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Expression of selected proteins in breast cancer brain metastases

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Abstract. The aim of the study was to assess the immunohistochemical (IHC) profiles of SRC3, Pax2, ER, PgR, Her2, EGFR, CK5/6, and Ki67 proteins in breast-cancer brain metastasis. The study utilized tumor samples from 30 metastatic patients and calculated correlations between all IHC variables. In fourteen cases, primary breast cancers paired with secondary deposits were analyzed. We evaluated the association between IHC status in the primary and secondary deposits, grade, and histotype of the tumors. The examination of the metastatic deposits in all 30 patients resulted in positive detection in the following cases: SRC3 in 20 cases (66.6%), Pax2 in 22 (73.3%), ER in 22 (73.3%), PgR in 25 (83.3%), Her2 in 10 (33.3%), EGFR in 12 (40%), CK5/6 in 7 (23.3%), and Ki67 in 23 (76.6%). Grade 2 was found in 13.3% of all patients, and grade 3 in 86.7%. SRC3 and Pax2 were positive in both G2 and G3. Invasive lobular carcinoma and invasive ductal carcinoma were diagnosed in 23.3% and 76.7% of cases, respectively. There were no differences between the IHC expression of the studied proteins in either grading or histotype of the tumors. In the IHC profiles, which included SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, and EGFR, we found no statistically significant differences between the primary cancer and the brain metastasis. In our study of metastatic breast carcinoma deposits, there was no correlation between SRC3, Pax2 status and histotype, and tumor grade. The IHC status of the paired primary and metastatic deposits did not differ in a statistically significant manner. *(Folia Histochemica et Cytobiologica 2013, Vol. 51, No. 3, 213–218)*

Key words: breast cancer, brain metastases, immunohistochemistry, SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, EGFR

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Introduction

Breast cancer is the second most common cause of brain metastasis. Central nervous system (CNS) metastatic deposits during the course of breast cancer have unfavorable prognosis, and are associated with indicators such as a young age at diagnosis, a disease-free interval of less than one year, estrogen receptor negativity, and Her2 receptor positivity. The incidence may be caused by the clonal selection of tumor cells induced by new treatment modalities such as trastuzumab (Herceptin). The development of breast-cancer brain metastasis depends on numerous factors, such as invasiveness, migration, adhesion, extracellular matrix degradation, revascularization, and proliferation. One of the most significant characteristics of malignant breast-cancer cells is their ability to penetrate the blood-brain barrier and subsequently invade the brain tissue.

Estrogens are major factors in the pathogenesis, progression, growth, differentiation, and survival of breast cancer. The cellular effects of estrogen are primarily mediated by estrogen receptor alpha (ERα). ERα is expressed in 70% of breast cancers [1], and endocrine treatment is a key modality in the treatment of the vast majority of breast-cancer patients.

ERα is a transcription factor that acts by regulating gene expression, either through direct binding to estrogen response elements, or by recruitment of gene promoters through interactions with other transcription factors. The p160 coactivators — steroid receptor coactivator-1 (SRC-1), SRC-2, and SRC-3 — are critical to ERα functioning. SRC-3 is also known as AIB1 (Amplified In Breast cancer 1). A large proportion of patients who develop resistance to endocrine therapies respond to alternative endocrine agents, demonstrating that ERα continues to play a critical role in breast-cancer cell proliferation in breast tumors. Steroid Receptor Coactivator-3 (SRC-3/AIB1/ACTR/pCIP/RAC3) is a member of the p160 coactivator family and plays an important role in cell growth, reproduction, metabolism, and cytokine signaling [1–3]. There is growing evidence for the importance of SRC-3 in cell growth and the oncogenesis of breast cancer [3, 4]. The SRC-3 gene is amplified in up to 10% of human breast cancers, and its high expression is found in 64% of cases [5, 6]. SRC-3 amplification is preferentially found in breast tumors that are positive for ERα and Progesterone Receptor (PgR) [7]. It has been shown that the high levels of SRC-3 expression in breast cancer are associated with a decreased risk of relapse in untreated patients [8]. However, in breast-cancer patients treated with tamoxifen, SRC-3 overexpression in tumor tissu e acts as a marker of disease relapse. Furthermore, when the expression of SRC-3 and epidermal growth factor receptor 2 (Her2) were considered together, those patients whose tumors expressed high levels of both SRC-3 and Her2 experienced worse outcomes with tamoxifen therapy than all other patients combined [8]. This indicates that tamoxifen antitumor activity in breast cancer may be partly determined by SRC-3 and Her2 tumor levels [8]. The Pair box (Pax) gene family consists of nine genes that encode transcription factors [9]. The Pax2 protein competes with SRC-3 for the binding and regulation of Her2 transcription, which determines tamoxifen response in breast-cancer cells [10]. Epidermal growth-factor receptor (EGFR) activates the tumor cells through its specific surface receptor, and a higher expression of EGFR has been found in metastatic brain disease [11].

