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Original Article

Alteration of *GLIS3* gene expression pattern in patients with breast cancer

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Abstract

Background: The GLIS family members are zinc fingers with transcriptional repression and activation function. *GLIS3* is one of these family members, which aberrant expression of it revealed to be related to several different cancer types. Regarding to the role of *GLIS3* in tumor genesis and its probable connection with β-catenin signaling pathway, one of the pathways that involves in both normal development and tumor genesis of breast tissue, the aim of this study is investigating the alteration of *GLIS3* mRNA expression level in breast cancer.

Materials and Methods: Real-time polymerase chain reaction performed with *GLIS3* and *GAPDH* genes primer on the RNA which extracted from 15 fresh frozen breast tumor tissue samples and also 15 normal samples with slight distance from site of tumor.

Results: The relative expression of *GLIS3* in breast cancer tissues revealed a 4 times increase comparing normal breast tissues; with a significant difference between cancer and normal samples ($P = 0.027$) and in patients without lymph node involvement and tissues that had estrogen receptor (ER^-) and progesterone receptor (PR^-) statuses. We see no significant difference between cancer and normal tissues based on lobular or ductal origin of the tumor as well as the tumor grade.

Conclusions: Our study suggested a probable relationship between *GLIS3* overexpression and breast cancer. Furthermore, detection of a probable association between *GLIS3* overexpression and triple-negative breast cancer (ER^-/PR^-/human epidermal growth factor receptor 2^-) might be useful for prognostic and diagnostic uses or as a probable target for treatment of these patients.

Key Words: Breast cancer, gene expression, *GLIS3*

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INTRODUCTION

Breast cancer is the most invasive form of cancer and the second leading cause of death in women in developed countries.[1,2] This cancer also causes the most cancer deaths in each year (besides lung, stomach, liver and colon cancer)[3] and it is expected that the number of affected people will have a global rise within the next two decades.[3,4] Cancer is a multifactorial disease that genetic, environmental, medical, and lifestyle factors interact in producing it.[5] Although, the precise pathogenesis of the disease remains poorly understood,[2] the genetic factors including: Loss or aberrant function of

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DNA repair genes, tumor suppressor genes[6] and signaling molecules shown to have an important role in breast cancer.[7,8] The Wnt/β-catenin signaling pathway is one of these pathways that involves in cell proliferation, migration and differentiation regulation[1] and also has shown to play key roles in both normal development and tumor genesis of breast tissue.[1,9,10] Aberrant activation of these pathways has shown in many of tumors including breast cancer.[10] β-catenin has a critical role in this signaling pathway.[11] Wnt proteins are extracellular signals that activate the Wnt/β-catenin signaling pathway by connecting with the cell surface receptors such as FZD and the LRP5/6. Subsequent activation of β-catenin in the cell cytoplasm and its nucleus entrance to induce the expression of downstream target genes with cell cycle and growth regulation actions are the next steps of Wnt/β-catenin signaling pathway.[1]

The GLIS family members are zinc fingers and can have transcriptional repression and activation functions.[11,12] Characteristic feature of GLIS family is the presence of two or more conserved C_2H_2 zinc finger domains. Members of this family involve in a broad range of cellular activities including proliferation, differentiation and development.[11] *GLIS3* is one of these family members which aberrant expression of it, revealed to be related to some pathological statuses such as osteopenia and rib fracture,[11,13] hypothyroidism (in %85 of cases witch result of abnormal development of thyroid gland),[11,14] pancreatic insufficiency and diabetes,[11,15,16] degenerative liver disease, cystic renal dysplasia,[11,13] polycystic kidneys, facial dysmorphism and bilateral sensorineural deafness.[11] Increased *GLIS3* expression also has been detected in several different cancer types such as ependymomas[11,17] and chromophobe renal cell carcinoma.[11,18] *GLIS3* gene amplification has been observed in proneural glioblastomas.[11,19]

Recently was reported that *GLIS3* interacts with the tumor suppressor and negative regulator of hedgehog (Hh) signaling pathway (SUFU).[11,20] SUFU also interacts with β-catenin in Wnt signaling pathway.[20] The interaction of *GLIS3* with SUFU makes the possibility of connection between the *GLIS3*, Hh, and Wnt pathways.[11]

Regarding to the role of *GLIS3* in tumor genesis and its probable connection with β-catenin; the aim of this study is investigating the alteration of *GLIS3* mRNA expression level in breast cancer for the 1st time.

MATERIALS AND METHODS

Patients and tissue samples

Totally 15 tissue samples provided by national tumor bank of Iran had been obtained from women who are getting surgery because of breast cancer. Furthermore, 15 normal breast tissues were taken from the same patients that had partial or total mastectomy, with a slight distance from the site of tumor. Sampling performed based on convenient sampling method. Tissue samples were stored at – 80°C, immediately after collection. Histopathology examination performed on tissue samples to confirm their cancer diagnosis and grade of disease; the statuses of human epidermal growth factor receptor 2 (HER2) and estrogen receptor/progesterone receptor (ER^-/PR^-) also detected using immunohistochemistry method. For sample collection, consent was obtained from patients before surgery. None of these patients had treatment with radiation or chemotherapy before surgery.

