Zinc-α2-glycoprotein: a New Biomarker of Breast Cancer?

V. DUBOIS[1,2], L. DELORT[1,2], F. MISHELLANY[3], T. JARDE[1,2], H. BILLARD[1,2], C. LEQUEUX[5],
O. DAMOUR[5], F. PENAULT-LLORCA[3], M.P. VASSON[2,4,6] and F. CALDEFIE-CHEZET[1,2,6]

[1]Laboratoire SVFp, Clermont-Ferrand, France;
[2]EA 4233 'Nutrition, Cancérogenèse et Thérapie Anti-Tumorale',
UFR Pharmacie, Université d'Auvergne, CRNH-Auvergne, IFR Santé 79, France;
[3]Laboratoire d'Anatomopathologie, [4]Unité de Nutrition, Centre Jean-Perrin, Clermont-Ferrand, France;
[5]Banque de Tissus et Cellules, Hôpital Edouard-Herriot, 69000 Lyon, France;
[6]CLARA, Région Lyon Auvergne Rhône-Alpes, France

Abstract. *Background/aim: Obesity increases the risk of breast cancer. It is established that adipocyte secretions, i.e. adipokines, may play a role in mammary carcinogenesis. We have shown that two major adipokines, leptin and adiponectin, were expressed in mammary adenocarcinoma. Patients and Methods: Here, we evaluated zinc-α2-glycoprotein (ZAG) expression in tumor (n=55) and healthy (n=6) breast tissue by immunohistochemistry and examined whether it was correlated with that of major adipokines, usual tumor biomarkers (sex steroids receptors, i.e. estrogen (ER) and progesterone; Ki-67; cErb2), or apoptosis markers (Bcl2 and Bax). Results: ZAG expression was detected in ductal carcinoma and normal epithelial adjacent tissue but not in normal tissue of healthy women. In cancer tissue, its expression was correlated positively to leptin receptor and negatively to adiponectin receptor and ER. Conclusion: These preliminary results suggest both a relationship between ZAG expression and pathways involving adipokines or estrogen and that ZAG may be a potential breast cancer biomarker.*

By the year 2000, 300 million adults were obese worldwide (1). Obesity is now recognized as a real risk factor for postmenopausal breast cancer development. Nevertheless, the specific mechanisms implicating obesity in the breast cancer process are not well understood (2, 3). After menopause, aromatization processes that convert androstenedione into estrogen, take place primarily in the adipose tissue whose

Correspondence to: Virginie Dubois, Faculté de Pharmacie, Laboratoire SVFp, 28 place Henri-Dunant, B.P. 38, 63000 Clermont-Ferrand, France. Tel: +33 473177972, Fax:+33 473178438, e-mail: virginie.dubois@u-clermont1.fr

mass is increased in the case of obesity (4). The mammary epithelial microenvironment (adipose tissue, fibroblasts, myoepithelial cells) could also be implicated in the development of this tumoral pathology and especially adipose tissue. Indeed, in healthy or tumoral breast, epithelial cells are directly in contact with adipocytes which could also influence them.

Adipocytes secrete a large number of molecules named adipokines (5). Approximately 250 adipokines have been described to date including new adipokines such as zinc-α2-glycoprotein, apelin, visfatin, adiponutrin, resistin and hepatocyte growth factor. Some of them, such as adiponectin and leptin, are known to play a role in breast cancer development (6). We have previously established that leptin had pro-carcinogenic effects and adiponectin anti-carcinogenic effects (7, 8). Indeed, in ductal tumors, we showed a strong expression of leptin and conversely a low expression of adiponectin. Moreover, normal tissue within normal breast, *e.g.* non-cancerous lesion holders, does not express leptin, contrary to the normal tissue surrounding a breast ductal carcinoma. Additionally, we have established a strong expression of adiponectin in myoepithelial cells known for their tumor suppressor properties (9). *In vitro*, we have reported on the expression of adiponectin and leptin receptors on the breast cancer cell line MCF7 by qRT-PCR, and that adiponectin induces an anti-proliferative response to these cells in contrast to leptin. In addition, we have shown that adiponectin decreases aromatase activity and expression of the estrogen receptor in MCF7 tumor cells (8, 9). These results suggest an important local role of the adipocyte microenvironment in the mammary gland, which could be of importance in the case of obesity.

