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S Phase Kinase Associated Protein 2 Expression in Breast Cancer and Its Prognostic Implications

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Background : S Phase Kinase Associated Protein 2 (Skp2), an F-box protein necessary for DNA replication, has recently been demonstrated to be an oncogene. The purpose of this study was to examine the Skp2 expression and to investigate its association with expressions of estrogen receptor (ER), androgen receptor (AR) and HER-2, as well as clinicopathological variables including tumor recurrence. **Methods :** The expressions of Skp2, ER and AR were examined by immunohistochemistry and HER-2 amplification by chromogenic in situ hybridization (CISH) in 117 cases of breast carcinoma. **Results :** Skp2 was expressed in 26 patients (22.2%) and was significantly correlated with tumor type (p=0.031), tumor grade (p=0.017) and ER expression (p=0.038). Twenty four (20.5%) of 117 patients had a tumor recurrence, and 6 patients (5.1%) died of multifocal metastases. Tumor recurrence was significantly correlated with histological grade (p=0.041) and lymph node status (p<0.001). **Conclusions :** Although Skp2 expression was statistically insignificant in association with tumor recurrence, it might be useful as a biologic predictor in breast cancer. The simple and reliable immunohistochemical assay presented in this study can be a routine part of breast cancer evaluation and may influence patient management.

Key Words : Androgen receptor; Breast cancer; Estrogen receptor; Her 2 gene; S-Phase Kinase-Associated Proteins

Patients with breast carcinomas can display a different response to therapy and clinical outcomes, despite of the similar stage and grade of disease. In the last few years, intense efforts have been made to elucidate the deregulation pathways of the cell cycle, which result in the formation of most malignant tumors. Recently, subgroups of breast carcinomas have been identified with distinct molecular signatures and biologic behavior.[1] The importance of G_1-S progression phase has gained much significance due to high incidence of aberrations in genes involved in a wide variety of tumors. The mechanisms that drive the cell from the G_0-G_1 phase into the S-phase have been discovered.[2] As in the case of other common carcinomas, a series of multiple alterations in cell cycle, control-related, gene products are believed to be involved in breast cancer pathogenesis. It is therefore important to explicate the mechanisms controlling the cell cycle, involved in tumor development and to assess their prognostic value.

S Phase kinase Associated Protein 2 (Skp2) is a member of F-box family of SCF (Skp1-Cullin-F-box protein) ubiquitin-protein ligase complexes that contain several constant subunits and a variable subunit known as F-box protein.[3,4] F-box proteins are characterized by an approximately 40-amino acid domain called F-box because it was first identified in cyclin F.[5,6] The F-box proteins play a crucial role in the ubiquitin-mediated degradation of cellular regulatory proteins.[6] In a normal cell cycle, levels of Skp2 are reported low in the G_0-G_1 phase and high in the S phase.[7] Studies on mice have demonstrated that Skp2 may play an important role in regulating normal cell proliferation, because Skp2-deficient mice grew more slowly than littermate controls and had smaller organs.[8,9] Overexpression of Skp2 has been reported in many cancers, including breast, gastric, lung, prostate, oral squamous cell, ovarian carcinoma and lymphoma.[1,2,7,10-14] Shigemasa *et al*.[12] reported that Skp2 expression might play an important role in the development and progression of ovarian carcinoma.

It is evident that the progression of cells through the cell cycle is regulated by both positive (Cdks) and negative (Cdk inhibitors) signals. Expression of Skp2 is required for the ubiquitination and subsequent degradation of the Cdk-inhibitor, p27.[6,15] Although a variety of prognostic factors have been tested,[16-18] only tumor stage, grade, size, hormone receptor status, and S-phase fraction are used on a routine basis for the disease prognosis. The prognos-

tic significance of estrogen receptor (ER) and HER-2 expression in breast cancer and their therapeutic roles are well recognized,[19] however, the significance of Skp2 expression in carcinomas is not well known. Kudo *et al*.[20] reported that high Skp2 expression was correlated with poor prognosis in oral squamous cell carcinoma. Thus, Skp2 may have a great significance in human carcinogenesis. In the present study, we examined the expression of Skp2 in 117 breast cancer tissues and investigated its correlation with other prognostic variables as well as clinicopathologic data, including tumor recurrence.

MATERIALS AND METHODS

One hundred and seventeen tissue samples were obtained from the surgical pathology files of the Department of Pathology, Our Lady Mercy Hospital, Catholic University of Seoul, from the period 1998 to 2001. The following information was obtained from each patient's medical records: age, grade and type of tumor, tumor size, lymph node status, and clinical follow-up data. Tumors were graded according to the modified Scarff-Bloom-Richardson system.[16] In situ lesions, like mucinous carcinomas or secretory carcinoma, etc. were categorized as nongraded. All patients had primary breast carcinoma and received current standard therapy. Chemotherapy and hormone therapy treatments were based on the characteristics of the tumor.

