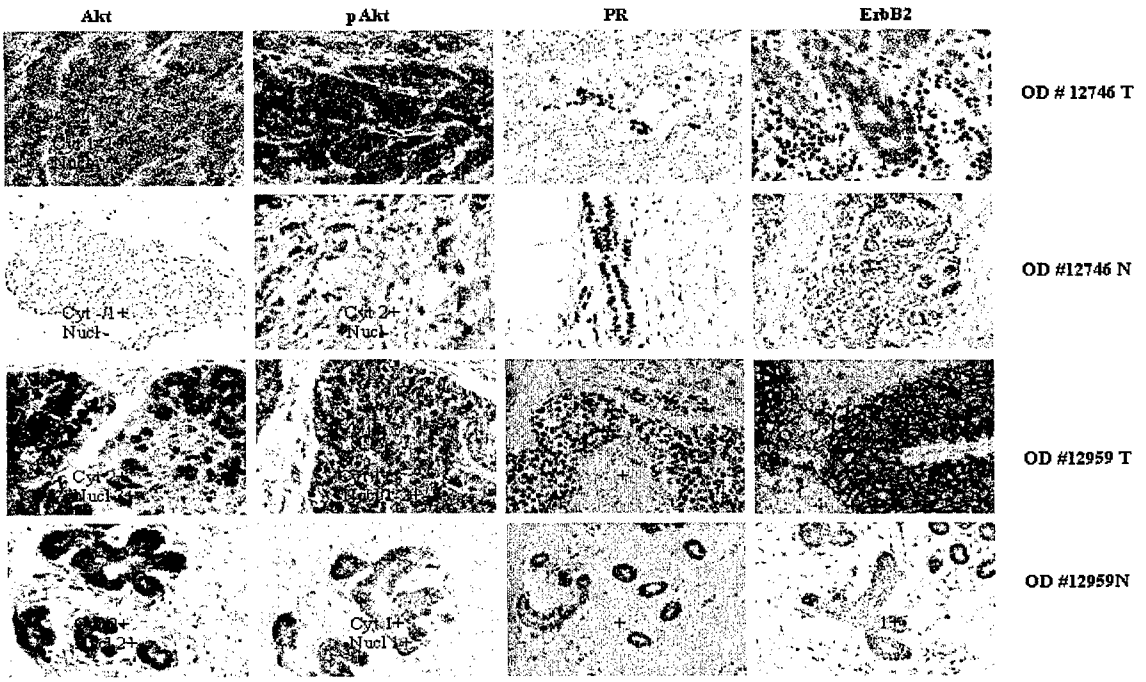

Figure 3. Immunohistochemistry for ErbB2, Akt, pAkt, and PR in Human Breast Tumors (T) and Adjacent Normal Tissue (N). *Immunohistochemical staining was performed using an anti-PR, ErbB2, Akt, or P-Akt antibody (Table 4). The immunohistochemical staining intensity was categorized into four grades (- = negative, 1=weak, 2=moderate, 3=strongly positive). The final score resulted from the product of staining intensity and percentage of stained cells.*

