Original Article

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Tissue Expression of Neutrophil Gelatinase-Associated Lipocalin and Kidney Injury Molecule-1 in Breast Cancers

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ABSTRACT

Objective: Breast cancer is the most common cancer among women worldwide. Neutrophil gelatinase-associated lipocalin (NGAL) has important roles in immunity, cell proliferation, and carcinogenesis. Kidney injury molecule-1 (KIM-1) is a transmembrane glycoprotein also known as hepatitis A virus cellular receptor 1 and T-cell immunoglobulin and mucin, has restricted expression in immune cells and healthy epithelial cells, but it is up-regulated in several human cancers. The aim of this study was to determine the prognostic values of NGAL and KIM-1 expression in tumor cells and to detect the presence of NGAL-positive neutrophils (PNL) in the tumor microenvironment.

Materials and Methods: The expression of NGAL and KIM-1 protein were assessed by immunohistochemical staining in tissue specimens from 412 primary breast cancer cases.

Results: In this series, the mean age of the patients was 55.6±12.4 years. In 218 (52.9%) cases, there was NGAL expression in tumor cells. In 104 (25.2%) cases there was KIM-1 expression in tumor cells. NGAL-positive inflammatory cells were seen in tumors of 45 (10.9%) cases. There was no significant relationship between NGAL-positive PNL presence in the tumor microenvironment and other clinicopathological features. However, there was a significant association between the presence of *in situ* carcinomas and NGAL expression (p = 0.008) and KIM-1 expression (p = 0.020) in tumor cells.

Conclusion: This study has demonstrated positivity of NGAL and KIM-1 in breast cancer cells. Considering the development of anti-KIM-1 therapies, the presence of KIM-1 expression may be a new treatment option in breast cancer, especially in *in situ* component-rich tumors. These findings should be confirmed in larger series.

Keywords: Breast carcinomas; ductal carcinoma *in situ*; neutrophil gelatinase-associated lipocalin; kidney injury molecule-1; prognosis

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Key Points

- This study has demonstrated that the positivity of neutrophil gelatinase-associated lipocalin (NGAL) and kidney injury molecule-1 (KIM-1) will be effective on breast cancer, especially *in situ* component-rich ones.
- This result showed that NGAL and KIM-1 may be effective during the early carcinogenesis of breast cancer.
- Recently the new immune modulatory drug for TIM-1.
- Considering the development of anti-KIM-1 therapies, the presence of KIM-1 expression may be a new treatment option in breast cancer.

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Introduction

Breast cancer is the most common malignancy in women around the world. The development of breast carcinoma is regulated by many factors, such as hormonal effects, advanced age, alcohol consumption, obesity, dietary habits, and genetic factors (1-3). Ductal carcinoma *in situ* (DCIS) is also considered to be a precursor to invasive breast carcinoma and in which the proliferation of tumor cells is confined within the lumen of the breast ductal system (4-6). While the traditional classification of malignant breast tumors by the World Health Organization (WHO) was made based on histological features of the tumor, currently some subtypes have been described according to molecular characteristics of the tumors (1-3, 7, 8).

As a member of the lipocalin superfamily, neutrophil gelatinase-associated lipocalin (NGAL), also called lipocalin 2 or 24p3, was first isolated as a 25 kDa glycoprotein covalently bound to matrix metalloproteinase 9 (MMP9) in neutrophils. NGAL has been initially classified as an acute phase protein, which is rapidly released, mainly from neutrophils, as a response to inflammation and tissue injury (9-11). Initially, NGAL was thought to be an antibacterial factor and a component of the innate immune system and present in a large variety of cell types including hemopoietic cells. During hematopoiesis, immature ($CD34^+$) bone marrow progenitor cells, granulocyte precursors, activated monocytes, macrophages and neutrophils express NGAL. In contrast, NGAL protein expression has never been reported in lymphocytes and plasmacytes (9-13). Circulating low levels of NGAL can be detected in the urine and blood of healthy people, possibly secreted by neutrophils and renal epithelial cells. Expression of NGAL may play several physiological roles, including transporting hydrophobic molecules across cell membranes, regulating immune responses, modulating iron metabolism, and promoting epithelial to mesenchymal translations. In summary, NGAL is involved in many functions during diverse processes of growth, development, and tumorigenesis (12, 13).

