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The Relationship between *KIT* Copy Number Variation, Protein Expression, and Angiogenesis in Sporadic Breast Cancer

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Abstract

Background: *KIT* is a protooncogene that encodes for the KIT oncoprotein, which is a transmembrane tyrosine kinase growth factor receptor that holds a critical role in a variety of normal physiological and pathological processes including angiogenesis. *KIT* has been shown to be involved in tumorigenesis, contributing to the development of gastrointestinal carcinoma and leukemia. A link between KIT overexpression and breast cancer development has previously been reported. In the current study, we explored *KIT* gene expression and exonic copy number variants (CNV) and the relationship with angiogenesis (CD34) and the clinicopathological features of breast cancer.

Methods: MLPA technique was used to determine the CNV in 64 breast cancer tumor samples from patients diagnosed with primary sporadic breast cancer. Results were confirmed by quantitative PCR. Expression of KIT and CD34 was determined using immunohistochemistry (IHC).

Results: Our results show that 28.1% of the tumor samples from patients with primary sporadic breast cancer had CNV in the *KIT* gene. Among the breast tumor samples, 54.7% showed positive *KIT* expression. The expression of the CD34 angiogenesis marker was reported in 43.8% of the tumor samples as low, 42.2% as moderate and 14.1% as high. A significant correlation between increased CNV of *KIT* exons, a high level of angiogenesis (CD34) and increased tumor grade was observed ($p< 0.05$).

Conclusions: A significant correlation between the *KIT* CNV and the angiogenesis marker was found. Examining *KIT* expression and CNV has the potential to function as a biomarker for tyrosine kinase inhibitor drugs in breast cancer.

Keywords: Angiogenesis, Breast Cancer, CD34, *KIT*.

Introduction

Breast cancer is the most commonly diagnosed malignancy in women worldwide, after non-melanoma skin cancer and is one of the leading causes of mortality. Of the diagnosed cases of breast cancer, 80%-85% are considered sporadic with no prior family history or hereditary cause (1-3). Angiogenesis, the formation of new blood vessels, is understood to hold an essential role in breast cancer progression and dissemination by promoting both local tumor growth and metastasis. In its absence, tumor growth and metastasis are restricted significantly limiting tumor growth to roughly 2 mm (4, 5). *KIT* (CD117) is a protooncogene that encodes for a type-III tyrosine kinase receptor involved in signal transduction in a variety of cell types. Normally, the KIT protein is activated upon binding to its ligand, the stem cell factor (SCF). This

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phosphorylation cascade leads to the activation of various transcription factors that regulate apoptosis, cell differentiation, proliferation and angiogenesis (6, 7).

CD34 is known as an angiogenesis marker, which can influence *KIT* gene expression (8). Of the final tyrosine kinase receptor, exons 1-9 of *KIT* gene encode for the extracellular domain, exon 10 encodes for a transmembrane domain, exon 11 encodes a juxtamembrane domain (JM), and exons 13-21 encode for the split tyrosine kinase domain (9).

Abnormalities in the *KIT* gene such as point mutations, deletions, and insertions and CNV leading to *KIT* overexpression have been shown to contribute to the development of various cancers including gastrointestinal carcinoma, leukemia, and melanoma (10-15). Previous research has demonstrated that the overexpression of the *KIT* gene holds a critical role in the development of different breast tumor subgroups such as phyllodes tumors and triple negative breast tumors. However, in most cases mutations were not found to cause *KIT* overexpression (16-20). In malignant breast tumors, it has been shown that point mutations in *KIT* are rarely present (16). Additionally, a study reported that point mutations occur with low frequency in triple negative breast cancer (21). In a separate study, *KIT* gene CNVs of exon 15 and 18 was observed to be common in patients with phyllodes tumors and associated with *KIT* overexpression (22).

