Loss of heterozygosity in ING3 and ING5 genes in breast cancer

Esra GÜNDÜZ1,*, Gökhan NAS1, Muradiye ACAR1, Eyyüp ÜÇTEPE1, Mikdat BOZER2, Murat ÖZNUR1, Reyhan BAYRAK3, Mehmet GÜNDÜZ1,4

1Department of Medical Genetics, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
2Department of General Surgery, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
3Department of Pathology, Faculty of Medicine, Turgut Özal University, Ankara, Turkey
4Department of Otolaryngology, Faculty of Medicine, Turgut Özal University, Ankara, Turkey

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Abstract: The tumor suppressor genes (TSGs) ING3 and ING5, members of the inhibitor of growth gene family, are effective in inhibition of cell growth and induction of apoptosis. However, in many cancer types, one of the alleles of a TSG is lost through carcinogenesis, while the remaining allele is usually inactivated through a process called loss of heterozygosity (LOH). Previous studies in head and neck cancer revealed that allelic loss and reduced expression is a common pattern of ING gene family members. Fifty paraffin-embedded breast cancer tissues were analyzed by polymerase chain reaction and denatured-polyacrylamide gel electrophoresis for LOH status. The allelic deletion frequency of ING3 and ING5 were detected as 14% and 17% in breast cancer patients, respectively. No significant relationship was detected between ING3 LOH status and clinicopathological variables. Our data also suggest that both ING3 and ING5 LOH statuses have no significant effect in overall survival and disease-free survival of breast cancer patients. These results provide a rational explanation and relative contribution for the complexity of tumor formation, whereby allelic loss of ING3 and ING5 genes is not a major factor for breast cancer but is rather a part of a larger complex mechanism.

Key words: Clinicopathological factors, early diagnosis, survival analysis, tumor suppressor genes

1. Introduction
Breast cancer is the most deadly form of cancer after lung cancer, and after skin cancers it is the most common cancer among women in the world. About 1 in 8 (12%) women in the United States will have breast cancer in their lifetime. According to the American Cancer Society, 232,340 new women had an invasive breast cancer diagnosis and about 40,000 women died from breast cancer in the United States in 2013. It is the cause of death for 1 out of every 36 women (about 3%) (American Cancer Society, 2013).

Breast cancer mortality has been decreasing since 1989, and this decline has been seen especially in women younger than 50. It is known that earlier detection through screening and increased awareness about the disease has led to these declines, as well as enhanced treatment (Andersson and Janzon, 1997). Early diagnosis of breast cancer before symptoms emerge is the most effective form of treatment. Thus, understanding the genetic background of breast cancer is essential for early diagnosis and finding new biomarkers.

Breast cancer is the end point of multiple genetic events in the breast tissue. One of these events, inactivation of tumor suppressor genes (TSGs), is considered one of the most important mechanisms in tumorigenesis. Hence, localization and identification of TSGs are of great interest for a better understanding of cancer and the development of molecular diagnostics as well as new drugs. One of the important steps for the identification of a TSG is loss of heterozygosity (LOH) analysis. LOH is commonly seen in cancer, where it denotes the lack of a functional TSG in the lost region. On the other hand, the other allele serves as a functional gene, so this loss does not cause illness. However, a point mutation, nondisjunction during mitosis, or deletion of a chromosome segment can inactivate the remaining copy of the TSG, leaving no TSG to protect the body, and then the individual goes on to develop cancer (Knudson, 1971).

The first member of the inhibitor of growth (ING) family was discovered by Riabowol's group in 1996 by using subtractive hybridization (Garkavtsev et al., 1996). Our group identified its genomic structure in 2000 (Gündüz et al., 2000). We showed that ING1 encodes a 33-kDa protein (p33ING1b). In that study, ING1 was also shown to be a tumor suppressor gene. The loss of ING1 gene expression
has been shown in numerous cancer types and *ING1* gene knockout mice have been found to be cancer-prone. Subsequently, we and other groups identified 4 additional human *ING* genes (*ING2–ING5*) (Gündüz et al., 2000; Gündüz et al., 2002; Shiseki et al., 2003; Gong et al., 2005; Soliman and Riabowol, 2007; Gündüz et al., 2008; Unoki et al., 2008; Coles and Jones, 2009). Among these genes, *ING3*, encoding a 46.8-kDa protein (p47ING3) with a C-terminal plant homeodomain (PHD) finger motif, was subsequently identified through a computational domain search in 2003 (Gündüz et al., 2000). It has been mapped on chromosome 7 at the locus 7q31 and encodes 2 variants (Figure 1). The other members of the *ING* family, including *ING5*, were identified through a computational sequence homology search in the same year (Shiseki et al., 2003). *ING5* has been mapped on chromosome 2 at locus 2q37.3 and encodes 1 variant (Figure 2).