Material and methods

Patients. We studied 30 brain metastatic breast-cancer deposits in cooperation with Imperial College London in the UK and the Charles University Teaching Hospital in Prague, Czech Republic. The project was approved by the local ethics committees. All of the patients had been previously treated for brain metastasis, and all of the brain tissue samples were obtained during neurosurgical procedures. 23 patients were treated for primary breast-cancer tumors with standard anticancer drugs, such as docetaxel, cisplatin, and etoposide, in the course of standard oncological regimes. None of these anticancer medications are capable of penetrating the blood-brain barrier.

Sections of paraffin-embedded tissues were studied through immunohistochemistry (IHC), using monoclonal antibodies against ER, PgR, Her2, SRC3, Pax2, CK5/6, Ki67, and EGFR proteins. All the primary and secondary breast-cancer deposits underwent IHC staining processes at one time. Five-micrometer sections of each paraffin-embedded specimen were stained with hematoxylin and eosin, in order to verify the presence of adequate numbers of invasive tumor cells and the tumor grade. Cases were considered positive for ER, PgR, Her2, SRC3, Pax2, CK5/6, Ki67, and EGFR when at least 10% of the tumor cells showed distinct positive staining. Immunohistochemistry for ER (clone SP1, Ventana, Tucson, AZ, USA), PgR (clone IE2, Ventana, Tucson, AZ, USA), and Her2 (clone 4B5, Ventana, Tucson, AZ, USA) was used to semiquantitatively measure ER, PgR, and Her2 protein expression (Ventana Benchmark XT, Ultraview detection kit, Tucson, AZ, USA).

Antigen retrieval was performed by heating the slides for 30 minutes in HCl. Antibodies were applied at room temperature: ER for 28 minutes, PgR for 24 minutes, and Her2 for 16 minutes. Immunohistochemistry for EGFR (Bondmax, Bucks, United Kingdom) was used to semiquantitatively measure EGFR protein expression (Bondmax, Leica, Bucks, United Kingdom), and a Bond Polymer Detection Kit DS9800 was employed (Leica, Bucks, United Kingdom). Antigen retrieval was performed by heating the slides in protease digestive enzyme (Leica,

Table 1. Source of primary antibodies used in the study

Antibody	Company
ER (clone SP1)	Ventana, Tucson, Arizona, AZ, USA
PgR (clone IE2)	Ventana, Tucson, Arizona, AZ, USA
Her2 (clone 4B5)	Ventana, Tucson, Arizona, AZ, USA
EGFR	Bondmax, Bucks, United Kingdom
Ki67	Bondmax, Bucks, United Kingdom
CK5/6	Bondmax, Bucks, United Kingdom
AIB1	BD Transduction Laboratories, San Diego, CA, USA
Pax2 (ab38738)	Abcam, Cambridge, United Kingdom

Bucks, United Kingdom) for 10 minutes. EGFR antibody was applied for 30 minutes in a 1:50 concentration. Immunohistochemical staining for Ki67 (Bondmax) and CK5/6 was used to semiquantitatively measure Ki67 and CK5/6 protein expression (Bondmax, Leica, and Bond Polymer Detection Kit DS9800). Antigen retrieval was performed by heating the slides for 30 minutes (Ki67) and 20 minutes (CK5/6) in ER1 (Bond Epitope Retrieval Solution 1, Leica, Bucks, United Kingdom). The antibodies were applied for 30 minutes at concentrations of 1:100 (Ki67) and 1:200 (CK5/6) [12].