In inhibiting genes involved in angiogenesis, such as *KIT* gene, the development, progression, and migration of malignant tumors such as breast cancer can be suppressed. Therefore, this gene may serve as a potential target for cancer therapy (23, 24). The purpose of this study was to determine the relationship between KIT expression, CNV and CD34. Furthermore, we explored the connection between *KIT* gene expression and the clinicopathological features of sporadic breast cancer in Iranian women.

Materials and methods

Ethical approval

All procedures performed in the study were in accordance with the ethical standards of the institutional research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards. This study has received approval from the ethics committee of "University of Social Welfare and Rehabilitation Sciences".

Informed consent was obtained from all individual participants included in the study.

Patients and breast tissue samples

We recruited 64 female patients diagnosed with primary sporadic breast cancer with no prior history of treatment, there were no restrictions on age or histopathological subtype. The referral hospital was the Mehrad Hospital in Tehran. Participants were provided with a written consent form explaining the procedures in the study. The consent forms were required to be signed prior to participation. Tumor samples were obtained from macroscopically visible tumor regions and confirmed by H&E stained slides. DNA from the breast tumor samples was extracted, and the quality and integrity of the DNA was evaluated by agarose gel electrophoresis. The concentration of high-quality extracted DNA was standardized to a final volume of 125 ng per reaction using a Nano Drop ND 2000 spectrophotometer.

Multiplex Ligation-Dependent Probe Amplification (MLPA)

We analyzed the presence of deletion and duplication mutations of *KIT* gene exons using the P354-A2 MLPA kit. The *KIT* gene contains 21 exons, spans ~83 kb of genomic DNA and is located on 4q12, 55 Mb from the p-telomere. The P354-A2 probemix contains two probes for exon 1 of the gene and one probe for each of the other exons. In addition, nine reference probes are included in this probemix, detecting several different autosomal chromosome locations. DNA extracted from the tumor and normal tissue samples, women without breast cancer, was preheated to 98 °C, then salt solution and probe mix was added to the DNA. After the ligation of annealed nucleotides, PCR was performed to amplify target genes. PCR products were separated on an ABI3730-XL capillary sequencer (Applied Biosystems, Foster City, CA, USA). *KIT* gene copy number was determined using Coffalyser (ver.

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140721.1958). Cut-off values between 0.7 and 1.3 were considered normal. Results below 0.7 or above 1.3 were interpreted as deletion or duplication mutations of exons, respectively.

Quantitative PCR

Quantitative PCR (qPCR) was used to confirm the results of the MLPA. The qPCR mixes were prepared according to the Takara SYBR Master Mix instructions (Shiga, Japan) and were carried out using an ABI7500 PCR machine (Applied Biosystems, Foster City, CA). Data was analyzed by the $2^{-\Delta\Delta Ct}$ method, using SYBR green qPCR. The relative copy number of exons and genes was estimated by comparison with normal breast tissue control samples. We used RPPH1 housekeeping gene in normal breast tissue. Primer sequences for q-PCR are shown in Table 1.

Table 1. Primer sequences for q-PCR confirmations.

Exon 1	Forward	GGATCCCATCGCAGCTACC
	Reverse	GAGTAGTCGCACGGTCGG
Exon 2	Forward	GACTTTTGAGATCCTGGATGAAA
	Reverse	GCCAAGCATTTACCTCTAACAA
Exon 19	Forward	GCTTTGCAAACTGTGTCTCA
	Reverse	TGGCTCTTACATTTCAGCAGG
Exon 21	Forward	GCTGTATTGACTATGGGCTTGT
	Reverse	TGACCCAAACACTGATTCTGC
RPPH1	Forward	GAGGTGAGTTCCCAGAGAACG
	Reverse	TTCGCTGGCCGTGAGTCTGTTC