*ING* genes are TSGs. They play roles in a wide variety of cellular processes. They are involved in cell cycle checkpoints and cell cycle progression as well as induction of apoptosis. *ING1* expression is significantly decreased in 44% of human primary breast cancers and 100% of established breast cancer cell lines. Decreased *ING1* expression has been identified in many solid and blood tumors (Gündüz et al., 2009). Additionally, *ING2*, *ING3*, and *ING4* gene expression is decreased in human melanoma cancer (Aguissa-Toure et al., 2011).

The role of *ING3* in regulation of the cell cycle and apoptosis has been determined. Ectopic expression of *ING3* in RKO cells, a colon cancer cell line, resulted in decreased colony formation, possibly by reducing the number of cells in S phase (Nagashima et al., 2003; Shiseki et al., 2003).

Although we have limited information about *ING5* function, very recent transfection experiments have demonstrated that *ING5* reduces colony-forming efficiency, inhibits S-phase progression, and induces apoptosis in a p53-dependent manner. *ING5* can also induce expression of the cyclin-dependent kinase inhibitor p21 (Shiseki et al., 2003), a p53-target gene. These results suggest that *ING5* has a role in regulation of cell growth and p53 activity, but further studies of *ING5* function are needed.

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**Figure 1.** *ING3* gene is composed of 13 exons and has 2 transcripts. Domains are as indicated above.

**Figure 2.** *ING5* gene is composed of 8 exons and has 1 transcript. Domains are as indicated above.
To our knowledge, there has been no prior study examining the LOH status of ING3 and ING5 in breast cancer. In this study, we examine for the first time the LOH status of ING3 and ING5 in breast cancer tissues. We also investigate the relation between the genes’ LOH statuses and breast cancer patient survival.

2. Materials and methods

2.1. Tissue samples

Formalin-fixed paraffin-embedded archival matched tumor and macroscopically normal samples of 50 patients (median age: 51.9 years; range: 27–78 years) diagnosed with invasive ductal breast cancer between 2006 and 2011 were obtained from the Department of Pathology, Faculty of Medicine, Turgut Özal University. All tissues were frozen in liquid nitrogen immediately after surgery and stored at −80 °C until the extraction of DNA and RNA. All tumors were confirmed as invasive ductal breast carcinoma in the Department of Pathology.

These samples were collected after acquisition of informed consent from each patient and approval of the study by the Institutional Human Ethics Committee of Turgut Özal University.

Tumor histology was determined according to the 2003 criteria of the World Health Organization. Tumors were graded according to the Bloom–Richardson grading scale. Each tumor and normal breast tissue sample was histopathologically confirmed for the presence or absence of cancer cells. Tumor samples containing more than 70% tumor cells were selected. ER, PR, and HER2 statuses were determined by immunohistochemistry.

2.2. Extraction of genomic DNA

Tumor and normal tissue samples were deparaffinized and rehydrated in a decreasing alcohol series prior to DNA extraction by use of the Pure Link Genomic DNA Mini Kit (Invitrogen, USA). The extracted genomic DNA was finally eluted in 100 µL of Tris-HCl buffer (10 mM, pH 9.0).