Immunohistochemistry for AIB1 (BD Transduction Laboratories, San Diego, CA, USA) was used to semiquantitatively measure AIB1 protein expression (Bond Polymer Detection Kit DS9800, Leica, Bucks, United Kingdom). In summary, antigen retrieval was performed by heating the slides for 30 minutes in ER2 (Bond Epitope Retrieval Solution 2, Leica, Bucks, United Kingdom). AIB1 antibody was applied for 30 minutes at a concentration of 1:500 [13]. PAX2 immunohistochemistry was performed on an automated BondMax Immunostainer (Leica, Bucks, United Kingdom) using anti-PAX2 antibody (ab38738; Abcam, Cambridge, United Kingdom) at a dilution of 1:100 (Table 1).

Statistical analysis. Histological data and patients' ages were used for the statistical analysis. All histological slides were independently checked by two pathologists. To evaluate the association between all the immunohistochemical variables, Spearman's and Kendall's correlation coefficients were calculated. To compare the immunohistochemical profiles of SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, and EGFR among primary and brain metastasis, both the Wilcoxon paired signed-rank test and McNemar's chi-square test were utilized (STATISTICA v. 9, StatSoft Inc., Tulsa, OK, USA).

Results

Thirty breast-cancer patients with brain metastasis were included in the present study. The median age at the time of primary tumor diagnosis was 54 years of age (range 42–83) and the average age was 57. Grade 2 was diagnosed in 4 out of 30 cases (13.3%) and grade 3 in 26 out of 30 cases (86.7%). Invasive lobular carcinoma was diagnosed in 7 out of 30 cases (23.3%), and invasive ductal carcinoma in 23 out of 30 cases (76.7%). One case of invasive ductal carcinoma was mixed with ductal carcinoma in situ. All patients had at least one lymphatic node test positive for metastatic tumor cells.

To assess the association between all immunohistochemically observed variables, both Spearman's and the more suitable Kendall's correlation coefficients were calculated. Spearman's correlation coefficient only showed one statistically significant correlation, between EGFR and CK5/6 ($P < 0.004$); Kendall's correlation coefficient indicated statistically significant associations in the following cases: EGFR *vs.* CK5/6 ($P < 0.001$), ER *vs.* PR ($P < 0.01$), AIB1 *vs.* CK5/6, PAX2 *vs.* Her2, and PAX2 *vs.* EGFR ($P < 0.04$ in last 3 cases).

Of the metastatic deposits taken from the 30 patients, SRC3 was positive in 20 cases (66.6%), Pax2 in 22 (73.3%), ER in 22 (73.3%), PgR in 25 (83.3%), Her2 in 10 (33.3%), EGFR in 12 (40%), CK5/6 in 7 (23.3%), and Ki67 in 23 (76.6%) (Table 2). In fourteen cases, primary breast cancers paired with secondary deposits were analyzed. In sixteen cases, the primary breast-cancer deposits were not available. Out of 14 cases of primary tumors, SRC3 was positive in 8 (57.1%), Pax2 in 11 (78.5%), ER in 9 (64.2%), PgR in 7 (50%), Her2 in 4 (28.6%), EGFR in 2 (14.3%), CK5/6 in 6 (42.9%), and Ki67 in 10 (71.4%).

Among the metastatic deposits, almost all of the samples were positive for Ki67 (92.9%); more than one half of the samples were positive for SRC-3, Pax2, and EGFR (57.1%); CK5/6 was positive in 42.9%; Her2 in 35.7%; ER 21.4%; and PgR 14.3% of cases (Table 3).