Immunohistochemistry

The expression of *KIT* (CD117) at the tissue level was determined using Immunohistochemistry (IHC). Sections of paraffin blocks with 4 μm thickness were used. IHC was performed using the CD117 antibody from DAKO (10103820, Glostrup, Denmark). IHC-Paraffin (ICH-P) refers to the staining of tissues that have been fixed (usually in neutral buffered formalin) and then embedded in paraffin before being sectioned. The basic steps of the IHC-P protocol are as follows: 4 μm-thick mounted sections on slides from buffer (25) formaldehyde-fixed paraffin-embedded tissue blocks were deparaffinized and rehydrated in graded alcohols. The slides were then incubated with pre-diluted anti-CD117 antibody. Follow the incubation period, the sections were washed in PBS and incubated with horseradish peroxidase conjugated secondary antibody. Color development was performed using 3, 3'-diaminobenzidine, and the tissue samples were counterstained with hematoxylin. Positive (positive gastrointestinal stromal tumor (GIST) slides) and negative external controls (normal skin tissue slides) were used to validate the test results. Samples were interpreted according to the ASCO/CAP guidelines: negative (0, 1+); weakly positive (2+); and strongly positive (3+) (Fig. 1). The slides were examined by a pathologist.

Statistical analysis

For data analysis, we used the SPSS 19.0 statistical package. Fisher's exact tests were used to analyze the association between CNV of *KIT* exons and gene expression, *KIT* expression and clinicopathological features, *KIT* expression and CD34 expression. A *p*-value of <0.05 was considered statistically significant.

Results

Clinicopathology

Clinicopathological data of the 64 patients included in this study is listed in Table 2.

MLPA and qPCR

MLPA analysis using SALSA P354-A2 kits was carried out successfully in all 64 breast tumor samples. Deletion and duplication mutations were present in 18 of the 64 tumor samples, examining all *KIT* gene exons (Table 3). In the remainder of the patient tumor samples, the CNV were not detected. Deletion and duplication mutations for 1, 2, 19, 21 exons were confirmed by qPCR.

Immunohistochemistry

Protein expression of the KIT receptor in the tumor tissue samples was determined using IHC staining. Our findings show 45.3% of breast

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tumor samples to have no *KIT* expression, 34.4% of tumor samples with low expression, 17.2% with moderate expression and 3.1% with high expression. IHC staining is shown in Figure 1 and the results are shown in Table 3. The scoring method for protein expression is as follows: score 1, expression of at least 10% of cells with mild intensity; score 2, expression of at least 10% of cells with moderate intensity; score 3, expression of at least 10% of cells with strong intensity. All of the normal breast tissue samples were found to be positive for the KIT protein.

Table 2. Clinicopathological data

Characteristic		N (%)
Stage	I	4 (6.3)
	II	47 (73.4)
	III	8 (12.5)
CD34	low	28 (43.8)
	moderate	27 (42.2)
	high	9 (14.1)
Histologic grade	I	10 (16.1)
	II	46 (74.2)
	III	6 (9.7)
ER status	Positive	43 (67.2)
	Negative	19 (29.7)
PR status	Positive	38 (59.4)
	Negative	24 (37.5)
HER2 status	Positive	29 (45.3)
	Negative	33 (51.6)
Tumor size	<2 cm	5 (7.8)
	2-5cm	54 (84.4)
Age	<40 years	8 (12.5)
	≥40 years	52 (81.3)
Histological Type	[a]IDCA	57 (89.1)
	[b]ILCA	4 (6.3)
Node status	Positive	29 (45.3)
	Negative	30 (46.9)
Ki67	Positive	10 (15.6)
	Negative	52 (81.3)
Annexin V	Positive	10 (20.8)
	Negative	38 (79.2)
Subtype	luminal A	36 (56.3)
	luminal B	11 (17.2)
	Basal Like	13 (20.3)
	ERBB2	2 (3.1)

[a]IDCA = Invasive Ductal Carcinoma.
[b]ILCA= Invasive Lobular Carcinoma.
* Clinicopathological data of a number of patients not available.