Other adipokines may also play an important role in breast cancer progression. For example, hepatocyte growth factor (HGF) has been associated with proliferation, migration and invasion of tumor cells, and several tumors, including breast cancer, produce HGF and overexpress its

receptor (10). In a Korean study, the frequency of tumors with the highest histological grade was significantly increased in patients presenting a higher resistin level than the median, suggesting that high resistin levels were likely to be associated with increased breast cancer risk in Korean women (11). Another study suggested that apelin behaved as a potent activator of tumor neoangiogenesis by a paracrine effect (12). Overall, several of the adipokines mentioned seem to have pro-carcinogenic effects, and it would be wise to identify a molecule with anti-carcinogenic properties such as adiponectin. Among these adipokines, zinc-α2-glycoprotein (ZAG) seems to be of potential interest for breast cancer cases.

ZAG, also named GCDFP-44 (gross cystic disease fluid protein) (13), is a 44 kDa single chain polypeptide (14-16), identified in 1961 (17, 18). A high degree of similarity between ZAG and class I MHC molecules has been shown both at sequence and structural levels (14). ZAG is secreted in various body fluids and is present at high concentrations in human seminal plasma (14) and breast cyst fluid (19). It has been shown that ZAG can bind to the adrenoreceptor (14) and its expression can be regulated through TNF-α and the PPARγ nuclear receptor (20). Interestingly, the ZAG gene and protein has been shown to be down-regulated (−70%, $p<0.05$) in adipose tissue of obese compared to lean subjects (21-23). ZAG, also called lipid-mobilizing factor (14, 15, 24), stimulates lipolysis (25) and is associated with an extensive reduction of fat in the human body (14).

To the best of our knowledge, only few studies reported a link between ZAG and cancer process. In breast fluids, the presence of ZAG was demonstrated in about 40% of breast carcinomas (26). Moreover, in breast tumor cytosols, the concentration of ZAG ranged from 0 to 23.5 μg/mg of total soluble protein, with an average value of 2.4 μg/mg (19). In *in situ* ductal carcinoma, ZAG was expressed in 40% of the studied cases (13). Moreover, ZAG was also described as a potential serum marker of prostate cancer that may be early elevated in tumor growth (14) and seem to be expressed in cancer cachexia (14, 15, 20, 24, 27, 28).

Cancer is characterized by a dysfunction of different cell mechanisms including apoptosis, cell cycle, angiogenesis, proliferation and migration. Dysregulation of normal programmed cell death pathways plays an important role in the pathogenesis and progression of breast cancer but no relationship between ZAG and apoptosis has been described to date. Among many apoptosis-inducing factors, Bax and Bcl2 have been identified functionally antagonistic proteins which control this mechanism. Bax is a 21 kDa proapoptotic protein with extensive amino acid homology with Bcl2 and is suggested as a good prognostic marker (29). Bax has been shown to form heterodimers with Bcl2 and the ratio of Bcl2 to Bax determines the survival or cell death following an apoptotic stimulus such as removal of a growth factor (29).

Bcl2 gene encodes a 26 kDa protein, which appears to play a key role in cell regulation by inhibiting apoptosis (29). These proteins are expressed differentially depending on cell types and stage of differentiation; and their biological effects depend on their level, selective expression and dimerisation status (30).

In this study, in an attempt to elucidate the relationship between ZAG and breast carcinogenesis, we investigated ZAG expression in different types of breast tumor, in normal tissue adjacent to breast tumor and in tissue of healthy women by immunohistochemistry. We also evaluated the relationship between ZAG expression and the expression of two major adipokines, leptin and adiponectin, with apoptosis and clinical classical markers.

Patients and Methods

All chemicals were purchased from Sigma (Saint-Quentin-Fallavier, France) except for the anti-ZAG primary antibody (Santa Cruz Biotechnology, Santa Cruz, United States), the biotinylated anti-goat IgG (Vector Laboratories, Burlingame, United States), the avidin/biotin blocking kit, the Vectastain ABC kit, the diamino-benzidine (DAB) substrate and the Vectamount mounting medium (Vector Laboratories, Abcys, Paris, France).

Patients. Primary tumors from 39 women, aged 30-80 years, not treated previously by radiotherapy or chemotherapy, were surgically resected in the Department of Surgery, Centre Jean-Perrin, France. Control tissue samples were collected from 6 healthy women aged 33-63 years with mammary hypertrophy by the Tissue and Cell bank, Hôpital Edouard-Herriot, France. Tissue samples were immediately paraffin-embedded and cut into 3 μm wide sections.