Kidney injury molecule-1 (KIM-1) was first described in I996, as a mucin-like membrane glycoprotein type I, homologous to the immunoglobulin family proteins and which facilitated the intracellular penetration of the hepatitis A virus. Therefore, it was named hepatitis A virus cellular receptor 1 (HAVcr-1). Two years later, it was found to be a very sensitive and specific predictor of renal proximal tubule injury and was redesignated as KIM-1. In the 2000s, a group of proteins, belonging to the T-cell immunoglobulin and mucin (TIM) domain family, which are especially expressed in T cells functioning in the respiratory system was identified. TIM-1, one of these proteins, is homologous to KIM-1. In summary, the definitions of HAVcr-1, KIM-1 and TIM-1 (CD365) mentioned in the biological databases today describe the same protein (14-16). KIM-1 is normally expressed at a low level in the healthy kidney. However, cell-associated KIM-1 expression increases dramatically in post-ischemic kidney tissue and KIM-1 exerts an anti-inflammatory role following kidney injury (16, 17). Its expression is also up-regulated in several human cancers, most notably in renal and ovarian carcinomas, but has very restricted expression in healthy tissues, thus representing a promising target for antibody-mediated therapy. Recently, a human monoclonal IgG1 antibody specific for the extracellular domain of TIM-1 was developed. This antibody (CDX-O14) was shown to bind purified recombinant chimeric TIM-1-Fc protein and TIM-1 expressed on a variety of transformed cell lines. However, it has not been included in the routine treatment regimen to date (14-19).

Hitherto, as relevant markers for assessing the proliferative activity and tumor cell dynamics of breast carcinomas, many parameters have been suggested. However, among these parameters NGAL and KIM-1 have not been investigated extensively. In this study we aimed to explore the clinical importance NGAL and KIM-1 expressions in breast cancers.

Materials and Methods

The expression of NGAL and KIM-1 protein was investigated by immunohistochemical staining in tissue specimens from 412 primary breast cancer cases who underwent mastectomy, and excisional breast biopsy between the years 2011 and 2018, and were subsequently diagnosed as breast carcinoma in the Pathology Laboratory of İzmir Tepecik Training and Research Hospital. Patients' files were retrospectively evaluated. This study was approved by the local Ethics Committee of the Hospital. Hematoxylin-Eosin (H&E) stained, archived slides were re-evaluated, based on 2012 breast tumor classification of the WHO. For immunohistochemistry (IHC), H&E staining was used to select appropriate paraffin blocks and to identify the viable tumor areas. The paraffin block most suitable for IHC evaluation was selected, and labeled firstly on the slide, and then the block, and 2 mm thick cylindrical paraffined tissue samples were harvested from donor blocks. Then multiple blocks were prepared using mapping and addressing techniques. Then IHC was performed using diluted (1:300) monoclonal rabbit antibodies against NGAL (Novus Biologicals, Littleton, USA; NDP1- 90331) and KIM-1 (Bioss, Philadelphia, USA; HAVCRI). Histopathologists, blinded to the clinical features of the patients, examined the slides and staining patterns were classified according to the intensity of staining. NGAL positivity was defined as diffuse cytoplasmic and/or nuclear staining in both invasive and *in situ* components of tumor (Figure 1). KIM-1 positivity was defined as diffuse cytoplasmic staining in both invasive and *in situ* components of tumor (Figure 2). For both antibodies, focal staining occupying less than 1–2% of the high-power field of view or weak staining visible under a microscope was considered as NGAL or KIM-1 negativity. In addition, the presence of NGAL-positive neutrophils and/or macrophages that infiltrated the tumors was evaluated (Figure 3).