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Table 3. The Mutation frequency in each of the *KIT* Exons

CNV		Exons
Yes N (%)	No N (%)	
15 (23.4)	49 (76.6)	1
7 (10.9)	57 (89.1)	2
4 (6.2)	60 (93.8)	3
4 (6.2)	60 (93.8)	4
6 (9.4)	58 (90.6)	5
4 (6.2)	60 (93.8)	6
4 (6.2)	60 (93.8)	7
3 (4.7)	61 (95.3)	8
6 (9.4)	58 (90.6)	9
7 (10.9)	57 (89.1)	10
7 (10.9)	57 (89.1)	11
7 (10.9)	57 (89.1)	12
5 (7.8)	59 (92.2)	13
2 (3.1)	62 (96.9)	14
5 (7.8)	59 (92.2)	15
3 (4.7)	61 (95.3)	16
5 (7.8)	59 (92.2)	17
6 (9.4)	58 (90.6)	18
11 (17.2)	53 (82.8)	19
7 (10.9)	57 (89.1)	20
9 (14.1)	55 (85.9)	21

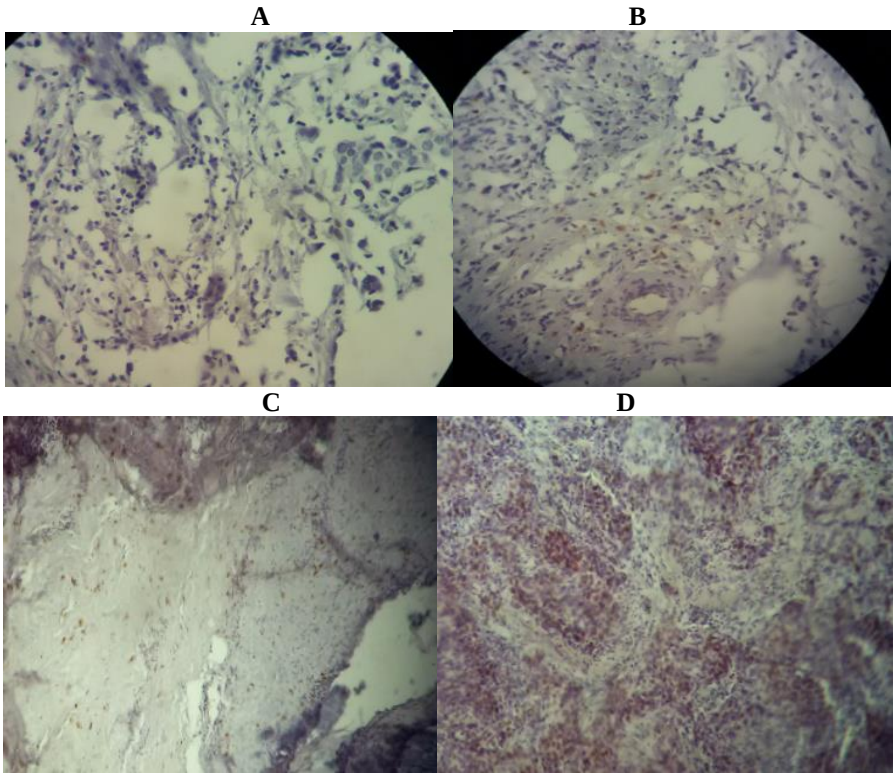

Fig. 1. *KIT* expression in the breast cancer.
A) Breast cancer tissue without *KIT* expression.
B) Breast cancer tissue with low level of *KIT* expression.
C) Breast cancer tissue with moderate level of *KIT* expression.
D) Breast cancer tissue with high level of *KIT* expression.

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Statistical analysis

Table 4 shows the results of the comparisons between copies of *KIT* exons, its expression level, CD34 expression and histologic grade. Our results indicate a significant correlation between increased copy number in exon 1, increase of CD34 expression and histological grade ($p< 0.05$). In addition, we found a significant correlation between increased copy number in exons 19 and 21 and histological grade ($p< 0.05$). The remaining exons did not show a significant relationship with the histological grade, CD34, or *KIT* expression ($p> 0.05$). Correlation between *KIT* gene copy number, expression level and clinicopathological features are presented in Table 5. Our findings indicate that *KIT* gene CNVs are significantly correlated with increased CD34 expression and high histological grade ($p< 0.05$).