Tissue classification. The diagnosis was made on formalin-fixed paraffin-embedded tissue sections after haematoxylin-eosin-saffron staining. Routinely, the expression of estrogen receptors (ER), progesterone receptors (PR) and Ki-67 were evaluated and scored as previously described (31). Tissues were classified according to histological subtypes as malignant lesions corresponding to *in situ* ductal carcinoma (n=16) and invasive ductal carcinoma of different grades [grade 1 (n=11), 2 (n=14) and 3 (n=14)]. Invasive tumors were evaluated according to the SBR grade classification modified by Elston and Ellis (32).

Immunohistochemistry. The expression of ZAG was investigated by immunohistochemical staining using affinity-purified goat poly-clonal biotinylated antibodies against ZAG. Sections were deparaffined in toluene through graded concentrations of ethanol to distilled water. Sections to be stained with antibodies were pretreated by boiling them for 40 minutes in citrate buffer. Non-specific binding sites were blocked using the avidin/biotin kit for 30 min. Slides were then incubated overnight at 4°C in a humid chamber with the primary anti-ZAG antibody (0.5 μg/ml) and 4 hours with the secondary anti-ZAG biotinylated antibody. Endogenous peroxidase activity was inhibited with 0.3% hydrogen peroxide for 5 min. Visualisation was carried out using a Vectastain ABC peroxidase-conjugated streptavidine kit for 30 min. The sections were then treated with DAB substrate for 10 min. Finally,

Table I. *ZAG expression in breast cancer, normal tissue adjacent to breast cancer and normal tissue of healthy women.*

Tissue type	Total number of cases	Cases with positive ZAG expression		ZAG expression in positive cases		
	n	n'	(%)	Staining intensity		Stained cells (%)
In situ ductal carcinoma	16	13	81	(+)	11 cases	25-100
				(++)	1 case	100
				(+++)	1 case	100
Invasive ductal carcinoma	39	31	79	(+)	19 cases	5-100
				(++)	10 cases	90-100
				(+++)	2 cases	95-100
Grade I	11	9	82	(+)	4 cases	25-75
				(++)	3 cases	90-100
				(+++)	2 cases	95-100
Grade II	14	10	71	(+)	7 cases	5-100
				(++)	3 cases	100
Grade III	14	12	86	(+)	8 cases	10-100
				(++)	4 cases	100
Normal tissue adjacent to carcinoma	33	31	94	(+)	24 cases	10-100
				(++)	4 cases	75-100
				(+++)	3 cases	70-100
Normal tissue	6	0	0			

n, Number of cases studied; number (n') and percentage (%) of cases expressing ZAG. Staining intensity was graded as + (mild), ++ (moderate), +++ (intense).

slides were contrasted using haematoxylin, dehydrated and mounted using the Vectastain mounting medium. For each assay, control samples without the anti-ZAG antibody were used to establish the specificity of the immunohistochemical analysis.

The expressions of leptin, leptin receptor (Ob-R), adiponectin and adiponectin receptors (adipoR1 and R2) were determined by immunohistochemistry as previously reported (8). By addition, the expressions of Bax and Bcl2 were evaluated with the same technique.

Microscopic examination. Assessment of immunostaining was performed by a anatomopathologist blinded to the clinical data. The expression of ZAG in cancerous and normal adjacent tissues was classified as negative (<5% labeled cells) or positive (≥5% labeled cells). The total percentage of cases with ZAG expression was determined.

Statistical analysis. The statistical analysis was performed using StatView statistical software (SAS Institute, Carry, NC, USA). Relation-ships between ZAG and others adipokines, histological features and usual biomarkers were analysed using the Spearman rank correlation. In this study, we combined ductal, *in situ* and invasive, carcinomas for Spearman rank correlation. Differences with $p < 0.05$ were considered to be statistically significant.

Results

ZAG expression in breast cancer tissue, in normal tissue adjacent to tumor and in normal tissue from healthy women. In ductal carcinoma, positive ZAG expression was noted in

81% of *in situ* (DCIS) cases studied (Figure 1A) and 79% of invasive (IDC) cases (Table I, Figure 1B). Concerning IDC cases, ZAG expression was detected in 82%, 71% and 86% of grade I, II and III, respectively. In normal tissue adjacent to breast cancer, positive ZAG immunostaining was observed in 94% of studied cases (Figure 1C). Nevertheless, ZAG expression was undetectable in normal cells from healthy breast (Figure 1D).