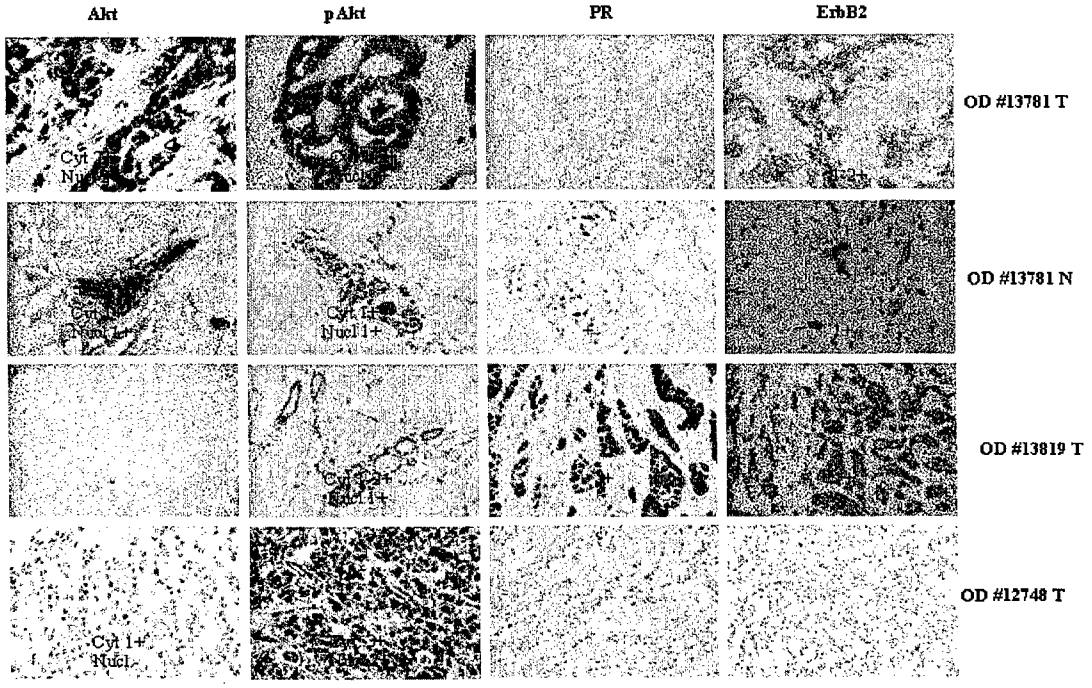

Figure 4. Immunohistochemistry for ErbB2, Akt, pAkt, and PR in Human Breast Tumors (T) and Adjacent Normal Tissue (N). *Immunohistochemical staining was*

performed using an anti-PR, ErbB2, Akt, or P-Akt antibody (Table 4). The immunohistochemical staining intensity was categorized into four grades (- = negative, 1=weak, 2=moderate, 3=strongly positive). The final score resulted from the product of staining intensity and percentage of stained cells.
use the following primers (Table 5) and PCR reaction characteristics: RT at 42 °C for 50 minutes, 70 °C for 15 minutes; PCR – 35 cycles: denaturation at 94 °C for 1 minute, annealing at 55 °C for 1 minute, and extension at 72 °C.

Table 5. RT-PCR Reaction

#	AKT Isoform		Primers
1	AKT1	forward	5'GTCGGACGATAGCTTGGA 3'
		reverse	5'GATGACAGATAGCTGGTG 3'
2	AKT2	forward	5'GGCCCCTGATCAGACTCTA 3'
		reverse	5' TCCTCAGTCGTGGAGGAGT 3'
3	AKT3	forward	5'GCAAGTGGACGAGAATAAGTCTC3'
		reverse	5'ACAATGGTGGGCTCATGACTTCC3'
4	actin	forward	5'AGCAAGAGAGGCATCCTCACCCTGAAGTACC3'
		reverse	5'CAGATTCTCCTTAATGTCACGCACGATTTCCC3'