In order to evaluate large numbers of tumors, low-density tissue microarrays blocks were made at the hospital. One block contained 20 cores (single core per tumor), each measuring 3 mm in diameter. Four-micrometer sections were mounted onto the positively charged slides.

Immunohistochemical analyses for Skp2 (Zymed, South San Francisco, CA, USA), AR (Santa Cruz Biotechnology, Santa Cruz, CA, USA), ER (Dako, Glostrup, Denmark), and HER-2 amplification by CISH (SPOT-LIGHT, Zymed, South San Francisco, CA, USA) were performed on formalin-fixed, paraffin-embedded material.

Immunohistochemistry for Skp2, ER, and AR

A heat-induced, epitope retrieval procedure was carried out by heating the slides in a conventional pressure cooker for three minutes, and subsequently they were placed in Couplin jars filled with either 0.1 M EDTA, pH 8.0 (for Skp2) or a solution of 0.01 M trisodium citrate, pH 6.0 for ER and AR. A standard avidin-biotin-peroxidase complex (ABC) technique was used for visualization, with diaminobenzidine as the chromogen (Histostain Plus-kit; Zymed, South San Francisco, USA). The staining was considered negative, when there were no tumor cells with nuclear staining, or when tumor cell nuclei up to 5% were stained. When more than 5% of the tumor cells showed positive nuclear staining, the staining was considered to be positive. Immunostaining results were scored independently by two of the authors (Chang E and Lee E) with no knowledge of patient's status.

Chromogenic in situ hybridization (CISH) for HER-2 amplification

A digoxigenin-labeled genomic probe for HER-2 was obtained from Zymed, using the manufacturer's reagent kits. In brief, the sections were deparaffinized and incubated in pretreatment buffer in a temperature-controlled microwave oven 92℃ for 15 min. Enzymatic digestion was carried out with pepsin at room temperature for 10-30 min. The slides were washed with PBS and dehydrated in graded dilutions of ethanol. The HER-2 probe (5-10 μL/slide) was applied to the slides under coverslips. The slides were co-denatured on a hot plate (94℃ for 3 min), hybridized overnight at 37℃, and washed with 0.5× SSC for 5 min. The hybridized probe was detected using the CISH detection reagents (anti-digoxigenin-FITC, anti-FITC-peroxidase, and diaminobenzidine as chromogen), according to the manufacturer's instructions. The sections were counterstained with hematoxylin and mounted. The slides were analyzed by two of the authors using an ordinary light microscope under 20× magnification. Generally, 1-4 copies were considered as no amplification.[21] As described by Tanner *et al*.[22] amplification was defined when more than 5-10 discrete copies per nucleus or large gene copy clusters in at least 50% of cancer cells were seen.

Statistical analysis

Statistical analysis was performed using SPSS for Windows (Version 8.0; SPSS, Chicago, IL. USA). The significance of the associations was determined using Fisher's exact probability test and the 2-tailed t test. Probability values, $p<0.05$ were considered to be statistically significant.

RESULTS

The characteristics of a patient and tumor and immunohistochemical expressions of Skp2 are summarized in Table 1. Skp2

Table 1. Skp2 expression and its relationship with clinicopathologic variables, ER, AR and HER-2 expressions of breast carcinomas.

Characteristics		No. of cases (%)	No. of Skp2		p value
			Pos (%)	Neg (%)	
Age (year)	<40	26 (22.2)	7 (26.9)	19 (73.1)	0.685
	40-55	57 (48.7)	13 (22.8)	44 (77.2)	
	>55	34 (29.1)	6 (17.6)	28 (82.4)	
Tumor type	Ductal	112 (95.7)	23 (20.5)	89 (79.4)	0.031
	Lobular	1 (0.9)	0 (0.0)	1 (100)	
	Others	4 (3.4)	3 (75.0)	1 (25.0)	
Tumor grade	I	20 (17.1)	0 (0.0)	20 (100)	0.017
	II	35 (29.9)	6 (17.1)	29 (82.9)	
	III	37 (31.6)	11 (29.7)	26 (70.3)	
	Nongraded (in situ, etc)	25 (21.4)	9 (36.0)	16 (64.0)	
Tumor size (cm)	<2	22 (18.8)	6 (27.2)	16 (72.8)	0.376
	2-4	50 (42.7)	8 (16.0)	42 (84.0)	
	>4	45 (38.5)	12 (26.6)	33 (73.4)	
Lymph nodes	Neg	54 (46.2)	12 (22.2)	42 (77.8)	0.331
	Pos (1-3)	29 (24.8)	4 (13.7)	25 (86.3)	
	Pos (>3)	34 (29.0)	10 (29.4)	24 (70.6)	
ER	Pos	66 (56.4)	10 (15.1)	56 (84.9)	0.036
	Neg	51 (43.6)	16(31.3)	35 (68.7)	
AR	Pos	51 (43.6)	11 (21.5)	40 (78.5)	0.881
	Neg	66 (56.4)	15 (22.7)	51 (77.3)	
HER-2	Pos	26 (22.2)	9 (34.6)	17 (65.4)	0.085
	Neg	91 (77.8)	17 (18.6)	74 (81.4)	
Recurrence	Yes	24 (20.5)	4 (16.6)	20 (83.4)	0.463
	No	93 (79.5)	22 (23.6)	71 (76.4)	
Skp2	Pos	26 (22.2)	-	-	-
	Neg	91 (77.8)	-	-	