Correlations between expression of ZAG and other adipokines. As previously reported, in breast cancer, leptin (79%) and Ob-R (81%) were strongly expressed whereas adiponectin (7%) and adipoR1 (15%) were weakly expressed (9). In ductal carcinoma (IDC and DCIS cases), ZAG expression was correlated positively with the expression of leptin receptor ($p=0.015$, $r=0.341$) and negatively with the expression of adiponectin receptor R2 ($p=0.024$, $r=-0.315$). The expression of ZAG showed no statistically significant correlation with expression of leptin, adiponectin and adiponectin receptor R1 (Table II).

Correlations between expression of ZAG and apoptosis biomarkers, Bax and Bcl2. All of the DCIS cases were Bax negative while 87.5% of cases were Bcl2 positive. Concerning IDC, 25% of cases were Bax positive and 60% of cases expressed Bcl2. Normal tissue adjacent to breast cancer were Bax negative whereas 94% of cases were Bcl2 positive. In
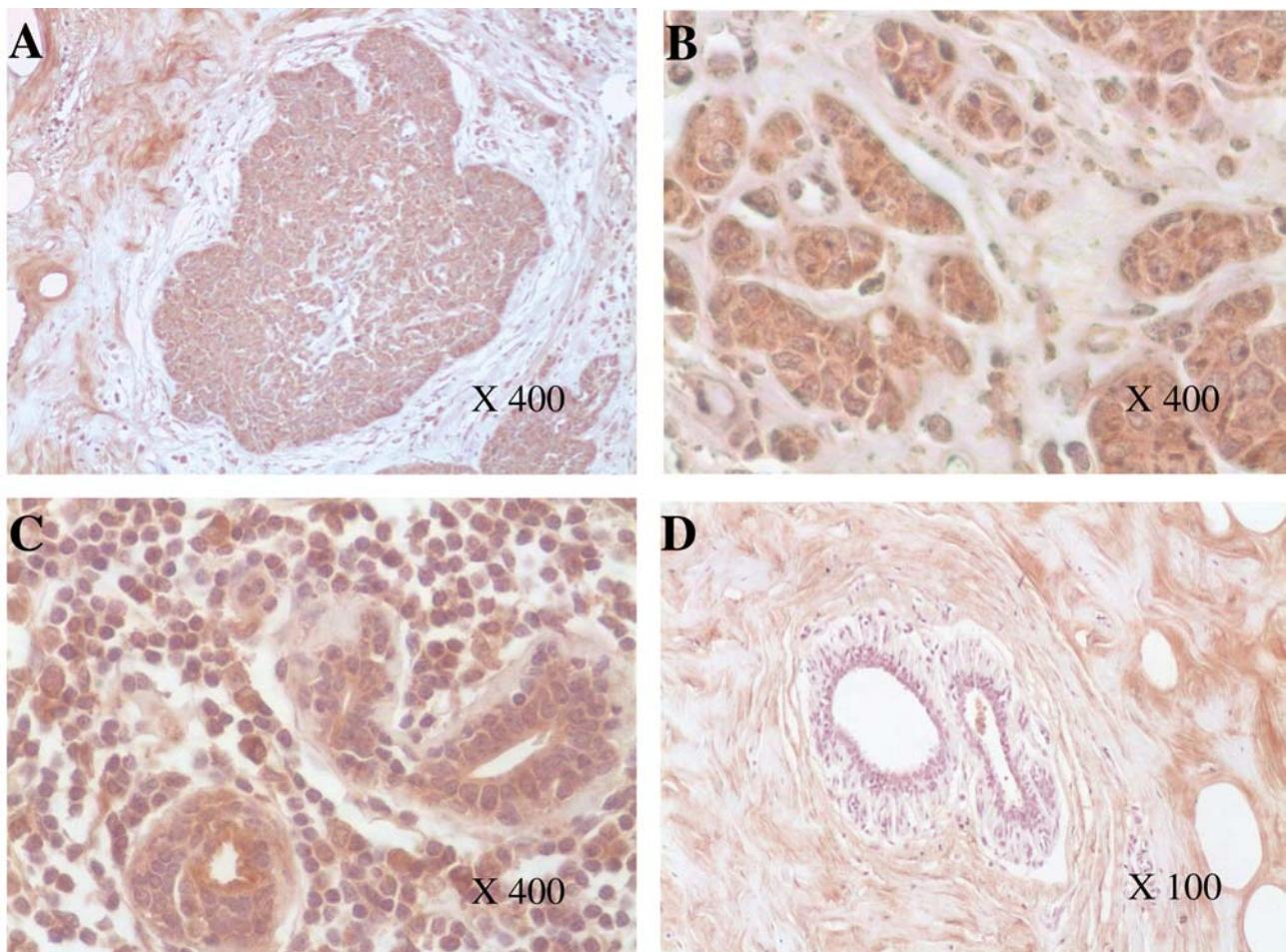
Figure 1. *Immunohistochemical detection of ZAG. In these images, ZAG detection is observed in in situ carcinoma (A), invasive ductal carcinoma (B), normal tissue adjacent to carcinoma (C) and in normal tissue of healthy breast (D).*