Table 4: The relationship between the CNV in *KIT* gene exons, expression level, angiogenesis (CD34) and histologic grade. A value of $p< 0.05$ was considered statistically significant.

	Histologic Grade N(%)				**CD34 expression N(%)**				**Expression level of *KIT* N(%)**				**Copy Number Variation/ Exons**	
p value	3	2	1	*P*-value	>40	20-40	<20	*P*-value	3	2	1	0		
0.02	2 (4.2)	37 (77.1)	9 (18.8)	0.04	9 (18.4)	17 (34.7)	23 (46.9)	0.95	2 (4.1)	9 (18.4)	16 (32.7)	22 (44.9)	No	1
	4 (28.6)	9 (64.3)	1 (7.1)		0 (0)	10 (66.7)	5 (33.3)		0 (0)	2 (13.3)	6 (40)	7 (46.7)	Yes	
0.07	3 (5.8)	40 (76.9)	9 (17.3)	0.19	9 (17)	20 (37.7)	24 (45.3)	0.56	2 (3.8)	10 (18.9)	16 (30.2)	25 (47.2)	No	19
	3 (30)	6 (60)	1 (10)		0 (0)	7 (63.6)	4 (36.4)		0 (0)	1 (9.9)	6 (54.5)	4 (36.4)	Yes	
0.02	3 (5.7)	40 (75.5)	10 (18.9)	0.08	9 (16.4)	20 (36.4)	26 (47.3)	1.00	2 (3.6)	10 (18.2)	19 (34.5)	24 (43.6)	No	21
	3 (33.3)	6 (66.7)	0 (0)		0 0%	7 (77.8)	2 (22.2)		0 (0)	1 (11.1)	3 (33.3)	5 (55.6)	Yes	

Discussion

KIT gene regulates cell differentiation, proliferation and angiogenesis pathways and is involved in the development of a variety of human malignancies, it has been a target of inhibitory drugs used for cancer treatment (6,7). *KIT* expression has been observed to be increased in gastrointestinal cancers (90%), leukemia (68%), mast-cell tumors (70%), and melanomas (26, 27). In these cancers, mutations such as point mutations, insertions, and deletions have been observed to be present in exons 9, 11, 13, and 17 of *KIT*. Of the mutations in *KIT*, an increase in copy number was the least reported (10-15). Conversely, in breast cancer increased copy number is more commonly reported than point mutations, insertions and deletions in exons (16-20). To date, no study has simultaneously investigated the CNV in all exons of the *KIT* gene. Thus, in this study all *KIT* gene exons were investigated simultaneously by using MLPA (P354-A2 kit, MRC Holland). This kit was designed and routinely used for Peibaldism and contains probes for all exons. Therefore, it can be used to investigate CNV of exons and genes in patients with breast cancer. Our results show that 28.1% of the tumor samples from breast cancer patients had CNVs in the exons of *KIT* gene. Using this method, our study found exons 1, 2, 19, and 21 of the *KIT* gene to have the highest ratio of CNV.

Within the *KIT* gene, exons 1 and 2 encode for the extracellular domain of the tyrosine kinase receptor that interacts with the SCF ligands involved in promoting angiogenesis. Therefore, increased copy number of these exons would lead to increased affinity of the SCF ligand to the tyrosine kinase receptor. Exons 19 and 21 encode for the tyrosine kinase domain involved in phosphorylation of the KIT receptor and therefore the activation of downstream factors. We studied

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KIT expression in 64 breast tumor samples isolated from women diagnosed with primary

Table 5. Clinicopathological variables in correlation to CNV of *KIT* gene and its expression level.