The paired deposits (total $n = 14$) allow a direct comparison of the primary tumor and the corresponding metastasis. Surprisingly, the majority of cases revealed unstable marker expression (Table 2). Constant SRC3 status was observed in 10 out of 14 cases (71%), while 2 cases converted to SRC3 negative (14.3%). Newly acquired SRC3 expression was detected in 2 cases (14.3%). Constant Pax2 status was only observed in 7 of the 14 cases (50%). Five cases converted to Pax2 negative (35.7%), and 2 cases converted to Pax2 positive (14.3%). The constancy of ER was observed in only 4 cases (28.6%), with switching observed in 10 cases (71.4%). The switching of PgR, Her2, EGFR, CK5/6, and Ki67 was observed in 50%, 21.4%, 57.1%, 28.6% and 21.4% of cases, respectively. Interestingly, the highest constancy was observed in Her2 and Ki67 staining. For Her2, a loss of expression was found in two cases (14.3%) and

Table 2. Summary of positive primary and metastatic breast-cancer deposits (n = 30)

Antigen	% of positive primary deposits	% of positive metastatic deposits
SRC3	57.10%	66.60%
Pax2	57.10%	73.30%
ER	21.40%	73.30%
PgR	14.30%	83.30%
Her2	35.70%	33.30%
EGFR	57.10%	40%
CK5/6	42.90%	23.30%
Ki67	92.90%	76.60%

a gain was encountered in one case (7.1%). For Ki67, gains were encountered in all three cases of switching (21.4%) (Table 3). To determine whether the changes between primary tumors and secondary metastatic deposits were statistically significant, the Wilcoxon paired signed-rank test and McNemar's chi-square test were used. There were no statistically significant differences among the observed variables at a p-value of 0.05. Differences at a significance level of $\alpha = 10\%$ (*i.e.* $P < 0.1$) were only found for EGFR ($P = 0.059$), PR ($P = 0.091$), and ER ($P = 0.093$) (Table 3).

Discussion

The incidence of brain metastasis in breast cancer is increasing. The percentage of breast cancer cases involving the CNS is between 10 and 40%, depending on the study [14]. Only 5% of patients with ER-positive primary tumors developed CNS secondary deposits, while 9% of ER negative breast-cancer patients developed CNS metastasis [15]. The rate of discordance between primary tumor and secondary deposits has been reported to range from 28% to 42% for ER, and was 17% for PgR [14, 15]. Depending on the study and the techniques applied, the discordance rates of Her2 ranged between 0% and 37% [16–20]. Hurtado et al. showed that PAX2 competes with SRC-3 for binding and regulation of HER-2 transcription. Human breast cancers that were PAX2 positive and SRC-3 negative had the lowest recurrence rate, and the relationship between PAX2 and SRC-3, with regard to levels that determine relapses, were found to be inversely dependent ($P < 0.03$) [10]. This suggests a transcriptional link between the two subtypes of breast cancer, namely ER-positive and HER2-positive tumors. This mechanism may also lead to the subsequent activation of SRC-3 *via* phosphorylation.

At present, there is no publication comparing SRC3 and Pax2 protein expression in primary and secondary deposits of breast cancer. Our study involved only a small number of patients, but it is nevertheless remarkable that the biological characteristics of the CNS deposits were sometimes transformed with respect to that of the primary tumor.

Immunohistochemical profiles were performed for 30 metastatic deposits using antigens for SRC3, Pax2, ER, PgR, Her2, CK5/6, Ki67, and EGFR. SRC was positive in 66.6% of cases and Pax2 in 73.3%. No correlation was seen between the patient's age, cancer grade, histotype, lymphatic node status, and protein expression (Figures 1–8).

Using all seven antigens, the primary tumor's protein expression differed from that of the brain deposits in 13 cases out of 14 (93%). Constant expression of SRC3 and Pax2 was seen in 71% and 50% of cases, respectively. The highest level of protein detection was observed with Ki67, in 10 cases out of 14 (71.4%); the greatest gain was observed with EGFR in 7 cases; and the biggest loss of protein was observed with ER in 8 cases.