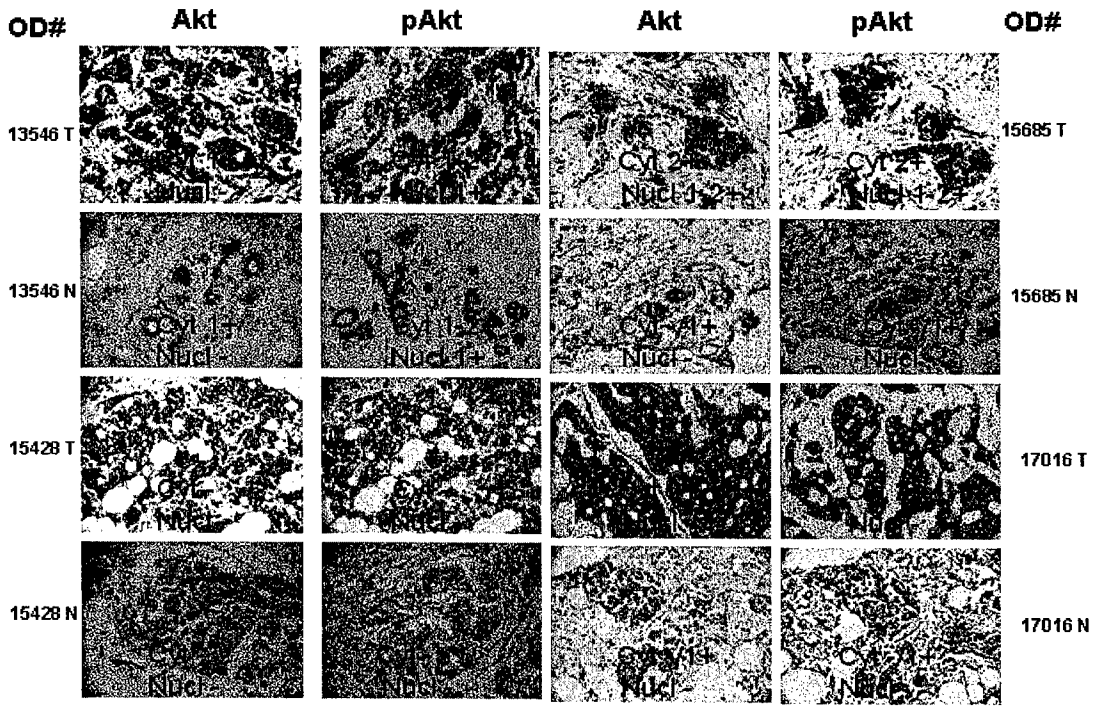

Figure 5. Immunohistochemistry for Akt and pAkt in Human Breast Tumors (T) and Adjacent Normal Tissue (N). *Immunohistochemical staining was performed using an anti- Akt, or P-Akt antibody (Table 4). The immunohistochemical staining intensity was categorized into four grades (- = negative, 1=weak, 2=moderate, 3=strongly positive). The final score resulted from the product of staining intensity and percentage of stained cells.*

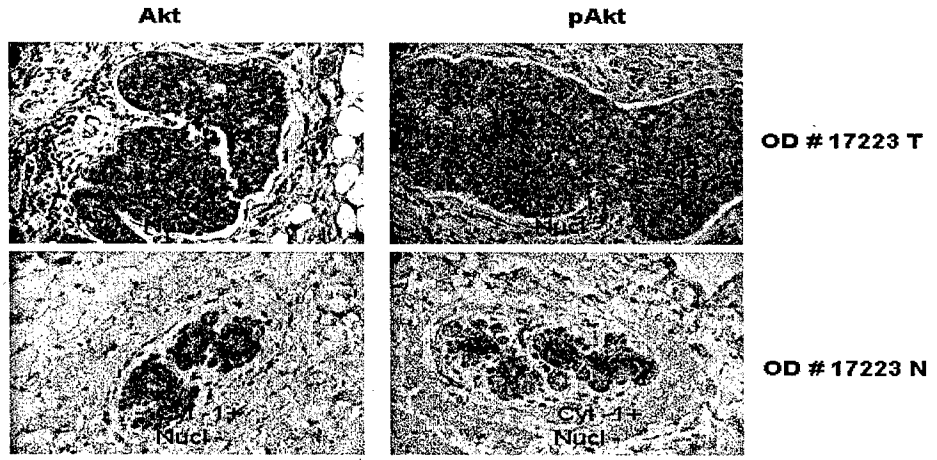

Figure 6. Immunohistochemistry for Akt and pAkt in Human Breast Tumors (T) and Adjacent Normal Tissue (N). *Immunohistochemical staining was performed using an anti- Akt, or P-Akt antibody (Table 4). The immunohistochemical staining intensity was categorized into four grades (- =negative, 1=weak, 2=moderate, 3=strongly positive). The final score resulted from the product of staining intensity and percentage of stained cells.*

Table 6. Akt and pAkt

A. Immunohistochemistry – $n_T = 31$; $n_N = 22$

Localization	Akt				pAkt			
	T		N		T		N	
Score	n	%	n	%	n	%	n	%
cytoplasmic: -	1	3	4	18	1	3	5	23
1+	11	35.5	14	64	18	58	15	68
2+	17	55	4	18	11	35.5	2	9
3+	2	6.5	0	0	1	3	0	0
total +	30	97	18	82	30	97	17	77
nuclear: -	11	35.5	16	73	20	64.5	14	64
1+	7	22.5	3	14	4	13	7	32
2+	11	35.5	3	14	7	22.5	1	4.5
3+	2	6.5	0	0	0	0	0	0
total +	20	64.5	6	27	11	35.5	8	36

c) We detected protein expression for total Akt by immunostaining of paraffin sections of 31 tumors and their adjacent normal tissues with total anti-Akt antibody. Scores as intensity staining were assigned – this reflects level of expression low (1+) to high (3+). Also percent of tumor positivity (entire, partial, or localized) were noted (Figures 3-6) and Table 6A.

Akt staining occurred in ductal areas of tumors and in cell groups surrounded by lymphocytes and inflammatory cells. Staining heterogeneity was observed. Akt expression was higher in the cytoplasm than in the nucleus. Normal breast tissue revealed mostly a cytoplasmatic Akt staining pattern, but the staining intensity and percentage of

stained cells was lower. From 31 analyzed tumors, 97% were positively stained for Akt in the cytoplasm and 64.5% were positively stained in the nucleus as compared to 82% and 27% of normal tissues positively stained in the cytoplasm and nucleus, respectively. Some of the adjacent tissues received as "normal" were either contaminated with tumor tissue or contained entirely collagen and/or adipose tissue, lacking breast lobules, ducts, and/or breast epithelial cells. These were not included in the results from Table 5A (this is the reason for a discrepancy between the number of T and N).