Total RNA extraction

RNA extracted from 10 mg frozen tissue using high pure RNA tissue kit (Roche) following manufacturer's instructions. The quality of extracted RNAs checked by nano-spectrophotometer (German precision NANOLYTIK®) based on absorbance density in 260 nm/280 nm. Extracted RNAs were stored at –80°C before use.

cDNA synthesis

cDNA synthesis performed on 11 μg of extracted RNA, using F RevertAid™ First Strand cDNA Synthesis Kit (Fermentas) based on manufacturer's instructions. Oligo-dT primer was used to amplify all RNAs with the polyadenylate tail. A template free reaction used as negative control. Real-time polymerase chain reaction (PCR) performed on prepared cDNA using *GAPDH* primers for the analysis of quality and quantity of cDNA.

Real-time polymerase chain reaction

Real-time PCR performed with *GLIS3* and *GAPDH* genes specific forward and reverse primers. The *GLIS3* specific primers were: 5'-ACGTTTGAAGGTTGCGAGAAG-3' (forward) and 5'-AGGTTTGGTGTCCAGATGCG-3' (reverse) that detected both transcript variants of *GLIS3* gene. The reaction efficiency was 100.22% for *GAPDH* primer and 99.5% for *GLIS3* primer. The reaction performed on synthetized cDNA as template. SYBR Green master mix (thermo science; #RO581) used for quantitative investigation of reactions. Real-time PCR performed in a StepOnePlus™ v2.2 Real-time PCR system based on manufacturer's instructions and in the following conditions: 94°C for 2 min, 35 cycles of 94°C for

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30 sec, 55°C for 30 sec, 72°C for 30 sec. The expression of *GLIS3* studied in comparison with *GAPDH* as housekeeping the gene;[17,18] and also in tumor samples in comparison with normal samples.

Statistical analysis

Statistical analysis performed using the REST 2009 (Qiagen) software based on software instruction for efficiency and *P* value calculation.[21] In this study, $P < 0.05$ has been considered statistically significant.

RESULTS

Alteration of *GLIS3* mRNA expression level in breast tumor

We analyzed *GLIS3* mRNA expression level in 15 breast cancer tissues and 15 normal breast tissues. All of the patients were women between 33 and 74 years old with a mean of 48.73 years. Two of patients had family history of an unrelated cancer (one with gastric cancer in her father and sister, the other with renal cancer in her father) and none of them had history of breast cancer in her family. The patients hadn't any treatment for their cancer disease before surgery. Cancer tissues classified in two subtypes based on their lobular or ductal origin. The tissues also had been analyzed by immunohistochemistry method for the detection of ER, PR and HER2 statuses. In this study, all of our tissue samples were negative for HER2 status.

The quantitative analysis of *GLIS3* performed in comparison to *GAPDH* mRNA expression level as a control endogenous gene. All of the *P* values in this study calculated with comparison of normal and tumor samples in each of groups (e.g. group of ER/PR− samples). Besides, the differences between *GLIS3* expression and *GAPDH* expression are separately calculated in normal and tumor sample for each patient. The *P* values then obtained from ΔΔCTs results.

Based on these calculations, the relative expression of *GLIS3* in breast cancer tissues was 4 times higher than normal breast tissues [Figure 1] and there were a significant difference between cancer and normal samples ($P = 0.027$); although, no significant difference between cancer tissues obtained based on their lobular or ductal origin as well as their tumor grade (all of the ductal carcinomas was invasive ductal carcinoma type).

In turn, there was a significant difference between cancer and normal tissues in patients without lymph node involvement and tissues that were ER and PR negative. Table 1 summarized clinical data of patients based on the origin of the tumor, lymph node involvement, grade of tumor and ER/PR and HER2 statues of tumor.

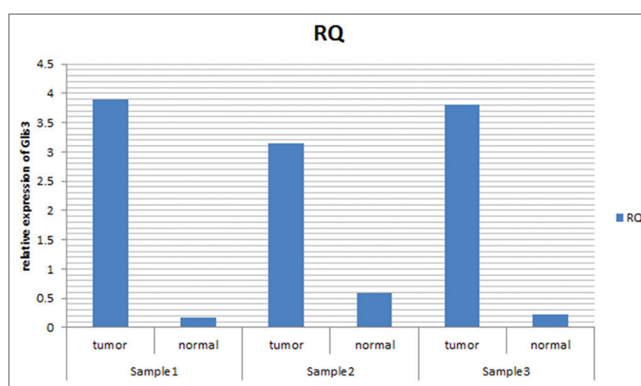

Figure 1: The relative expression of *GLIS3* gene in tumor and normal breast tissues (ductal tissue samples). The figure shows the ΔCT in 3 of our samples (both of tumor and normal samples obtained from one patient indicated as paired charts). The RQ means relative quantity of *GLIS3* gene expression in comparison to its control gene (*GAPDH*) in each sample that indicates the increased level of *GLIS3* gene expression in tumor samples in comparison to normal samples