Statistical Analysis

Statistical analysis was performed using SPSS, version 25.0 (IBM Inc., Armonk, NY, USA). For comparison of quantitative data the chi-square test was used. For the comparison of non-parametric data Mann–Whitney U test were used. For comparison of the measurements

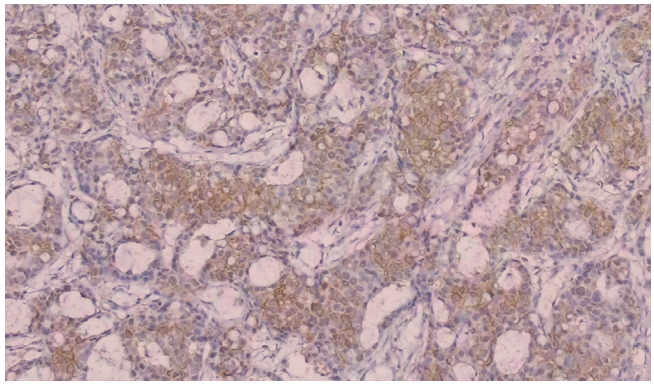

Figure 1. A case with immunohistochemically detected cytoplasmic and/or nuclear NGAL positivity in tumor cells (DAB x 200)

NGAL: neutrophil gelatinase-associated lipocalin; DAB: diaminobenzidine

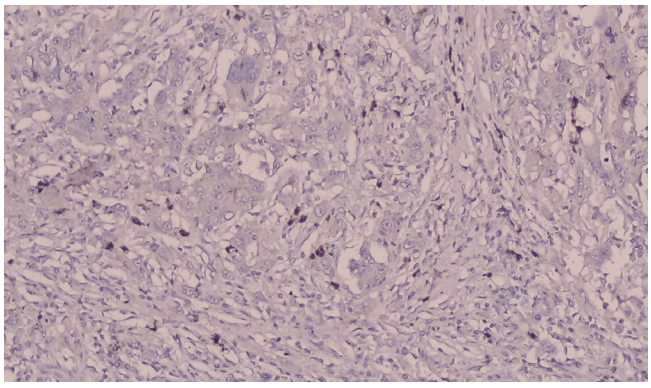

Figure 2. NGAL-positive inflammatory cells in tumor stroma (DAB x 200)

NGAL: neutrophil gelatinase-associated lipocalin; DAB: diaminobenzidine

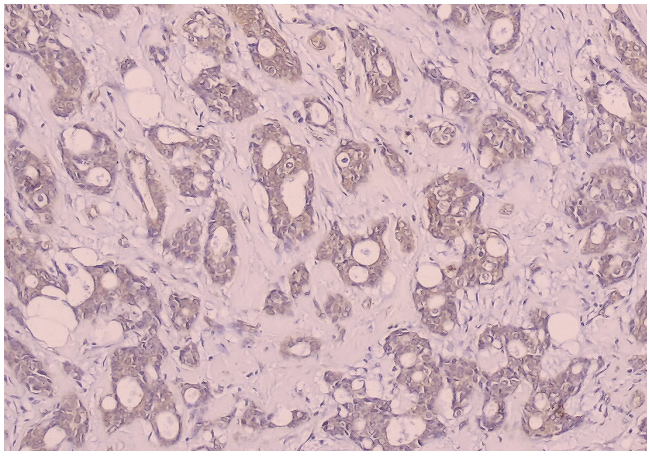

Figure 3. A case with immunohistochemically detected cytoplasmic KIM-1 positivity in tumor cells (DAB x 200)

KIM-1: kidney injury molecule-1; DAB: diaminobenzidine

in more than two groups the non-parametric Kruskal–Wallis test was utilized. Kaplan–Meier survival analysis was applied to compare the difference in survival between groups. A $p \leq 0.05$ was accepted as the level of statistical significance.