Skp2, S phase kinase protein 2; ER, estrogen receptor; AR, androgen receptor; HER-2, Her-2/neu oncogene.

was expressed in 26 cases (22.2%), which showed positive nuclear staining (Fig. 1). ER was found to be positively expressed in 66 cases (56.4%). Nuclear AR immunopositivity was seen in 51 cases (43.6%) (Fig. 2). HER-2 amplification was seen most often as large gene copy clusters in the majority of nuclei in 26 cases (22.2%) (Fig. 3).

Skp2 expression was correlated with tumor type (p=0.031), tumor grade (p=0.017) and ER expression (p=0.038), but not with the patients' age (p=0.685), tumor size (p=0.376), lymph node status (p=0.331), AR status (p=0.881), HER-2 amplification (p=0.085) and tumor recurrence (p=0.463).

The relationship between tumor recurrence and clinicopathologic factors are summarized in Table 2. The mean follow-up period for patients was 38.3 months (range, 19-65 months). Of the 117 cases, 24 cases (20.5%) had a tumor recurrence, and 6 cases (5.1%) died of multifocal metastases. The mean time of recurrence was 22.1 months (range, 0-62 months). Tumor recurrence was significantly correlated with histologic grade (p=0.041) and lymph node status (p<0.001), but not with age (p=0.127), tumor type (p=0.857), size (p=0.922), ER (p=0.241), AR (p=0.500), HER-2 (p=0.854) or Skp2 (p=0.463).

DISCUSSION

Using immunohistochemistry, we found that Skp2 protein was expressed in 22.2% of breast carcinoma cases. Skp2 expres-

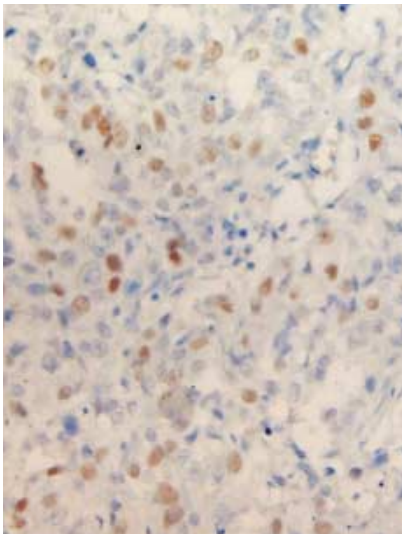

Fig. 1. Immunohistochemical staining for Skp2 shows positive nuclear reactions in more than 5% of the tumor cells.

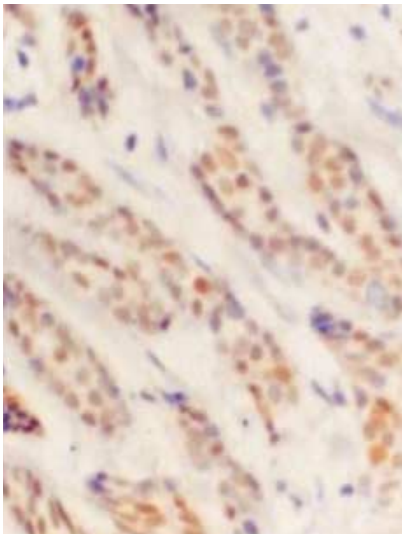

Fig. 2. Immunohistochemical staining for androgen receptor shows strong positive nuclear reactions.