In conclusion, our IHC study suggests that not all distant metastases have the same protein expression as the primary tumor. The reassessment of these protein

Table 3. Changes in immunohistochemically defined protein expression in paired samples from primary breast cancer and secondary CNS deposits (n = 14)

Primary/metastasis	Constant expression	Pos./pos.	Neg./neg.	Gain in relation to primary tumor	Loss in relation to primary tumor
SRC3	10 (71%)	6	4	2	2
Pax2	7 (50%)	6	1	2	5
ER	4 (28.6%)	1	3	2	8
PgR	7 (50%)	1	6	1	6
Her2	11 (78.6%)	3	8	2	1
EGFR	6 (42.3%)	1	5	7	1
CK5/6	10 (71.4%)	4	6	2	2
Ki67	11 (78.6%)	10	1	3	0

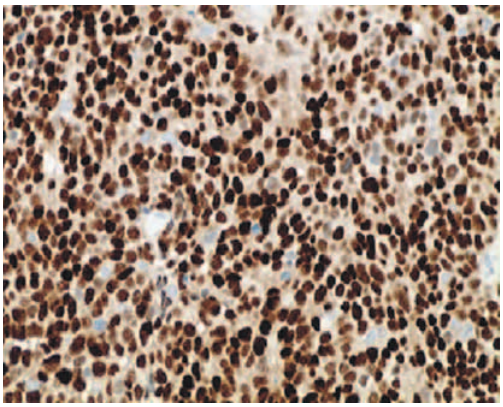

Figure 1. Expression of estrogen receptors (ER) in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

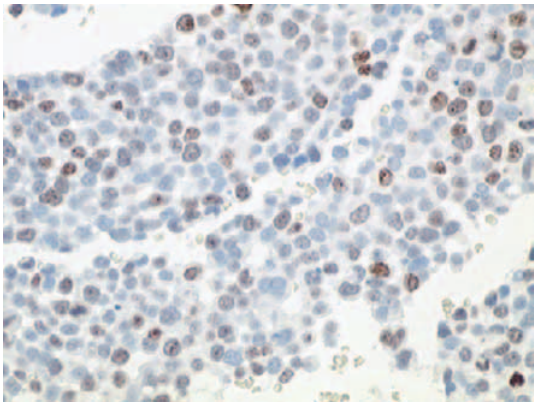

Figure 4. Expression of Ki67 receptors (Ki67) in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

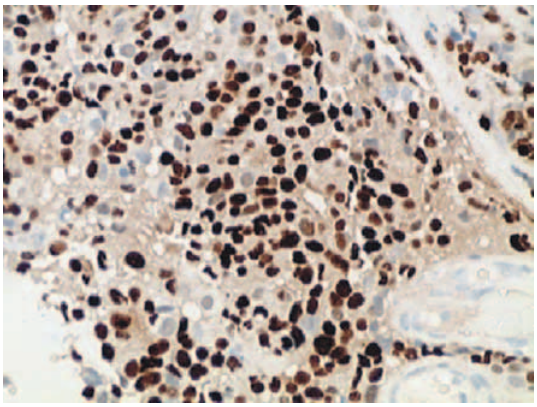

Figure 2. Expression of progersterone receptors (PgR) in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

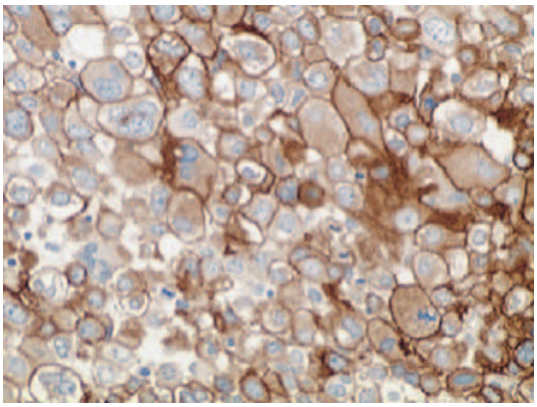

Figure 5. Expression of epidermal growth factor receptors (EGFR) in brain. The sections were stained by IHC as described in Methods. Original magnification ×20