Results

In this series, the mean age of the 412 patients was 55.6±12.4 years (range: 30–85 years) at the time the samples were obtained. The mean follow-up period was 37.75±21.4 (range: 0.83–111.07) months. Three hundred and sixty-five (88.6%) patients survived, and 47 (11.4%) patients died. Tumor location was reported in 342 cases, as follows. There were 163 (47.7%) tumors in the right and 178 (52%) in the left breast. There was only one case (0.3%) with bilateral breast tumor in the series. The mean tumor diameter was 2.94±1.79 cm (range: 0.5–10 cm). Pathological T staging could be evaluated in 339 patients (82.3%). According to pathological T staging these 339 cases were distributed as follows: pT1 (n = 139; 41%); pT2 (n = 144: 42.5%); pT3 (n = 41; 12.1%); and pT4 (n = 15; 4.4%). The tumor was multifocal in 40 (9.7%), and unifocal in all other cases. Among the cases with precisely known tumor location, the most common location was the upper outer quadrant (38.9%), followed by central (34.2%), upper inner (10.7%), lower inner (7.4%) and lower outer (8.7%) quadrants. Histopathologically tumors were classified as grade 1 in 27 (6.5%), grade 2 in 208 (50.4%), and grade 3 in 177 (42.9%) of cases. A DCIS component was present in 273 (66.3%) tumors and there were no cases of lobular carcinoma *in situ*. Of all the *in situ* components present, 40 (14.6%) were comedoes, 114 (41.8%) were non-comedoes and 119 (43.6%) were comedo+non-comedo mixed *in situ* carcinoma type. Axillary lymph node dissection was performed in 338 (82%) of the cases, and lymph node metastasis was detected in 163 (39.6%). In 112 (68.7%) cases with lymph node metastasis, capsular invasion was present in the metastatic lymph nodes (Table 1).

On IHC studies performed in 412 patients included in the study, estrogen receptor (ER)-positivity was detected in 330 (80.1%), and PR-positivity in 298 (72.3%) cases. Immunohistochemically, c-erbB2, which was applied to evaluate human epidermal growth factor receptor 2 (HER2)/neu amplification and was found to be 1+ or negative in 272 cases (66%), and both groups were considered as HER2-negative. In combined IHC-FISH evaluation, 92 cases (22.3%) were accepted as HER2-positive and all received targeted treatment. Ki67 proliferation index was studied in all cases, and the cut-off level for low/high Ki-67 expression was 15%. In this series the mean Ki67 index was found to be 22.74±18.76% (range: 1-95%). Based on molecular classification, respective number of cases with luminal A (n = 142; 34.4%), luminal B (n = 139; 33.7%), HER2-positive (n = 92: 22.3%), and triple-negative (n = 39; 9.5%) were detected (Table 2). Mean ages of the patients and survival time in different molecular groups were similar ($p = 0.377$). In this series, the longest survival time was found in the luminal A group ($p = 0.003$).

In 218 (53%) cases, there was NGAL expression in tumor cells. In 104 (25.2%) cases, there was KIM-1 expression in tumor cells. NGAL-positive inflammatory cells were seen in tumors of 45 (10.9%) cases. There was no difference in expressions of the two markers between *in situ* and invasive components of the tumors. When comparisons were made by chi-square test, the rate of cases with NGAL expression was higher in HER2 positive tumors compared to other molecular groups ($p = 0.019$). However, there was no significant difference in KIM-1 ($p = 0.100$) expression in tumor cells based on molecular subtype. Similarly, there were no statistical significance in the rate of expression of NGAL or KIM-1 according to the types of *in situ* components. Neither was there a significant relationship between NGAL-positive PNL presence in the tumor microenvironment and other clinicopathological features. However, there was a significant association between the presence of *in situ* carcinoma and the expression of both NGAL ($p = 0.008$) and KIM-1 ($p = 0.020$) in tumor cells (Table 3).