ductal carcinoma, Bax and Bcl2 were correlated negatively (p=0.025, r=–0.430). Bax and Bcl2 were not significantly correlated with ZAG expression (Table III).

Relationships between expression of ZAG and usual breast tumor biomarkers. In ductal carcinoma, ZAG expression was correlated negatively with ER (p=0.046, r=–0.279). The expression of ZAG was not significantly correlated with the expression of PR, c-Erb2 or Ki-67 (Table IV).

Relationships between expression of ZAG and tumor size. ZAG expression was correlated negatively with tumor diameter (p=0.008, r=–0.377) in ductal carcinoma.

Discussion

Obesity is now recognized as a risk factor for breast cancer development. We previously reported that adipokines, such as leptin and adiponectin, were expressed in breast cancer

tissue. ZAG, a well known lipid mobilizing factor, which is down-regulated in obesity, is actually considered as an adipokine. In human breast cancer tissue, the implication of ZAG in mammary carcinogenesis has been explored in only a few studies. The aim of the present study was therefore to research the expression of this adipokine in breast cancer tissue and to determine its relationship with other adipokines and common biomarkers, in an attempt to better understand the impact of the tumor adiposity microenvironment on breast cancer development.

We showed that ZAG was expressed both in primary ductal breast cancer and in epithelial cells of normal tissue in the vinicity of a ductal breast lesion. The results presented here are consistent with the data of Selim *et al.* (13) who reported ZAG expression in DCIS. However, a significant association between the expression of ZAG and the degree of differentiation of CCIS could not be established, contrary to this author who demonstrated that in the cytosol of breast cancer cells, there was a positive

Table II. *Adipokines expressions in ductal (in situ and invasive) breast cancer and correlation with ZAG expression.*

	Total number of cases	Cases with positive expression		Correlations with ZAG expression (IDC+DCIS)	
	n	n'	(%)	*p*-Value	r
Leptin	58	46	79	0.252	0.160
Ob-R	58	47	81	0.015	0.341
Adiponectin	58	4	7	0.162	–0.196
AdipoR1	58	9	15	0.610	0.071
AdipoR2	58	46	79	0.024	–0.315

n, Number of cases studied; number (n') and percentage (%) of cases expressing adipokines or receptors.

Table III. *Apoptosis marker expressions in ductal (in situ and invasive) breast cancer and correlation with ZAG expression.*

	Total number of cases	Cases with positive expression		Correlations with ZAG expression (IDC+DCIS)	
	n	n'	(%)	*p*-Value	r
Bax	28	5	18	0.972	–0.007
Bcl2	28	19	67	0.336	0.196

n, Number of cases studied; number (n') and percentage (%) of cases expressing markers of apoptosis.