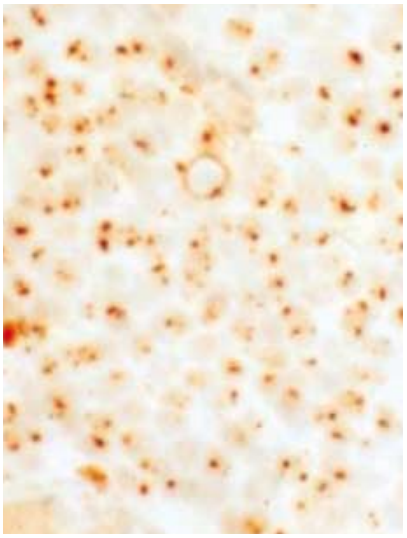

Fig. 3. A typical high-level HER-2/*neu* amplification appears as multiple large clusters of gene copies by chromogenic in situ hybridization.

Table 2. Relationship between tumor recurrence and clinicopathologic variables in breast carcinomas

Characteristics		No. of recurrence		p value
		Pos (%)	Neg (%)	
Age (year)	<40	9 (34.6)	17 (65.4)	0.127
	40-55	9 (15.7)	48 (84.3)	
	>55	6 (17.6)	28 (82.4)	
Tumor type	Ductal	23 (20.5)	89 (79.5)	0.857
	Lobular	0 (0.0)	1 (100)	
	Others	1 (25.0)	3 (75.0)	
Tumor grade	I	6 (30.0)	14 (70.0)	0.041
	II	11 (31.4)	24 (68.6)	
	III	6 (16.2)	31 (83.8)	
	Nongraded (in situ,etc)	1 (4.0)	24 (96.0)	
Tumor size (cm)	<2	4 (18.1)	18 (81.9)	0.922
	2-4	10 (20.0)	40 (80.0)	
	>4	10 (22.2)	35 (77.8)	
Lymph nodes	Neg	3 (5.5)	51 (94.5)	<0.001
	Pos (1-3)	7 (24.1)	22 (75.9)	
	Pos (>3)	14 (41.1)	20 (58.9)	
ER	Pos	11 (16.6)	55 (83.4)	0.241
	Neg	13 (25.4)	38 (74.6)	
AR	Pos	9 (17.6)	42 (82.4)	0.500
	Neg	15 (22.7)	51 (77.3)	
HER-2	Pos	5 (19.2)	21 (80.8)	0.854
	Neg	19 (20.8)	72 (79.2)	
Skp2	Pos	4 (15.3)	22 (84.7)	0.463
	Neg	20 (21.9)	71 (78.1)	

Skp2, S phase kinase protein 2; ER, estrogen receptor; AR, androgen receptor; HER-2, Her-2/neu oncogene.

sion was found to be in a significant correlation with tumor type, tumor grade and ER status. In addition, tumor recurrence was significantly correlated with histological grade and lymph node status, suggesting that Skp2 expression might be a useful prognostic marker and may play an important role in breast carcinoma development. Traditional biologic markers, such as ER expression and lymph node involvement can predict the recurrence of a disease. Other factors, such as shorter disease free interval and visceral involvement indicate the exacerbation of clinical outcome after recurrence.[23] In the present study, Skp2 expression was correlated with ER status and tumor recurrence was correlated with tumor grade and lymph node status. Our results were similar to those reported by other researchers.[16-19] Increased Skp2 protein levels were not always correlated with Ki-labeling indices, suggesting that alterations of Skp2 may contribute to the malignant phenotype without affecting tumor cell proliferation.[10]

A decreased level of p27 expression in human malignant tumors may be caused by increased expression of Skp2, which indicates degradation of p27.[2] It is well known that reduced p27 expression is frequently found in various human cancers [11,24-27] and is correlated with poor survival.[12,28] We did not perform the studies on expression of p27, because of inverse action between Skp2 and p27 level. Although the molecular mechanisms underlying increased Skp2 expression in many cancer cells have not been elucidated, the Skp2-p27 pathway may represent a novel molecular target for human cancer prevention or treatment.[8]

Skp2 is considered to be a potential and specific therapeutic target in aggressive breast cancers.[1] The expression of Skp2 induces resistance to antiestrogens, thus it is possible to infer that deregulated Spk2 expression in ER-positive tumors treated with antihormonal agents may play an important role in the development of resistance to antiestrogens.[1] Therefore, evaluation of Skp2 overexpression could be applicable to the clinical management of breast cancer patients, allowing identification of patients with poor prognoses.

In conclusion, our results support the hypothesis that Skp2 expression might offer an insight in understanding the aggressive behavior of breast tumors, although Skp2 expreesion was statistically insignificant with tumor recurrence. Skp2 was significantly correlated with tumor type, tumor grade and ER expression. The simple and reliable immunohistochemical assay, used in the present study may become a routine part of breast cancer evaluation and may influence patients management.

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