Discussion and Conclusion

Following a number of studies and meta-analyses, breast cancers began to be classified according to the molecular subtype in the 2000s (1-3, 7). It has emerged that 75% of breast tumors contain estrogen and/or progesterone receptors (ER/PR), and therefore belong to the luminal group. However, since tumors in the luminal group manifest diverse behaviors, this group is divided into luminal A and B subgroups according to the their proliferative index (1). Other subtypes are HER2-positive and triple-negative or basal cell-like tumors. HER2 amplification was known as a poor prognostic factor when it was first identified, but with the subsequent development of HER2-targeted therapeutic agents, cases with HER2-positive tumors no longer differ in terms of survival (7). As expected, in the present study, the longest survival time was found in the luminal A group. However, the survival of HER2 positive group was also close to the survival of luminal group

and we attributed this to the fact that all patients in this group received tailored therapy against HER2.

There is an established signaling network between tumor cells and stromal cells (20). This network plays an important role to constitute the tumor microenvironment. The tumor microenvironment can influence behavior of cancer cells in different ways and can promote cancer progression. The tumor microenvironment is composed of various cells of different origins that secrete several soluble factors, including cytokines, growth factors, and microRNAs as well as other factors. Adipocytes also secret NGAL, the main functions of which appear to be activation of the innate immune response and transportation of small hydrophobic molecules (20, 21). In addition, it was determined that NGAL secretion from breast adipose tissue can promote breast cancer progression by increasing EMT (20-25). Surprisingly, the roles of NGAL in carcinogenesis may be contrary. Pro-tumoral effects attributed to NGAL include acting as an intracellular iron carrier and protecting MMP9 from proteolytic degradation in different neoplasms of breast, stomach, esophagus, uterine cervix, and brain. NGAL was also associated with NF-kB which is an important factor involved both in tumor growth and in the link between chronic inflammation and neoplastic development. NGAL, paradoxically, has been reported to have an anti-tumoral and anti-metastatic effect in cancers of colon, ovary, and pancreas (22-27). Some studies have demonstrated that NGAL can inhibit angiogenic factors, such as HIF-1 alpha and vascular endothelial growth factor. In a recent study using a three dimensional spheroid model, it was shown that NGAL contributes to the early events of metastasis *in vitro*. The release of NGAL from macrophages induced an epithelial-mesenchymal transition program in the MCF-7 breast cancer cell line and enhanced local migration as well as invasion into the extracellular matrix. Thus,

Table 1. Demographic and histopathologic data

		n	%
Prognosis	Survived	365	88.6
	Died	47	11.4
Tumor Location	Right	163	47.7
	Left	178	52
	Bilateral	1	0.3
Diagnosis	Invasive ductal carcinoma (IDC)	249	60.4
	Invasive lobular carcinoma	28	6.7
	Invasive papillary carcinoma	11	2.6
	IDC with dominant *in situ* component	102	24.7
	Other histologic variants	22	5.3
Grade	Grade 1	27	6.5
	Grade 2	208	50.4
	Grade 3	177	42.9
Pathologic T stage	pT1	139	41
	pT2	144	42.5
	pT3	41	12.1
	pT4	15	4.4
In situ component	Present	273	66.3
Type of *in situ* component (if any)	Comedo	40	14.6
	Non comedo	114	41.8
	Mixed	119	43.6
Lymph node metastasis	Present	163	39.6
	Absent	175	42.5
Capsular invasion in the lymph node	Present	112	68.7
	Absent	51	31.3
Multifocality	Solid	272	91.3
	Multifocal	40	9.7
Nipple involvement	Present	24	5.8
Dermal/epidermal invasion	Present	38	9.2
Lymphovascular invasion	Present	144	35
Perinueral invasion	Present	104	25.2

Table 2. Immunohistochemical, and molecular findings

Parameters	Status	n	%
ER status	Positive	330	80.1
PR status	Positive	298	72.3
c-erbB2 expression (according to ASCO/CAP 2013 criteria)	Negative or 1+	272	66
	2+	88	21.3
	3+	52	12.6
HER2 amplification (FISH method)	Positive	47	11.4
	Negative	41	10
Molecular type	Luminal A	142	34.4
	Luminal B	139	33.7
	HER2- positive	92	22.3
	Triple negative (Basal-like)	39	9.5
NGAL expression	Positive in inflammatory cells	45	10.9
NGAL expression	Positive in tumor cells	218	53
KIM-1 expression	Positive in tumor cells	104	25.2