Table IV. *Classical biomarker expressions in ductal (in situ and invasive) breast cancer and correlation with ZAG expression.*

	Total number of cases	Cases with positive expression		Correlations with ZAG expression (IDC + DCIS)	
	n	n'	(%)	*p*-Value	r
ER	58	39	67	0.046	–0.279
PR	58	33	57	0.248	–0.162
cErb2	26	8	31	0.194	–0.277
Ki-67	58	25	43	0.086	–0.240
Tumor size	58	-	-	0.008	–0.377

n, Number of cases studied; number (n') and percentage (%) of cases expressing biomarkers.

significant association between ZAG concentration and the histological grade of the tumors. Indeed, it has been reported that ZAG levels are higher in well-differentiated tumors (mean 4.6 μg/mg) than in moderately (1.8 μg/mg) or poorly (0.9 μg/mg) differentiated tumors (19). Interestingly, in the present study, ZAG was undetectable in normal cells from healthy breast in all the cases studied. We report here for the first time that ZAG was expressed by all malignant cells, as well as in normal tissue in the vicinity of a ductal breast lesion but not in normal tissue of healthy breast. Given this observation, we may postulate that ZAG uptake may be increased by tumor cells and/or that ZAG may be directly synthesized by these malignant cells. Moreover, Sanchez *et al*. showed that ZAG was found in all the breast fluids obtained from most healthy women (93%) as well as in most patients diagnosed with duct ectasia, fibroadenoma, and gross cystic breast disease (88%) than in fluids from women diagnosed with breast carcinoma (43%) (26). Consequently, these data suggest that ZAG expression was enhanced in breast cancer compared to normal tissue in healthy women and that ZAG expression was induced in normal tissue adjacent to breast cancer during carcinogenesis.

Marrades *et al*. showed statistically significant positive correlations between ZAG gene expression and serum adiponectin (r=0.89, *p*<0.01) and a negative correlation with the plasma level of leptin (r=–0.82, *p*<0.05) in obese subjects (21). Previously, we evaluated the protein expression of these two major adipokines, leptin and adiponectin, and we reported that leptin and its receptor were strongly expressed whereas adiponectin and its receptor adipoR1 were weakly expressed. In our study, we showed that ZAG was positively correlated with leptin receptor (Ob-R) and negatively with adipoR2. These data suggest that there may be a link in the regulation of various adipokines as previously reported by Jarde *et al*. (8).

In the development of cancer, it is well known that apoptosis plays a key role, particularly through secretion of factors such as Bax and Bcl2. We reported that there was a low expression of Bax and a high expression of Bcl2 in ductal breast cancer and in normal tissue adjacent to lesions. These data are consistent with the results of Veronese *et al*. showing positive Bax immunostaining in 45% (grade 1 and 2) and 55% (grade 3) and Bcl2 in 67% of cases (30), and with those of Baccouche *et al*. demonstrating an overexpression of Bax (12%) and Bcl2 (41%) in tumors (33). Rehman *et al*. showed Bax expression in 66% of DCIS and 56% of IDC and Bcl2 expression in 40% of cases (29). In the present study, Bax was signi-ficantly negatively correlated with Bcl2, which is in contrast with the results of Rehman *et al*. showing a positive correlation between Bax and Bcl2 (29). Moreover, ZAG was not associated with Bax and Bcl2 apoptosis biomarkers, but was negatively correlated with estrogen receptor (ER). ZAG and estrogen seemed not to be associated even though a link between leptin and estrogen receptor has previously been reported (8). The data presented here suggest that the adipokines could interact with the estrogen pathway. ZAG was also negatively correlated with tumor size. Consequently, it could be suggested that ZAG may have anticarcinogenic effects. Nevertheless, these data are not consistent with the results of Diez-Itza *et al*. which demonstrated that there was no significant correlation between ZAG and tumor size or ER (19).

In conclusion, our study demonstrated that ZAG was expressed in breast cancer cases and in normal tissue adjacent to lesions but not in normal tissue of healthy women. This data supports a role for ZAG as a potential biomarker in breast cancer. ZAG correlated positively with Ob-R and negatively with adipoR2, ER and tumor size. Consequently, we hypothesize that ZAG may be linked with other adipokines and the estrogen pathway and that it could have anticarcinogenic effects. The presented data confirm our interest to study the tumoral microenvironment, particularly adipose tissue, in relation to breast cancer development. Nevertheless, further studies are necessary in order to better understand the mechanisms involved in carcinogenesis.

References

1 Calle EE and Kaaks R: Overweight, obesity and cancer: epidemiological evidence and proposed mechanisms. Nature Rev *4*: 579-591, 2004.

2 Chu SY, Lee NC, Wingo PA, Senie RT, Greenberg RS and Peterson HB: The relationship between body mass and breast cancer among women enrolled in the Cancer and Steroid Hormone Study. J Clin Epidemiol *44*: 1197-1206, 1991.