2.3. Polymerase chain reaction and denatured-polyacrylamide gel electrophoresis

Specific primers were designed for ING3 and ING5 gene regions for allelic deletion. Polymerase chain reaction (PCR) was carried out in 25 µL of total reaction volume with 12.5 µL of master mixture (QIAGEN), 0.5 µL (20 pmol) of each primer, 2 µL of genomic DNA, and 9.5 µL of ddH2O. Initial denaturation at 94 °C for 5 min was followed by 35 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 54 °C (ING3) or 60 °C (ING5) for 30 s, and an extension step at 72 °C for 1 min. A final extension step at 72 °C for 5 min was added. Amplicons were run in agarose gel electrophoresis and an appropriate amount of PCR amplicon for both normal and tumor samples was loaded into denatured-polyacrylamide gel. Next, 2–4 µL of the PCR mixture was mixed with 2X loading dye (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, and 0.05% xylene cyanol, ddH2O), heat denatured, chilled on ice, and then electrophoresed through an 8% polyacrylamide gel containing 8 M urea. DNA fragments were visualized by the silver staining method (Bassam and Gresshoff, 2007).

LOH was determined if one of the heterozygous alleles showed at least 50% reduced intensity in tumor DNA as compared with the corresponding normal DNA (Figure 3). This decision was made by direct visualization in cases with clear deletion as compared to normal allele.

2.4. Statistical analysis

The Mann–Whitney U test as a nonparametric method and Pearson’s chi-square test were used to evaluate the correlation between ING3 and ING5 genes and the clinicopathological characteristics of the patients (Table). Survival curves were calculated according to the Kaplan–Meier method. For comparison of survival between LOH-negative and LOH-positive ING genes, the log-rank test was used. Overall survival in months was calculated from the day after surgery to the last follow-up examination or death. The survival periods of the patients who were still alive were noted along with the date of the most recent follow-up appointment. The duration of disease-free survival (DFS) was determined from the day after surgery to the initial recurrence of the surgically resected cancer, as evaluated by clinical examination. SPSS 15 for Windows (SPSS, Chicago, IL, USA) was used for all statistical manipulations and all results with a P-value of less than 0.05 were considered to be statistically significant.

3. Results

We included 50 breast cancer samples in the study. We were unable to carry out PCR for the ING5 gene in some samples. Our results showed that 14% of the patients presented LOH of ING3, 58% of the patients were normal for ING3, and 28% of the patients’ results were not informative (Figures 4 and 5). Likewise, 17% of the patients presented LOH of the ING5 gene, 62% of the patients had no deletion at the ING5 gene locus, and 21% of the patients’ results were not informative (Figures 4 and 5). Lymphovascular invasion was identified in 35% of the patients. Nipple invasion was identified in 70% of the patients, while ER was identified in 60.5% of the patients and PR in 60.5% of the patients. Fifty-eight percent of the patients were smokers and 2.3% of patients consumed alcohol.

First, we investigated the relationship between LOH status of ING3 and ING5 genes and most clinical markers. No significant relationship was detected between ING3 LOH status and tumor grade (P = 0.967), absence or presence of lymph-node metastasis (P = 0.185),
lymphovascular invasion (P = 0.782), nipple invasion (P = 0.199), age (P = 0.845), smoking (P = 1.000), alcohol consumption (P = 0.739), and application of chemotherapy (P = 0.201) or radiotherapy (P = 0.626).

Likewise, no significant relationship was detected between LOH status of ING5 and tumor grade (P = 0.711), absence or presence of lymph-node metastasis (P = 0.107), lymphovascular invasion (P = 0.100), nipple invasion (P = 0.213), age (P = 0.166), smoking (P = 1.000), alcohol consumption (P = 0.564), and application of chemotherapy (P = 0.112) or radiotherapy (P = 0.619).

### 3.1. ING3 and ING5 genes and survival analysis

Both overall survival (OS) and DFS were determined. All patients were enrolled in a follow-up program. The follow-up time was between 22 and 90 months. Valid follow-up data were available for 42 (84%) of 50 breast cancer patients.