ASCO: American Society of Clinical Oncology; CAP: College of American Pathologists; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2; FISH: fluorescence *in situ* hybridisation; NGAL: neutrophil gelatinase-associated lipocalin; KIM-1: kidney injury molecule-1

Table 3. Association between NGAL and KIM-1 expression and prognosis

	Presence of NGAL expression (n = 218)	*p*	Presence of KIM-1 expression (n = 104)	*p*
Molecular type	Luminal A (n = 73) Luminal B (n = 69) HER2-positive (n = 62) Triple negative (Basal-like) (n = 14)	**0.019**	Luminal A (n = 35) Luminal B (n = 41) HER2-positive (n = 24) Triple negative (Basal-like) (n = 4)	0.100
Type of *in situ* component (if any)	Comedo (n = 25) Non-comedo (n = 68) Mixed (n = 67)	0.755	Comedo (n = 9) Non-comedo (n = 40) Mixed (n = 30)	0.157
Presence of *in situ* component	Absent (n = 58) Present (n = 160)	**0.008**	Absent (n = 25) Present (n = 79)	**0.020**
Lymph node metastasis	Absent (n = 90) Present (n = 87)	0.720	Absent (n = 43) Present (n = 45)	0.525
Grade of tumor	Grade 1 (n = 14) Grade 2 (n = 110) Grade 3 (n = 94)	0.993	Grade 1 (n = 6) Grade 2 (n = 57) Grade 3 (n = 41)	0.591
Survival	Deceased (n = 30) Alive (n = 188)	0.111	Deceased (n = 12) Alive (n = 92)	0.961

NGAL: neutrophil gelatinase-associated lipocalin; KIM-1: kidney injury molecule-1; HER2: human epidermal growth factor receptor 2

an association between macrophage-released NGAL and breast cancer progression was explored. Therefore one aim of the present study was to attempt an evaluation of the utility of NGAL levels in making an early diagnosis, establishing a prognosis, and predicting response to different treatments (23-30).

A recent study reported a difference in serum levels of NGAL according to breast cancer subtypes with elevated levels of MMP9/NGAL complex in luminal subtypes (31). In contrast, the serum levels of MMP9/NGAL were found to be substantially decreased in Triple Negative and HER2 positive group (31, 32). In contrast, the NGAL-positivity rate increased in HER2 positive group in our study, although we did not measure blood concentrations of NGAL. It has been reported that high cytoplasmic and low nuclear localization of NGAL was associated with the worst survival outcome in breast cancer patients (27). In our study, prominent nuclear NGAL expression was

absent and most NGAL expression was cytoplasmic in the tumor cells. Therefore, although we found a strong correlation between the presence of *in situ* carcinoma and the presence of both nuclear and cytoplasmic NGAL expression, we cannot draw any conclusions about the relationship between the location of NGAL expression and prognosis.