3 Lahmann PH, Hoffmann K, Allen N, van Gils CH, Khaw KT, Tehard B, Berrino F, Tjonneland A, Bigaard J, Olsen A, Overvad K, Clavel-Chapelon F, Nagel G, Boeing H, Trichopoulos D, Economou G, Bellos G, Palli D, Tumino R, Panico S, Sacerdote C, Krogh V, Peeters PH, Bueno-de-Mesquita HB, Lund E, Ardanaz E, Amiano P, Pera G, Quirós JR, Martínez C, Tormo MJ, Wirfält E, Berglund G, Hallmans G, Key TJ, Reeves G, Bingham S, Norat T, Biessy C, Kaaks R and Riboli E: Body size and breast cancer risk: findings from the European Prospective Investigation into Cancer and Nutrition (EPIC). Int J Cancer *111*: 762-771, 2004.

4 Key TJ, Allen NE, Verkasalo PK and Banks E: Energy balance and cancer: the role of sex hormones. The Proc Nutr Soc *60*: 81-89, 2001.

5 Fain JN, Madan AK, Hiler ML, Cheema P and Bahouth SW: Comparison of the release of adipokines by adipose tissue, adipose tissue matrix, and adipocytes from visceral and subcutaneous abdominal adipose tissues of obese humans. Endocrinology *145*: 2273-2282, 2004.

6 Dizdar O and Alyamac E: Obesity: an endocrine tumor? Med Hypoth *63*: 790-792, 2004.

7 Caldefie-Chezet F, Damez M, de Latour M, Konska G, Mishellani F, Fusillier C, Guerry M, Penault-Llorca F, Guillot J and Vasson MP: Leptin: a proliferative factor for breast cancer? Study on human ductal carcinoma. Biochem Biophy Res Comm *334*: 737-741, 2005.

8 Jarde T, Caldefie-Chezet F, Damez M, Mishellany F, Penault-Llorca F, Guillot J and Vasson MP: Leptin and leptin receptor involvement in cancer development: a study on human primary breast carcinoma. Oncology Rep *19*: 905-911, 2008.

9 Jarde T, Caldefie-Chezet F, Damez M, Mishellany F, Perrone D, Penault-Llorca F, Guillot J and Vasson MP: Adiponectin and leptin expression in primary ductal breast cancer and in adjacent healthy epithelial and myoepithelial tissue. Histopathology *53*: 484-487, 2008.

10 Filigheddu N, Cutrupi S, Porporato PE, Riboni F, Baldanzi G, Chianale F, Fortina E, Piantanida P, De Bortoli M, Vacca G, Graziani A and Surico N: Diacylglycerol kinase is required for HGF-induced invasiveness and anchorage-independent growth of MDA-MB-231 breast cancer cells. Anticancer Res *27*: 1489-1492, 2007.

11 Kang JH, Yu BY and Youn DS: Relationship of serum adiponectin and resistin levels with breast cancer risk. J Kor Med Sci *22*: 117-121, 2007.

12 Sorli SC, Le Gonidec S, Knibiehler B and Audigier Y: Apelin is a potent activator of tumor neoangiogenesis. Oncogene *26*: 7692-7699, 2007.

13 Selim AA, El-Ayat G and Wells CA: Immunohistochemical localization of gross cystic disease fluid protein-15, -24 and -44 in ductal carcinoma *in situ* of the breast: relationship to the degree of differentiation. Histopathology *39*: 198-202, 2001.

14 Hassan MI, Waheed A, Yadav S, Singh TP and Ahmad F: Zinc alpha 2-glycoprotein: a multidisciplinary protein. Mol Cancer Res *6*: 892-906, 2008.

15 Bing C, Bao Y, Jenkins J, Sanders P, Manieri M, Cinti S, Tisdale MJ and Trayhurn P: Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed in adipocytes and is up-regulated in mice with cancer cachexia. Proc Natl Acad Sc USA *101*: 2500-2505, 2004.

16 Descazeaud A, de la Taille A, Allory Y, Faucon H, Salomon L, Bismar T, Kim R, Hofer MD, Chopin D, Abbou CC and Rubin MA: Characterization of ZAG protein expression in prostate cancer using a semi-automated microscope system. Prostate *66*: 1037-1043, 2006.

