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Research Article
Deletion of the *Alu* Repeat in the Tissue Plasminogen Activator (*tPA*) Gene For Protection of Breast Cancer

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Abstract

Background and Objective: Breast cancer is the most common malignant tumor and the major cause of death from cancer among women worldwide. Objective of the current study was to investigate the possible association between an *Alu* polymorphism in the Tissue Plasminogen Activator (*tPA*) gene (*PLAT*) with breast cancer. **Methodology:** Using the Polymerase Chain Reaction (PCR) on genomic DNA isolated from breast cancer patients (n = 73) and an age-matched normal individuals (n = 44), a region polymorphic for an *Alu* element insertion in the *tPA* gene was amplified. **Results:** The percentage of normal Jordanian individuals who were homozygotes for the absence of the *Alu* insert ($Alu^{-/-}$) was 84.1%, while 15.9% were homozygotes for the presence of the *Alu* insert ($Alu^{+/+}$). No heterozygosity ($Alu^{+/-}$) was detected in this study group. On the other hand, 22 (30.14%) breast cancer patients exhibited the $Alu^{-/-}$ genotype, 29 (39.73%) were $Alu^{+/-}$ and 22 (30.14%) were $Alu^{+/+}$. The $Alu^{-/-}$ genotype occurred 2.8 times more frequently in the normal individuals than in the breast cancer patients ($p<0.001$). **Conclusion:** The predominance of the $Alu^{-/-}$ genotype of the tissue plasminogen activator (*tPA*) gene within the normal group represents a protective deletion with respect to breast cancer.

Key words: Tissue plasminogen activator, *Alu* element, metastasis, breast cancer, *Alu* insert ($Alu^{+/+}$)

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Cancer metastasis represents an advanced stage of malignancy and is the leading cause of cancer-related deaths[1]. Metastasis is a multistep process that includes migration and invasion of cancer cells[1,2]. These processes involve a wide array of cellular mechanisms led by cytoskeleton dynamics as well as molecular alterations such as expression of adhesion and proteolytic enzymes[1,3,4].

Degradation of the extracellular matrix (ECM) is a prerequisite for cancer cell invasion, which requires the action of several proteolytic enzymes such as the serine proteases of the plasminogen activator (PA) system[5]. These proteases [urokinase plasminogen activator (uPA) and Tissue Plasminogen Activator (tPA)] convert the inactive plasminogen to the active plasmin which in turn breakdown (directly or indirectly) different ECM components[6,7]. The uPA has been implicated in cell migration and tumor invasion[8-11] while tPA is best known for its activity during thrombolysis[12-14].

The role of tPA during malignancy has been less investigated. Elevated levels of intratumoral plasma tPA have been associated with a poor prognosis in colorectal[15] and pancreatic cancers[16]. However, a better prognosis in breast cancer was reported with elevated levels of circulating tPA[17-19]. This suggests a complex yet not fully understood role for the PA system in cancer.

Tissue Plasminogen Activator (tPA) is encoded by the *tPA* gene (*PLAT*) which is located on chromosome 8p12-p11.2[20]. Numerous studies found a common insertion of a 300 bp sequence (*Alu* repeat) within intron 8 of this gene[21,22]. The *Alu* polymorphism of the *tPA* gene consists of the presence (insertion, Alu^{+}) or absence (deletion, Alu^{-}) of this element. Different populations have been found to be dimorphic for its presence or absence[23,24].

It was found that the *Alu* polymorphism in intron 8 of *tPA* gene is involved in tPA plasma levels[25]. However, others did not find an association between genetic variations at the tPA locus and plasma levels of tPA indicating the involvement of other genetic factors[26]. Nevertheless, a study by Jern *et al.*[27] reported an association between *tPA* gene polymorphism and the forearm vascular release rate of tPA *in vivo.* Subjects homozygous for the insertion ($Alu^{+/+}$) had a significantly higher release than both heterozygotes ($Alu^{+/-}$) or subjects homozygous for deletion ($Alu^{-/-}$). Moreover, hyperfibrinolysis due to elevated levels of tPA has been associated with metastatic breast cancer[28].

Up to researchers' knowledge, the association between *Alu* polymorphism in intron 8 of *tPA* gene and breast cancer has not been studied before, thus the aim of the current study was to investigate a possible association between the genetic variants of the *Alu* element at the *tPA* locus with breast cancer in Jordanian patients. The findings of this study will add to the pre-existing knowledge about the association between genetic polymorphism and breast cancer. The *Alu* polymorphism of the *tPA* gene could be used as a genetic biomarker for breast cancer.