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**Table. Clinicopathological characteristics of patients.**

<table>
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<th>Criteria</th>
<th>n</th>
<th>%</th>
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<td>Node status</td>
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<td>N0</td>
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<td>28</td>
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<tr>
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<td>5</td>
<td>11</td>
<td>N2</td>
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</tr>
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<td>Radiotherapy</td>
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<td>32.6</td>
</tr>
</tbody>
</table>

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**Figure 3.** Primary LOH data from 6 representative breast cancer samples. N, Normal DNA; T, tumor DNA. Case 5 shows LOH of ING5 and case 10s show LOH of ING3; case 6 and case 11 are not informative; case 4 and case 13 show retention of heterozygosity.
Correlations between ING3 and ING5 LOH statuses and the patients' OS and DFS were analyzed using the univariate Kaplan–Meier method. The mean overall survival in the ING3 gene LOH-negative group was 54 months (range: 22–90 months) and that in the LOH-positive group was 40 months (range: 28–71 months) (P-value for log-rank test = 0.824). The log-rank test showed that patients in the ING3 LOH-positive group did not have significantly shorter OS than those in the ING3 LOH-negative group (P = 0.824; Figure 6a). The mean DFS in the ING3 gene LOH-negative group was 49 (range: 22–90) months and that in the ING3 gene LOH-positive group was 40 (range: 21–51) months (P-value for the log-rank test = 0.767; Figure 6b). The mean OS in the ING5 gene LOH-negative group was 61 months and that in the ING5 gene LOH-positive group was 51.5 months (P-value for log-rank test = 0.102; Figure 7a). The mean DFS in the LOH-negative group was 54 months and that in the LOH-positive group was 51.5 months (P-value for the log-rank test = 0.125; Figure 7b). These data suggest that neither ING3 nor ING5 LOH status has significant effect on OS and DFS of breast cancer patients.

4. Discussion
Although the exact functions of the ING genes have not been fully clarified, the gene products mainly have roles in transcriptional regulation, apoptosis, the cell cycle, angiogenesis, and DNA repair through p53-dependent and -independent pathways, and they form complexes with histone acetyltransferases and histone deacetylases (Campos et al., 2004).

Loss of heterozygosity in ING3 was found in ameloblastoma, renal cell carcinoma, and head and neck squamous cell carcinoma (HNSCC; Shridhar et al., 1997; Borkosky et al., 2010). Decreased ING3 mRNA expression was also found in HNSCC and melanoma (Wang and Li, 2006; Gündüz et al., 2008).
Figure 6. Overall and disease-free survival in the groups of breast cancer patients with LOH-negative and LOH-positive ING3 gene. Kaplan–Meier survival curves for the total number of cases (n = 26) were stratified by ING3 LOH states. The cases were divided into LOH-negative and LOH positive groups. (a) The mean overall survival in the LOH-negative group was 54 months and that in the LOH-positive group was 40 months (P-value for log-rank test = 0.824). (b) The mean disease-free survival in the LOH-negative group was 49 months and that in the LOH-positive group was 40 months (P-value for the log-rank test = 0.767).

Figure 7. Overall and disease-free survival in the groups of breast cancer patients with LOH-negative and LOH-positive ING5 gene. Kaplan–Meier survival curves for the total number of cases (n = 37) were stratified by ING5 LOH states. The cases were divided into LOH-negative and LOH-positive groups. (a) The mean overall survival in the LOH-negative group was 61 months and that in the LOH-positive group was 51.5 months (P-value for log-rank test = 0.102). (b) The mean disease-free survival in the LOH-negative group was 54 months and that in the LOH-positive group was 51.5 months (P-value for the log-rank test = 0.125).
Our previous study on head and neck cancer showed that about half of 71 tumor samples demonstrated downregulation of ING3 compared to their matched normal counterparts. Even if most clinicopathological variables were not significantly related to ING3 downregulation or p53 mutation status, a significant relationship was determined in terms of OS between the cases with low and normal to high ING3 expression. At 5 years of follow-up, approximately 60% of the patients with normal to high ING3 expression had survived, whereas this rate was 35% in the patients with low ING3 expression. Multivariate analysis also showed downregulation of ING3 as an independent prognostic factor for poor OS. These results reveal that ING3 would function as a potential tumor suppressor molecule and that low levels of ING3 may point to an aggressive nature of head and neck cancer (Gündüz et al., 2008).

Loss of heterozygosity in ING5 was found in HNSCC and oral cancer. Decreased ING5 mRNA expression was also identified in ovarian cancer and HNSCC (Cengiz et al., 2007, 2010). Our group determined that loss of the ING5 chromosome locus was related to oral cancer. In our study, a high rate of LOH in oral cancer was demonstrated. Decreased expression of ING5 mRNA was also revealed in 61% of oral squamous cell carcinomas compared to the matched normal samples. Because of tumor-specific mutation and downregulation of ING5 mRNA, it was suggested as a TSG in oral squamous cell carcinoma (Cengiz et al., 2010).