17 Burgi W and Schmid K: Preparation and properties of Zn-alpha 2-glycoprotein of normal human plasma. J Bio Chem *236*: 1066-1074, 1961.

18 Skipworth RJ, Stewart GD, Bhana M, Christie J, Sturgeon CM, Guttridge DC, Cronshaw AD, Fearon KC and Ross JA: Mass spectrometric detection of candidate protein biomarkers of cancer cachexia in human urine. Int J Oncol *36*: 973-82, 2010.

19 Diez-Itza I, Sanchez LM, Allende MT, Vizoso F, Ruibal A and Lopez-Otin C: Zn-alpha 2-glycoprotein levels in breast cancer cytosols and correlation with clinical, histological and biochemical parameters. Eur J Cancer *29A*: 1256-1260, 1993.

20 Bao Y, Bing C, Hunter L, Jenkins JR, Wabitsch M and Trayhurn P: Zinc-alpha2-glycoprotein, a lipid mobilizing factor, is expressed and secreted by human (SGBS) adipocytes. FEBS Lett *579*: 41-47, 2005.

21 Marrades MP, Martinez JA and Moreno-Aliaga MJ: ZAG, a lipid mobilizing adipokine, is down-regulated in human obesity. J Physiol Biochem *64*: 61-66, 2008.

22 Mracek T, Gao D, Tzanavari T, Bao Y, Xiao X, Stocker C, Trayhurn P and Bing C: Down-regulation of zinc-{alpha}2-glycoprotein in adipose tissue and liver of obese ob/ob mice and by tumor necrosis factor-alpha in adipocytes. J Endocrinol *204*: 165-72, 2010.

23 Selva DM, Lecube A, Hernández C, Baena JA, Fort JM and Simó R: Lower zinc-alpha2-glycoprotein production by adipose tissue and liver in obese patients unrelated to insulin resistance. J Clin Endocrinol Metab *94*: 4499-507, 2009.

24 Russell ST, Zimmerman TP, Domin BA and Tisdale MJ: Induction of lipolysis *in vitro* and loss of body fat *in vivo* by zinc-alpha2-glycoprotein. Biochim Biophys Acta *1636*: 59-68, 2004.

25 Bing C and Trayhurn P: New insights into adipose tissue atrophy in cancer cachexia. Proc Nutr Soc *68*: 385-92, 2009.

26 Sanchez LM, Vizoso F, Diez-Itza I and Lopez-Otin C: Identification of the major protein components in breast secretions from women with benign and malignant breast diseases. Cancer Res *52*: 95-100, 1992.

27 Byerley LO, Lee SH, Redmann S, Culberson C, Clemens M and Lively MO: Evidence for a novel serum factor distinct from zinc alpha-2 glycoprotein that promotes body fat loss early in the development of cachexia. Nutr Cancer *62*: 484-94, 2010.

28 Kamoshida S, Watanabe K, Suzuki M, Mizutani Y, Sakamoto K, Sugimoto Y, Oka T, Fukushima M, Tsutsumi Y: Expression of cancer cachexia-related factors in human cancer xenografts: an immunohistochemical analysis. Biomedical Res (Tokyo, Japan) *27*: 275-281, 2006.

29 Rehman S, Crow J and Revell PA: Bax protein expression in DCIS of the breast in relation to invasive ductal carcinoma and other molecular markers. Pathol Oncol Res *6*: 256-263, 2000.

30 Veronese S, Mauri FA, Caffo O, Scaioli M, Aldovini D, Perrone G, Galligioni E, Doglioni C, Dalla Palma P and Barbareschi M: Bax immunohistochemical expression in breast carcinoma: a study with long-term follow-up. Int J Cancer *79*: 13-18, 1998.

31 Penault-Llorca F, Cayre A, Bouchet Mishellany F, Amat S, Feillel V, Le Bouedec G, Ferriere JP, De Latour M and Chollet P: Induction chemotherapy for breast carcinoma: predictive markers and relation with outcome. Int J Oncol *22*: 1319-1325, 2003.

32 Elston CW and Ellis IO: Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology *19*: 403-410, 1991.

33 Baccouche S, Daoud J, Frikha M, Mokdad-Gargouri R, Gargouri A and Jlidi R: Immunohistochemical status of p53, MDM2, bcl2, bax, and ER in invasive ductal breast carcinoma in Tunisian patients. Ann NY Acad Sci *1010*: 752-763, 2003.