Characteristic		CNV of *KIT*			Expression level of *KIT*				
		Yes N (%)	**No N (%)**	**[c]*P*-value**	**0 N (%)**	**1 N (%)**	**2, N (%)**	**3 N (%)**	***P*-value**
Expression level of *KIT*	0	9 (50)	20 (43.5)	0.87					
	1	7 (38.9)	15 (23.6)						
	2	2 (11.1)	9 (19.6)						
	3	0 (0)	2 (4.3)						
CD34 expression	<20	5 (27.8)	23 (50)	0.04	12 (41.4)	9 (40.9)	5 (45.5)	2 (100)	0.82
	20-40	12 (66.7)	15 (32.6)		12 (41.4)	11 (50)	4 (36.4)	0 (0)	
	>40	1 (5.6)	8 (17.4)		5 (17.2)	2 (9.1)	2 (18.2)	0 (0)	
Histologic grade	I	1 (5.9)	9 (20)	0.006	5 (17.2)	4 (19)	1 (10)	0 (0)	0.89
	II	11 (64.7)	35 (77.8)		21 (72.4)	14 (66.7)	9 (90)	2	
	III	5 (29.4)	1 (2.2)		3 (10.3)	3 (14.3)	0 (0)	0 (0)	
ER status	Positive	12 (70.6)	31 (68.9)	1.00	18 (62.1)	16 (76.2)	7 (70)	2 (100)	0.62
	Negative	5 (29.4)	14 (31.1)		11 (37.9)	5 (23.8)	3 (30)	0 (0)	
PR status	Positive	8 (47.1)	30 (66.7)	0.24	15 (51.7)	14 (66.7)	7 (70)	2 (100)	0.51
	Negative	9 (52.9)	15 (33.3)		14 (48.3)	7 (33.3)	3 (30)	0 (0)	
HER2 status	Positive	8 (47)	21 (46.7)	0.87	11 (41.4)	11 (57.1)	3 (30)	2 (100)	0.32
	Negative	9 (53)	24 (53.4)		17 (58.6)	9 (42.1)	7 (70)	0 (0)	
Tumor size	<2 cm	3 (18.8)	2 (4.7)	0.11	1 (3.7)	4 (20)	0 (0)	0 (0)	0.20
	2-5cm	13 (81.3)	41 (95.3)		26 (96.3)	16 (80)	10 (100)	2 (100)	
Age	<40 years	3 (17.6)	5 (11.6)	0.67	4 (14.8)	2 (9.5)	2 (20)	0 (0)	0.70
	≥40 years	14 (82.4)	38 (88.4)		23 (85.2)	19 (90.5)	8 (80)	2 (100)	
Histological Type	[a]IDCA	17 (100)	40 (90.9)	0.67	26 (92.9)	20 (95.2)	9 (90)	2 (100)	0.89
	[b]ILCA	0 (0)	4 (9.1)		2 (7.2)	1 (4.8)	1 (10)	0 (0)	
Node status	Positive	8 (50)	21 (48.8)	1.00	13 (48.1)	10 (50)	7 (70)	0 (0)	0.34
	Negative	8 (50)	22 (51.2)		14 (51.9)	10 (50)	3 (30)	2 (100)	
Ki67	Positive	15 (88.2)	37 (82.2)	0.71	23 (79.3)	19 (90.5)	8 (80)	2 (100)	0.69
	Negative	2 (11.8)	8 (17.8)		6 (20.7)	2 (9.5)	2 (20)	0 (0)	
Subtype	luminal A	8 (47.1)	28 (62.2)	0.65	13 (44.8)	15 (71.4)	6 (60)	2 (100)	0.74
	luminal B	3 (17.6)	8 (17.8)		7 (24.1)	2 (9.5)	2 (20)	0 (0)	
	Basal Like	5 (29.4)	8 (17.8)		7 (24.1)	4 (19)	2 (20)	0 (0)	
	ERBB2	1 (5.9)	1 (2.2)		2 (6.9)	0 (0)	0 (0)	0 (0)	
"Annexin V"	Positive	5 (29.4)	9 (20)	0.50	20 (69)	18 (85.7)	8 (80)	2 (100)	0.55
	Negative	12 (70.6)	36 (80)		9 (31)	3 (14.3)	2 (20)	0 (0)	
Stage	I	1 (6.3)	3 (7.0)	0.23	1 (3.7)	3 (15)	0 (0)	0 (0)	0.19
	II	11 (68.8)	36 (83.7)		23 (85.2)	13 (65)	10 (100)	1 (50)	
	III	4 (6.8)	49 (25)		3 (11.1)	4 (20)	0 (0)	1 (50)	