Most of the tumors that expressed Akt were also phosphorylated in both tumor and normal surrounding tissue, usually with a higher intensity and/or percentage in the tumor. Staining heterogeneity was apparent, especially at the invasive tumor front. In normal tissue, hyperplastic ducts and scattered end units were also positive for active Akt. From 31 tumors, 97% were scored positive for pAkt in the cytoplasm and 35.5 % were positive in the nucleus. The normal matched tissues stained lower for Akt activity in the cytoplasm (77%) but equally (36%) in the nucleus.

d) We prepared lysates from 44 frozen tumors and adjacent tissue and performed western blot analysis for Akt expression and activity (Figures 1,2,7-9) (Table 5B). There was a very good correlation between western blot scores and immunohistochemical scores (Table 6). Comparison between T and N revealed even a higher number of N that had equal and even larger Akt bands than their T counterparts, suggesting again that some adjacent tissue might have been contaminated with tumor tissue but also that some epithelial cells contain Akt expression and activity (Figures 7, 10). From 39 tumors (T) analyzed by western blot, 90% were positive for Akt as compared with 75% for adjacent tumor tissue (N). Again, Akt expression correlated well with Akt activity (pAkt) in almost all cases. 93% of the tumors were positive for p-Akt as compared to 89% for adjacent normal tissue (Table 6B). Moreover, Akt activity correlated well with GSK3 phosphorylation (Figures 2 and 9) and with MAPK activity (Figures 1, 7-9).

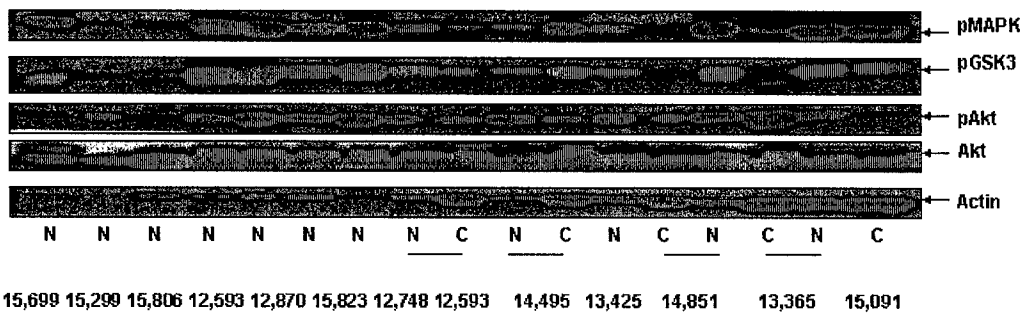

Figure 7. Expression/Activity of Akt, GSK3, and MAPK in Human Breast Tumors (C) and Adjacent Normal Tissue (N). *Western blot analysis: lysates of seven T and six N were analyzed by immunoblotting using the primary antibodies from Table 3. Actin was used as an internal control.*

Concerning AKT isoforms, Akt expression was observed as a mixture of all three AKT isoforms: AKT1, AKT2, and AKT3 (70% of analyzed cases) or in some cases of at least two isoforms. No tumor analyzed so far consisted of a single isoform. Tumors negative for Akt and pAkt were, in general, also negative for all Akt isoforms (12% of analyzed cases – examples: OD# 15, 299; 17,461, and 17,659). However, at this time, only western blots were performed without previous immunoprecipitation. Since our preliminary *in vitro* experiments revealed that MCF-7 cells only expressed AKT1 and

AKT2 and no AKT3 while MDA-MB231 expressed mainly AKT3 and very little AKT2 and no AKT1 and these western blot results were similar to kinase assays, we think that the AKT1, AKT2, and AKT3 antibodies are specific for each Akt isoform and do not cross react. Further kinase assays will confirm these findings.