A previous study suggested that a decline in nuclear ING5 localization and cytoplasmic translocation are involved in tumorigenesis and tumor differentiation in HNSCC. Nuclear ING5 may regulate the transactivation of target genes and may accelerate apoptosis and cell cycle arrest by interacting with the p300 and p21 proteins. ING5 may function as a TSG or oncogene tightly linked with p53 genes and may have an important role in the prognosis of HNSCC patients (Li et al., 2010).

Zhang et al. showed that overexpression of ING5 suppressed cell proliferation only in the presence of INCA1, while ING5 had no effect in INCA1 (-/-) murine embryonic fibroblasts (MEFs). ING5 overexpression also triggered a delay in S-phase progression, which required the inhibitor of cyclin A1 (INCA1). Furthermore, ING5 overexpression accelerated Fas-induced apoptosis in INCA1 (+/+ ) MEFs, while Fas antibody did not induce apoptosis in INCA1 (-/-) MEFs. Taken together, these results suggest that ING5 is a growth suppressor with reduced expression in AML whose functions depend on its interaction with INCA1 (Zhang et al., 2011).

Another study showed that among 18 frozen samples of colorectal carcinoma, significantly increased expression of ING5 protein was observed in carcinoma in comparison with adjacent mucosa in 14 cases (77.8%; P < 0.05), and 71.4% (10/14) of carcinoma cases exhibited upregulation of ING5 mRNA. Nuclear ING5 expression was negatively correlated with tumor size, depth of invasion, degree of dedifferentiation, and cancer staging (P < 0.05). In contrast, cytoplasmic ING5 expression was positively correlated with depth of invasion, lymphatic invasion, and cancer staging (P < 0.05). It was suggested that aberrant ING5 expression may have a role in pathogenesis, growth, and invasion of colorectal carcinomas and could be considered as a remarkable marker to estimate aggressiveness of colorectal carcinomas (Zheng et al., 2011).

Increased expression of ING5 mRNA was demonstrated in gastric carcinoma in comparison with paired mucosa (P < 0.05). Lower expression of nuclear ING5 was detected in gastric dysplasia and carcinoma as compared to that in nonneoplastic mucosa (P < 0.05). Gastric nonneoplastic mucosa and metastatic carcinoma showed more expression of cytoplasmic ING5 than did gastric carcinoma and dysplasia (P < 0.05) (Xing et al., 2011).

Survival analysis showed that nuclear ING5 was associated with favorable prognosis of gastric carcinoma patients (P < 0.05). It was proposed that aberrant ING5 expression may play a role in pathogenesis, growth, and invasion of gastric carcinomas and could be considered as a striking marker to estimate aggressiveness and prognosis of gastric carcinoma (Xing et al., 2011).

It has also been shown that ING5 can affect sensitivity to some drugs. Mendes-Pereira et al. (2012) showed that silencing ING5 causes sensitivity to tamoxifen.

This is the first study to analyze LOH status of ING3 and ING5 and examine its possible correlation with clinicopathological factors. In the current study, we analyzed the LOH status of the ING3 and ING5 genes; afterwards, we compared it with the clinicopathological characteristics of breast cancer patients. No significant relationship was detected between LOH statuses of the ING3 and ING5 genes and clinical markers including age, smoking and alcohol consumption, absence or presence of lymph-node metastasis, nipple invasion, and application of chemotherapy or radiotherapy. We did not find a relationship between LOH statuses of ING3 and ING5 genes and OS or DFS; other genetic and nongenetic factors could be considered to contribute to the factors related to OS and DFS, such as local recurrences, metastasis, and aggressive phenotype.

In conclusion, our current study has confirmed that patients with LOH in both ING3 and ING5 have shorter OS and DFS, but this is not significant statistically. There is also no significant relationship between the LOH
status of ING3 and ING5 genes and any clinical features. Comparative analysis of other genes, including various ING family members, in subsequent studies would give us more valuable and clinically applicable results.

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References