Earlier studies have suggested that the phagocytic function of KIM-1 to remove apoptotic bodies in injured proximal tubules reduced antigen exposure to inflammatory cells and prevented over-reaction of the immune system. However, as apoptotic bodies are phagocytosed by antigen presenting cells (APCs), these cells subsequently activate regulating T cells and cytotoxic T lymphocytes to attack target cells. In addition, renal cell carcinomas (RCCs), derived from the proximal tubules, express KIM-1, which implies some phagocytotic activity in RCC cells. Therefore, it was suggested that the phagocytotic function of KIM-1 may be adapted by RCC cells to clear tumor apoptotic bodies, thus preventing the activation of APCs and T lymphocytes against RCC cells. In other words, KIM-1 may play a scavenger role in RCC against potential immune reactions and may be a key factor in the tumor microenvironment for the survival and development of RCC (14-17). KIM-1 overexpression in the cells of clear cell and papillary RCC has for a long time been known as a special feature of kidney tumors, but data concerning the clinical significance of increased KIM-1 expression in the extra-renal tumors are ambiguous. For example, Liu et al. (17), reported that elevated expression of KIM-1 mRNA is associated with unfavorable prognosis and low sensitivity to chemotherapy in stomach cancer. Similarly, Zheng et al. (18) found that increased KIM-1 protein expression was also associated with worse survival in non-small cell lung cancers. Inactivation of KIM-1 in lung cancer cells suppresses proliferation, migration activity, and invasion and is also accompanied by a rise in the level of tumor suppressor protein PTEN and inhibition of the pro-oncogenic PI3K/Akt signaling pathway. In contrast, Wang et al. (19) reported that overexpression of KIM-1 mRNA in colon cancer tissue was associated with a longer recurrence-free survival of patients. In addition, high KIM-1 expression rates have been reported in clear cell carcinoma of the ovary (93.8%), nephroblastomas (74%), primary lymphomas of the central nervous system (54%), germ cell tumors (50%), and endometrium carcinomas (33.3%). However, there is no firm correlation between the level of KIM-1 expression in cancer cells and clinical and morphological characteristics of each specific malignant disease, which indicates independent prognostic significance of this indicator (12, 14-19). Similarly to these studies, we could not find a relationship between KIM-1 expression and invasive breast tumors. However, unlike the others, we found higher KIM-1 positivity in breast cancer with a ductal *in situ* component.

Today, widespread mammographic screening has led to the increasing diagnosis of DCIS and *in situ* carcinomas now comprise 20-25% of all breast carcinoma diagnoses. DCIS shares many of the epidemiological, hormonal and genetic risk factors with invasive breast cancer (IBC). Although DCIS is usually treated with surgical excision, chemoradiotherapy may be added depending on the extent of the lesion or the team that will administer the treatment. Despite the increase in the diagnosis and treatment of DCIS, there is no decrease in the diagnosis of IBC. This has led to the suggestion that the *in situ* carcinomas may never become invasive tumors and that the surgical wide-excision, hormone therapy, or radiotherapy are over-treatment (4-6).

Based on our results, we speculate that the reason for detecting high NGAL and KIM-1 expression in tumors with *in situ* carcinoma in this study may be associated with the behavior of DCIS. We think that the NGAL and KIM-1 positivity rates of tumor cells were found to the higher in the tumors with DCIS. Therefore, breast cancers expressing NGAL and/or KIM-1 may form a mass, may invade, and metastasize earlier. One of the most important limitations of this study is that NGAL and KIM-1 expressions were not investigated in DCIS cases without invasive cancer. If our speculation is correct, then it could be expected that NGAL and KIM1 positivity rates would be found to be significantly lower in patients without invasive carcinoma in their follow-up and repeat investigations.

This study has demonstrated higher positive expression rates of NGAL and KIM-1 in breast cancer with *in situ* components. Considering the development of anti-KIM1 therapies, the presence of KIM-1 expression may have increased importance in clinical practice, especially in *in situ* component-rich tumors. It remains for these findings to be confirmed in larger series which should also include DCIS with no evidence of invasion.

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Ethics Committee Approval: The study was approved by the Local Ethics Committee of the Hospital (2015/21/2-19 March 2015).

Informed Consent: Patients' files were retrospectively evaluated.

Peer-review: Externally and internally peer-reviewed.

Authorship Contributions

Surgical and Medical Practise: C.K.; Concept: G.D., A.G.P., D.S.K., U.V., S.S., D.A., C.K.; Design: G.D., A.G.P., D.S.K., U.V., S.S., D.A., C.K.; Data Collection and/or Processing: G.D., A.G.P., D.S.K., U.V., S.S., D.A.; Analysis and/or Interpretation: G.D.; Literature Search: G.D., A.G.P., D.S.K., U.V., S.S., D.A., C.K.; Writing: G.D., A.G.P., D.S.K., U.V., S.S., D.A., C.K.

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