Table 7. Akt and pAkt

B. Western Blot

Score	Akt				pAkt			
	T		N		T		N	
	n	%	n	%	n	%	n	%
-	4/39	10	9/36	25	3/41	7	4/37	11
1+	15/39	38	9/36	25	15/41	37	21/37	57
2+	9/39	23	13/36	36	14/41	34	9/37	24
3+	11/39	28	5/36	14	9/41	22	3/37	8
total +	35/39	90	27/36	75	38/41	93	33/37	89

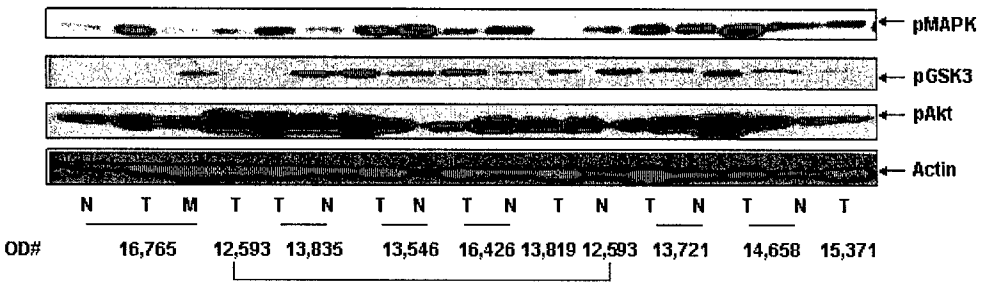

Figure 8. <u>Activity of Akt and MAPK in Human Breast Tumors (T), Adjacent Normal Tissue (N), and a metastasis (M).</u> *Western blot analysis: lysates of nine T, seven N, and 1 M were analyzed by immunoblotting using the primary antibodies from Table 3. Actin was used as an internal control.*

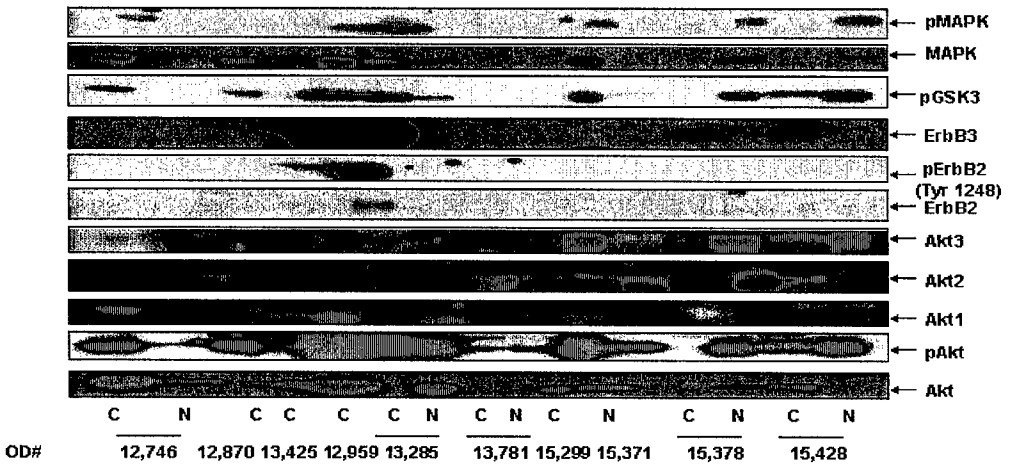

Figure 9. <u>Expression/Activity of ErbB2, ErbB3, Akt Isoforms, GSK3, and MAPK in</u>

Human Breast Tumors (C) and Adjacent Normal Tissue (N). *Western blot analysis: lysates of nine T and six N were analyzed by immunoblotting using the primary antibodies from Table 3.*