[a]IDCA = Invasive Ductal Carcinoma.
[b]ILCA= Invasive Lobular Carcinoma.
[c]*P*-value from Fisher's Exact Test.
*clinicopathological data of a number of patients not available.

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sporadic breast cancer. Of the samples, 54.7% were positive for *KIT* and the remainder were negative. Previous studies have shown that *KIT* expression in non-cancerous breast tissue is different from that of malignant tumors and high-grade breast cancer (18-20).

Our findings did not show a significant relationship between increased copy number and overexpression of *KIT*. However, previous research has reported an increase in gene copy number as the reason for *KIT* overexpression. In contrast with other cancers, few results have shown point mutations, insertions and deletions in breast cancer (21, 28- 30). Indeed, with the exceptions of the GISTs, no relationship between *KIT* activating mutations and *KIT* expression has been found in multiple types of tumors (31).

Similar to our findings, one study did not observe any significant correlation between increased *KIT* expression and gene copy number (20). This observation could be explained by translational, post-transcriptional and protein degradation regulation, indicating that some genes may be transcribed, however they are not displayed on the cell surface (32). Alternatively, this finding could be a result of epigenetic variation causing gene overexpression without alterations in the *KIT* gene. Therefore, it is other mechanisms may cause the increased *KIT* expression. It should be noted that the small sample size could also contribute to this discrepancy.

In the current study, we analyzed the relationship between CNV of all 21 exons in the *KIT* gene and protein expression. Our findings showed no significant correlation. Variations of exons that change the structure and function of proteins are important for administering tyrosine kinase inhibitor drugs, such as those for gastrointestinal tumors, as they are resistant to these drugs (12).

In a study by Chen et al, no correlation between CD34 expression, grade, tumor size and CD117 (*KIT*) expression was observed (33). The expression of *KIT*, CD34, and actin was investigated in 19 patients with breast phyllodes tumors. Compared with the benign tumors, malignant phyllodes tumor stromal lesions displayed an increased expression of *KIT*. Other genes may influence CD34 overexpression.

Furthermore, we examined the relationship between the *KIT* gene copy number, *KIT* expression and clinicopathological features of breast tumors. No significant correlation was found ($p > 0.05$). The small sample size may account for this lack of significance.

The current study demonstrated a significant correlation between an increase in CNV in *KIT* gene and a high level of CD34 expression as well as the high tumor grades ($p < 0.05$). Although there was no significant correlation between increased *KIT* expression and CD34 expression, *KIT* overexpression alone has the potential to be used as a biomarker for determining the effectiveness of tyrosine kinase inhibitor drugs in breast cancer therapy.

Many tyrosine kinase inhibitors exist which have been successfully administrated in various cancers such as gastrointestinal cancer and leukemia (34, 35). However, further studies with larger sample sizes are necessary to verify our findings and the effectiveness of tyrosine kinase inhibitors for breast cancer treatment.

A significant correlation between CNV in *KIT* gene exons and the CD34 angiogenesis marker within the breast tumor samples of patients with primary sporadic breast cancer. Thus, administration of the *KIT* inhibitor drugs may act to suppress breast cancer development and progression in patients with *KIT* overexpression and exonic CNV.

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