Table 1: Classification of samples based on their tumor property

Tissue feature	Number of samples	P
Origin		
Ductal	12	0.107
Lobular	3	0.079
Grade		
1	8	0.072
2	6	0.261
4	1	-
Stage		
II (A and B)	12	0.167
III (A and B)	3	0.020
HER2*		
Negative	15	0.027
LN*		
Negative	9	0.009
Positive	6	0.537
ER/PR*		
Negative	10	0.023
Positive	5	0.372
Total number of samples=15		

*HER2: Human epidermal growth factor receptor 2, ER/PR: Hormone receptors (estrogen/progesterone), LN: Lymph node involvement status

DISCUSSION

Although the mechanisms of pathogenesis in breast cancer haven't been understood clearly,[2] the key role of signaling pathways in tumor genesis has been recognized greatly.[7,22,23] One of these important signaling pathways, especially in breast cancer, is The Wnt/β-catenin signaling pathway.[1,3] Previous studies indicated that this essential pathway for breast cancer development has a probable relationship with *GLIS3*

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a transcription regulator zinc finger protein.[11] The exact position of *GLIS3* protein in signaling network of cells isn't clear;[11] but, obvious role of aberrant activation of Wnt/β-catenin signaling pathway in breast cancer development[1,24] and its less known relationship with a zinc finger protein like *GLIS3* with activator and repressor activity,[11] could be effective in a new recognition from both pathogenesis of cancer and *GLIS3* function in it, and also from the different roles of Wnt/β-catenin signaling pathway in tumor genesis itself.

GLIS3 is a regulator of transcription, zinc finger protein. The best known downstream factors that related to *GLIS3* are genes associated with different forms of diabetes[11] including (insulin) gene.[11,25] In addition, *GLIS3* is related to other proteins with other functions, including: SUFU (suppressor of fused, a tumor suppressor gene and negative regulator of Hh signaling pathway) and (transcriptional coactivator, related to polycystic disease).[11] It seems that *GLIS3* as a zinc finger protein can regulate transcription by interacting with GLIS-binding sites in the promoter of its target genes.[11]

Using the real-time PCR technique, we studied *GLIS3* gene expression in 15 normal and 15 breast cancer tissue samples in a case-control study. This study indicated a statistically significant difference in *GLIS3* gene expression between cancer and normal samples ($P = 0.027$) and we observed a 4 times higher expression of *GLIS3* gene in cancer samples in comparison to normal ones. This significant difference has shown also in tumor samples with HER2, PR and ER-negative statuses and patients without lymph node involvement.

Breast cancers can be classified in up to 21 distinct subtype based on cell morphology, growth, and architecture patterns.[26] Triple negative breast cancer (ER, PR and HER2 with negative results) is one subtype of breast cancer with the characteristic, e.g., difficulty in treatment because of lack of a distinct target for treatment.[24] Studies have shown that Wnt/β-catenin signaling pathway activation is in association with these subtype of breast cancer and, as a result, is related to the poor clinical outcome observation.[1,24] Our observation of a statistically significant difference between case and control group in *GLIS3* gene expression level in samples with negative statuses of ER, PR and HER2 might be related to triple negative subtype of breast cancer (TNBC) due to activation of Wnt/β-catenin signaling pathway. This result could be effective in finding a new target for facilitating treatment of TNBC. Although, it needs to more studies to be entirely validated.

Based on the TNM staging system, we also classified our samples to the 2 subgroup of stages as stage II or III. In this case, results of this study indicated a significant difference between *GLIS3* expression in normal and tumor samples with higher stage of tumor (stage III-samples with T3 or T4 for primary tumor size, N0, N1 or N2 for regional lymph nodes and M0 for any metastases).[27] It can indicate a probable relationship between higher extension of tumor in advanced stages and *GLIS3* gene overexpression. Having such an observation could be a result of *GLIS3* gene role in the progression of breast cancer as a member of Wnt/β-catenin signaling pathway which should be confirmed by other researches.

In this study, we couldn't show any significant difference in *GLIS3* gene expression between samples with different origins (lobular or ductal) and grades. This might be indicated that *GLIS3* gene isn't related to differentiation of breast tissue, despite of Wnt/β-catenin signaling pathways role in this process.[24]

Previously, *GLIS3* overexpression also had been shown to be related to some other types of cancer such as ependymomas[11,17] and chromophobe renal cell carcinoma.[11,18] These two types of cancer have ectodermal embryonic origin. Our study also suggested a probable relationship between *GLIS3* overexpression and breast cancer; another cancer with ectodermal embryonic origin. Regarding these results, the next studies can show if the effect of *GLIS3* expression enhancement is limited to cancers with this embryonic origin or not.

Results of previous studies and our study suggest a relationship between overexpression of *GLIS3* and conversion of a normal tissue to a cancer tissue. This might be useful in declaring the molecular pathogenesis of breast cancer and improving the treatment of the patient affected by breast cancer. Furthermore, detection of a probable association between *GLIS3* overexpression and TNBC might be useful for prognostic and diagnostic uses or as a target for treatment of these patients.

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Nil.

Conflicts of interest
There are no conflicts of interest.

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