Table 7. Akt and pAkt Comparison Between Immunohistochemistry and Western Blot

# OD#	Akt						pAkt					
	IHC				WB		IHC				WB	
	Cytoplasmic		Nuclear				Cytoplasmic		Nuclear			
	T	N	T	N	T	N	T	N	T	N	T	N
1. 12593-09	ND	ND	ND	ND	3+	2-3+	ND	ND	ND	ND	ND	ND
2. 12746-04	1-2+	1+	-	1-2+	3+	-/1+	2-3+	2+	2+	-	1-2+	-/1+
3. 12748-02	1+	ND	-	ND	ND	2+	2+	ND	-	ND	ND	-/1+
4. 12867-14	ND	ND	ND	ND	1-2+	1+	ND	ND	ND	ND	ND	ND
5. 12870-02	ND	ND	ND	ND	1+	3+	ND	ND	ND	ND	1-2+	3+
6. 13365-12	-	-	1+	-	2+	2+	2-3+	2+	2+	-	2+	1+
7. 13425-02	ND	ND	ND	ND	1+	2+	ND	ND	ND	ND	1+	1+
8. 13485-10	1+	-/1+	1-2+	1-2+	1+	2+	1+	1-2+	-	1+	ND	ND
9. 13546-03	2+	-	-	-	ND	ND	2+	-	2+	-	3+	2+
10. 13721-07	2+	1+	2+	1+	1+	-	1+	1+	2+	1+	1+	1+
11. 13819-01	2+	-/1+	1-2+	1+	ND	ND	1+	1+	-	1+	2+	ND
12. 13835-03	ND	ND	ND	ND	2+	-	ND	ND	ND	ND	3+	2+
13. 12959-06	2+	1+	3+	1+	3+	ND	1+	1+	1-2+	1+	3+	ND
14. 14495-01	1+	ND	1+	ND	2-3+	3+	-/1+	ND	-	ND	2+	1+
15. 13285-02	2+	2+	1+	2+	1-2+	2+	1+	1+	2+	2+	2+	3+
16. 13781-02	2+	1+	2+	1+	-	-	1+	1+	2+	1+	1+	1+
17. 14658-13	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	2+	1+
18. 14851-05	1+	ND	-/1+	ND	1+	2+	-/1+	ND	-	ND	1+	2+
19. 15091-05	ND	ND	ND	ND	2+	2+	ND	ND	ND	ND	1+	-/1+
20. 15299	ND	ND	ND	ND	1/1+	2+	ND	ND	ND	ND	1+	1+
21. 15371-07	2+	-/1+	-	-	2+	1-2+	1+	1+	-	-	1+	1+
22. 15378-03	2+	1+	-	-	2+	1+	-	-/1+	-	-	-	1+
23. 15428-05	-/1+	-	-	-	1+	-	-/1+	-	-	-	2+	3+
24. 15685-05	2+	1+	1-2+	-	1-2+	2-3+	2+	1+	1-2+	-	1+	2+
25. 15806-03	2-3+	ND	1-2+	ND	1+	2+	2+	ND	1+	ND	3+	1+
26. 15823-09	2+	ND	1+	ND	3+	3+	-/1+	ND	-	ND	3+	2+
27. 15699-01	ND	ND	ND	ND	2+	2+	ND	ND	ND	ND	-/1+	-/1+
28. 16426-05	ND	ND	ND	ND	1+	-	ND	ND	ND	ND	1+	1+
29. 16765-14	1-2+	ND	1+	ND	ND	ND	-/1+	ND	-	ND	2+	1+
30. 17016-06	2+	-/1+	-	-	3+	2+	2+	-/1+	-	-	2+	2+
31. 17223-14	1+	-/1+	-	-	2+	2+	1+	-/1+	-	-	3+	2+
32. 17174-17	ND	ND	ND	ND	3+	1+	ND	ND	ND	ND	2+	1+
33. 17453-11	1+	-/1+	1-2+	-	1+	-	2+	-/1+	-	-	1+	-/1+
34. 17467-03	1-2+	ND	2+	ND	-	1+	1+	ND	-	ND	-	1+

35. 17520-12	2+	1-2+	1-2+	-	2+	1+	1-2+	-	-	-	1+	1+
36. 17659-01	-/1+	ND	-	ND	-	-	-/1+	ND	-	ND	-	-
37. 17905-01	1-2+	1+	1-2+	-	2+	1+	1+	-	-	-	1+	-/1+
38. 17974-04	2-3+	2+	1+	-	1+	2+	1-2+	1+	2+	-	3+	-
39. 18205-01	2+	-/1+	1-2+	1-2+	1+	-	2+	-/1+	-	1+	1+	2+
40. 18175-06	ND	ND	ND	ND	2+	-	ND	ND	ND	ND	1+	-
41. 18333-04	1+	1-2+	2+	-	3+	1+	1+	-	-	-	2+	1+
42. 18470-01	1+	ND	1-2+	ND	3+	ND	-/1+	ND	-	ND	2+	ND
43. 18472-01	ND	ND	ND	ND	3+	ND	ND	ND	ND	ND	3+	ND
44. 18473-01	1-2+	ND	1+	ND	-/1+	ND	-/1+	ND	-	ND	2+	ND

Correlation between IHC and WB for Akt:	T	N
identical:	13/24 (54%)	10/18 (56%)
-/+1:	8/24 (33%)	7/18 (39%)
-/+2:	3/24 (12.5%)	1/18 (5.5%)
Correlation between IHC and WB for pAkt:	T	N
identical:	11/27 (41%)	8/18 (44%)
-/+1:	14/27 (52%)	7/18 (39%)
-/+2:	2/27 (7.5%)	3/18 (17%)