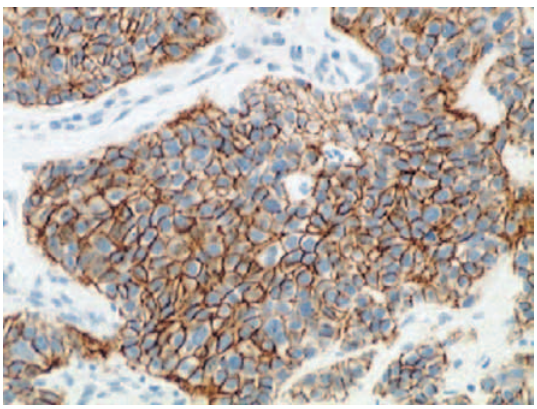

Figure 3. Expression of human epidermal growth factor receptor 2 (Her2) in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

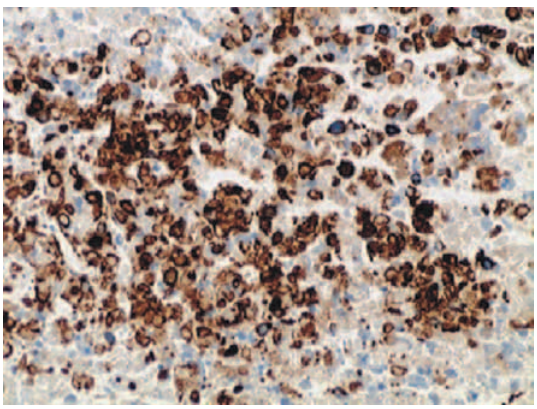

Figure 6. Expression of the presence of cytokeratin 5,6 (CK5/6) in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

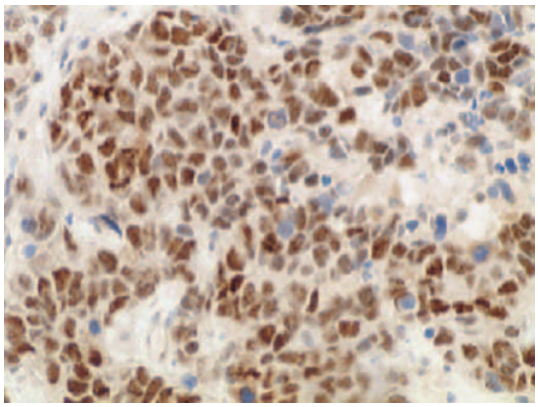

Figure 7. Expression of AIB1 (Amplified in Breast Cancer 1) in brain. The sections were stained by IHC as described in Methods. Original magnification ×20

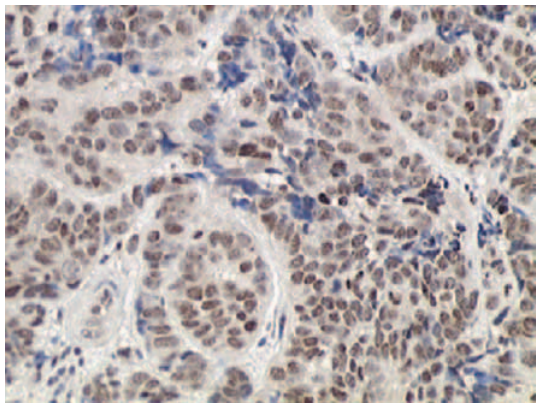

Figure 8. Expression of paired box 2 (Pax2) receptors in brain tissue. The sections were stained by IHC as described in Methods. Original magnification ×20

expressions in metastatic deposits may be useful for optimizing oncological treatments.