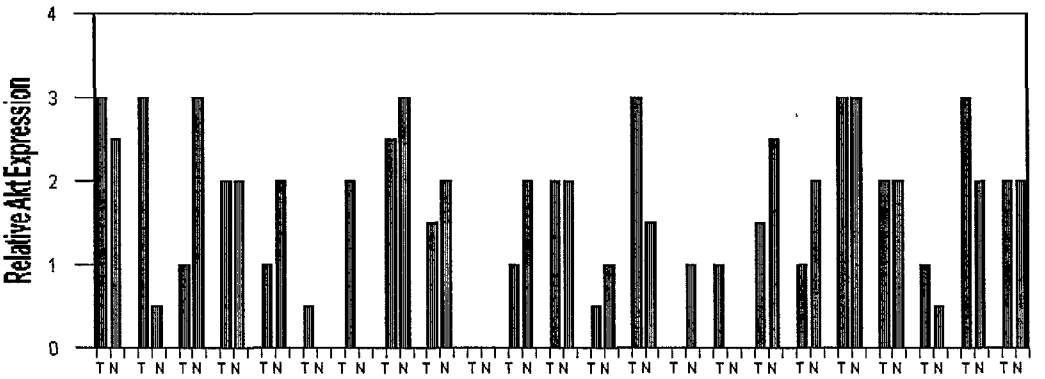

Figure 10. Relative Akt Expression of Matched Human Tumors (T) with Their Adjacent Normal Tissues (N). *Western blot analysis: lysates of 23 T and N were analyzed by immunoblotting using the primary antibodies from Table 3. Actin was used as an internal control. Akt is expressed in arbitrary units where 0-no Akt band or no Akt expression, 1+ weak band, 2+ moderate band, 3+ large band or very high Akt expression.*

Tasks 5 and 6. Correlation of activated AKT1 with ER-α activity, by patient subgroups and with other traditional prognostic markers.

The correlation between Akt expression/activity with the clinical and tumor characteristics of the patients is represented in Table 8. From a total of 44 analyzed tumors, 79.5% were from postmenopausal and 20.5% were from premenopausal women. The majority of the tumors (82%) were ductal carcinomas. Only 7% were lobular carcinomas and 4.5% were *in situ* ductal carcinomas (DCIS). Under "other histological tumor types (other)" we included a mucinous adenocarcinoma, a squamous-cell carcinoma, and a spindle-cell carcinoma (7%). Most of the tumors were invasive (82%

were poorly differentiated and 11% were moderately differentiated) and high grade (grade 3- 64%, grade 2 – 32%). 45% of the analyzed tumors were lymph node positive and 39% were lymph node negative. We had 48% ER-positive and 48% ER-negative tumors with 55% PR-negative and 43% PR positive.

Table 8. Clinical and Tumor Characteristics of Patients
Total number of patients: 44

#	Factor	n	%
1	Age: <50	9	20.5
	>51	35	79.5
2	Tumor type: in situ ductal	2	4.5
	ductal	36	82
	lobular	3	7
	other	3	7
3.	Invasiveness: poorly differentiated	36	82
	moderately differentiated	5	11
	unknown	3	7
4.	Nuclear Grade: 2	14	32
	3	23	52
	unknown	7	16
5.	Histological Grade: 2	15	34
	3	28	64
	unknown	1	2
6.	Lymph Nodes: negative	17	39
	positive	20	45
	unknown	7	16
7.	ER: negative	21	48
	positive	21	48
	unknown	2	4
8.	PR: negative	24	55
	positive	19	43
	unknown	1	2
9.	ErbB2: negative	21	48
	low (1+, 2+)	15	34
	high (>2)	8	18
10.	Akt: negative	3	9.5
	positive	41	93
11.	pAkt: negative	7	19
	positive	37	84

48% of the tumors were scored ErbB2 negative, 34% were ErbB2 low(score: 1+ and 2+), and 18% were ErbB2 over expressed (>2). Akt expression was positive in 93% of the tumors and negative only in 9.5% of the cases as measured by both immunohistochemistry and western blotting. From the Akt positive tumors, only 9 were

not active, resulting in a 19% pAkt negativity and 84% pAkt positivity. The value of 84% correlates well with a recent study which also analyzed invasive ductal carcinomas (95 cases) and found 81% pAkt positive cases (12). The pAkt negative tumors were mostly ER+ ErbB2- (group3 – 6 cases) or ER+ ErbB2 low (group 4 – 1 case). Since most of the Akt studies had a more heterogeneous tumor pool (in terms of invasiveness, lymph node status, and/or histological grade), the percentage of Akt activity reported was somewhat lower (~50%) (13-22), suggesting that, in our study pAkt may correlate with tumor aggressivity and/or worse prognosis. More cases are needed to confirm this finding and to be able to statistically analyze our data.

Task 7: Construct Kaplan-Meier curves to compare survival, recurrence-free survival, and response to tamoxifen among the six groups of patients

Since this has now become a prospective study and the tumors purchased from NDRI are from patients that had surgery recently, the information of survival and tamoxifen response is not available yet.

We expect, however that the patients with tumors in group 3 and in group 2 with pAkt negative will have the best prognosis and disease-free survival, being the best candidates for hormone therapy with no development of resistance to tamoxifen. In contrast, patients in groups 1 and 2 with high pAkt will probably develop resistance to hormone therapy and will need additional therapeutic intervention, e.g. inhibitors to interrupt the ErbB2/PI 3-K/Akt/ER signaling pathway.

We are intensely seeking more funding to be able to finish and eventually extend this study with the major goal of preventing hormone resistance in patients which have ER-positive tumors and have low-moderate ErbB2 values and are currently not receiving any inhibitors of the ErbB2/PI 3-K/Akt signal transduction pathway.

KEY RESEARCH ACCOMPLISHMENTS AND CONLUSIONS

- We have purchased and selected 44 human breast cancer and matched adjacent normal surrounding tissues for 6 tumor groups based on ER and ErbB2 status and gathered follow-up information about age of the patient, tumor histology, nuclear grade, ER, PR, and ErbB2 status
- We have prepared lysates from 44 frozen tumor-normal tissue pairs and have analyzed ErbB receptors, AKT isoforms, GSK3, and MAPK expression/activity by western blot with total and phospho-specific antibodies. We used actin as an internal control and PDGF-treated NIH 3T3 cells as our positive control.
    a) AKT expression/activity correlated well with ErbB2 expression, ErbB2 phosphorylation on Tyr1248, with ErbB3 expression, GSK3 phosphorylation, and MAPK activity. 90% of the analyzed tumors expressed Akt and 93% were positive for p-Akt.
    b) AKT expression was the result of expression of all three isoforms (AKT1, AKT2, and AKT3) in most of the cases (70%) or consisted of at least two isoforms

c) Normal surrounding tissue also contains AKT and some of this AKT is active. In general, AKT expression/activity is lower in normal tissue than in the tumor itself (several exceptions were however observed)

- To determine AKT localization, we have also analyzed 31 of the 44 tumor-normal surrounding tissue pairs by immunohistochemisty using total and phospho-specific antibodies.
    a) Akt staining heterogeneity was observed. Akt staining occurred in ductal areas of tumors and in cell groups surrounded by lymphocytes and inflammatory cells. Akt expression was higher in the cytoplasm than in the nucleus. 97% of the tumors were positively stained for Akt in the cytoplasm and 64.5% were positively stained in the nucleus
    b) Most of the tumors that expressed Akt were also phosphorylated on Ser 473. Again, staining heterogeneity was apparent, especially at the invasive tumor front. 97% of the tumors scored positive for pAkt in the cytoplasm and 35.5% in the nucleus
    c) Some of the adjacent tissue labeled as "normal" were either contaminated with tumor cells or contained collagen and/or adipose tissue, sometimes lacking lobules, ducts, and/or breast epithelial cells. In normal tissue, hyperplastic ducts and scattered end units were positive for active Akt
    d) A good correlation was found between the western blot results and immunohistochemical scores
- We have examined the correlation of expressed and activated Akt with some clinical and pathological characteristics of the patients
    a) In our study, we have analyzed mostly invasive, high grade ductal carcinomas (usually correlated with worse prognosis and higher incidence of recurrences) with an approximately equal distribution of ER, ErbB2, and lymph node positivity (~50%)
    b) Akt expression was positive in 93% of the tumors and in 84% of the tumors Akt was also active
- Taken together, our results suggest that, similar to our *in vitro* model and animal data, in invasive, high grade ductal carcinomas, Akt expression and activity is high, suggesting a worse prognosis. Akt activity correlates with Tyr 1248 phosphorylation of ErbB2, that can heterodimerize with ErbB3. ErbB3 may interact with PI3-K, activating Akt and phosphorylating its downstream target, GSK3. The ErbB2/PI 3-K/Akt and MAPK pathway activation may lead to ER-α activation via phosphorylation. This, in turn could lead to increased proliferation and resistance to tamoxifen. Therefore, in patients with ER-positive tumors with low-to-moderate ErbB2 expression, but active Akt, antiestrogen resistance may occur and several inhibitors of the key targets: ErbB2, Akt, MAPK as well as their downstream signaling molecules may be needed to be added to antiestrogens for efficient therapy.

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