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References

1. Bautista S, Valles H, Walker RL et al. In breast cancer, amplification of the steroid receptor coactivator gene AIB1 is correlated with estrogen and progesterone receptor positivity. *Clin Cancer Res*. 1998;4:2925–2929.
2. Wang H, Zhang D, Wu W et al. Overexpression and gender-specific differences of SRC-3 (SRC-3/AIB1) immunoreactivity in human non-small cell lung cancer: an in vivo study. *J Histochem Cytochem*. 2010;58:1121–1127.
3. Xu J, Liao L, Ning G et al. The steroid receptor coactivator SRC-3 (p/CIP/RAC3/AIB1/ACTR/TRAM-1) is required for normal growth, puberty, female reproductive function, and mammary gland development. *Proc Natl Acad Sci USA*. 2000;97:6379–6384.
4. Zhou G, Hashimoto Y, Kwak I et al. Role of the steroid receptor coactivator SRC-3 in cell growth. *Mol Cell Biol*. 2003;23:7742–7755.
5. Yan J, Yu CT, Ozen M et al. Steroid receptor coactivator-3 and activator protein-1 coordinately regulate the transcription of components of the insulin-like growth factor/AKT signaling pathway. *Cancer Res*. 2006;66:11039–11046.
6. Anzick SL, Kononen J, Walker RL et al. AIB1, a steroid receptor coactivator amplified in breast and ovarian cancer. *Science*. 1997;277:965–968.
7. List HJ, Reiter R, Singh B et al. Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res Treat*. 2001;68:21–28.
8. Osborne CK, Bardou V, Hopp TA et al. Role of the estrogen receptor coactivator AIB1 (SRC-3) and HER-2/neu in tamoxifen resistance in breast cancer. *J Natl Cancer Inst*. 2003;95:353–361.
9. Muratovska A, Zhou C, He S et al. Paired-Box genes are frequently expressed in cancer and often required for cancer cell survival. *Oncogene*. 2003;22:7989–7997.
10. Hurtado A, Holmes KA, Geistlinger TR et al. Regulation of ERBB2 by oestrogen receptor-PAX2 determines response to tamoxifen. *Nature*. 2008;456:663–666.
11. O-Charoenrat P, Rhys-Evans P, Modjtahedi H et al. Overexpression of epidermal growth factor receptor in human head and neck squamous carcinoma cell lines correlates with matrix metalloproteinase-9 expression and in vitro invasion. *Int J Cancer*. 2000;86:307–317.
12. Jiang J, Sarwar N, Peston D et al. Phosphorylation of estrogen receptor-alpha at Ser167 is indicative of longer disease-free and overall survival in breast cancer patients. *Clin Cancer Res*. 2007;13:5769–5776.
13. List HJ, Reiter R, Singh B et al. Expression of the nuclear coactivator AIB1 in normal and malignant breast tissue. *Breast Cancer Res Treat*. 2001;68:21–28.
14. Kamby C, Rasmussen BB, Kristensen B. Oestrogen receptor status of primary breast carcinomas and their metastases. Relation to pattern of spread and survival after recurrence. *Br J Cancer*. 1989;60:252–257.
15. Li BD, Byskosh A, Molteni A et al. Estrogen and progesterone receptor concordance between primary and recurrent breast cancer. *J Surg Oncol*. 1994;57:71–77.
16. Shimizu C, Fukutomi T, Tsuda H et al. c-erbB-2 protein overexpression and p53 immunoreaction in primary and recurrent breast cancer tissues. *J Surg Oncol*. 2000;73:17–20.
17. Gong Y, Booser DJ, Sneige N. Comparison of HER-2 status determined by fluorescence in situ hybridization in primary and metastatic breast carcinoma. *Cancer*. 2005;103:1763–1769.
18. Gancberg D, Di Leo A, Cardoso F et al. Comparison of HER-2 status between primary breast cancer and corresponding distant metastatic sites. *Ann Oncol*. 2002;13:1036–1043.
19. Regitnig P, Schippinger W, Lindbauer M et al. Change of HER-2/neu status in a subset of distant metastases from breast carcinomas. *J Pathol*. 2004;203:918–926.
20. Pectasides D, Gaglia A, Arapantoni-Dadioti P et al. HER-2/neu status of primary breast cancer and corresponding metastatic sites in patients with advanced breast cancer treated with trastuzumab-based therapy. *Anticancer Res*. 2006;26:647–653.

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