MATERIALS AND METHODS

Genomic DNA extraction: The current study was a case-control study conducted between February, 2016 and February, 2017. A total of 117 age-matched Jordanian individuals, 44 normal unrelated individuals (46.5±10.1 years) and 73 breast cancer patients (49.3±12.9 years) ($p>0.05$) at Al-Bashir governmental hospital (Amman, Jordan), were included in the current study. Three milliliters of peripheral blood were collected from all participating individuals in EDTA tubes and used to isolate genomic DNA from white blood cells using the Wizard Genomic DNA Purification Kit (Promega, USA) according to manufacturer's protocol. All individuals gave their informed consent and the study was approved by The Institute Review Board (IRB) of the hospital (AM/16/13/10/1503854) which conforms to the World Medical Association Declaration of Helsinki.

***tPA* genotyping:** Genomic DNA from normal and breast cancer patients was amplified by Polymerase Chain Reaction (PCR) in 30 µL volume which included: 1 µL of each forward GTAACCATTTAGTCCTCAGCTGTTCTCCT and reverse CCATGTAAGAGTAGAAGGAGACTCAGTCA primers[29], 8 µL of nuclease free water, 15 µL of the master mix (New England Biolabs, USA) and 5 µL of DNA sample. The PCR reaction was carried out as follows: 2 min at 96°C, followed by 35 cycles of denaturation (96°C for 30 sec), annealing at 65°C for 30 sec and synthesis at 65°C for 30 sec[29] in a thermal cycler (MyCycler, Bio-Rad, USA). The amplified products were electrophoresed on 2% (w/v) agarose gel with ethidium bromide. Homozygote individuals carrying the *tPA Alu* inserts are designated $Alu^{+/+}$, heterozygotes as $Alu^{+/-}$ and homozygotes for the absence of the insert as $Alu^{-/-}$.

Statistical analysis: Both genotypes and frequencies for insert or deletion of the recruited individuals were calculated according to the counting method. The observed genotypes and alleles frequencies were compared with those expected in order to verify the Hardy-Weinberg equilibrium. The Chi-square test and Fisher's exact test were performed for the polymorphism frequency using Statistica software, StatSoft Inc, Tulsa, OK, USA (version 10). A value of $p<0.05$ was considered statistically significant.

RESULTS

The *Alu* element and flanking sequences of the *tPA* gene were successfully amplified from genomic DNA extracted from peripheral blood of all individuals involved in the current study (Fig. 1).

The allelic and genotypic frequencies of the *Alu* insertion and deletion of the *tPA* gene were determined as described in Table 1. Table 1 shows that 84.1% of the normal Jordanian individuals studied were homozygotes for the absence of the *Alu* insert ($Alu^{-/-}$), while 15.9% were homozygotes for the presence of the *Alu* insert ($Alu^{+/+}$). No heterozygosity was detected in this study group. The allelic frequency of the Alu^- and Alu^+ in this group of individuals were determined at 0.84 and 0.16, respectively. On the other hand, 22 (30.14%) breast cancer patients exhibited the $Alu^{-/-}$ genotype, 29 (39.73%) were $Alu^{+/-}$ and 22 (30.14%) were $Alu^{+/+}$. Frequency of both Alu^- and Alu^+ in the breast cancer patients was calculated at 0.50 each. All genotypes and allelic frequencies for both study groups are in accordance with the Hardy-Weinberg equilibrium.

Statistical analysis of genotypic percentages of both $Alu^{-/-}$ and $Alu^{+/-}$ between the normal individuals and the breast cancer patients (Table 2) showed a significant difference ($p<0.05$). No significant difference was obtained when comparing the genotype percentage of homozygotes

Table 1: *Alu* genotypes and allelic distributions of Tissue plasminogen Activator (*tPA*) gene in normal and breast cancer Jordanian individuals

Genotype	Normal (n = 44)	Genotype (%)	Allele frequency	Breast cancer (n = 73)	Genotype (%)	Allele frequency
$Alu^{-/-}$	37	84.1	Alu^-: 0.84	22	30.14	Alu^-: 0.50
$Alu^{+/-}$	0	0.0	Alu^+: 0.16	29	39.73	Alu^+: 0.50
$Alu^{+/+}$	7	15.9		22	30.14	

Table 2: Statistical analysis of *Alu* genotypes and allelic distributions of Tissue Plasminogen Activator (*tPA*) gene in normal and breast cancer Jordanian individuals

Genotype (%)	Normal	Breast cancer	Odds ratio	Allele	Normal	Breast cancer
$Alu^{-/-}$	84.1	30.14 (0.0001)*	Vs ($Alu^{+/+}$+$Alu^{+/-}$) 12.25 (0.0001)* 95% CI: 4.74-31.68	Alu^-	0.84	0.50 (0.0002)*
$Alu^{+/-}$	0.0	39.73 (0.0001)*	Vs ($Alu^{-/-}$+$Alu^{+/+}$) 0.02 (0.0047)* 95% CI: 0.001-0.29	Alu^+	0.16	0.50 (0.0002)*
$Alu^{+/+}$	15.9	30.14 (0.0842)*	Vs ($Alu^{-/-}$+$Alu^{+/-}$) 0.44 (0.089)* 95% CI: 0.17-1.13			

*All statistical analysis was performed at $p<0.05$ level of significance

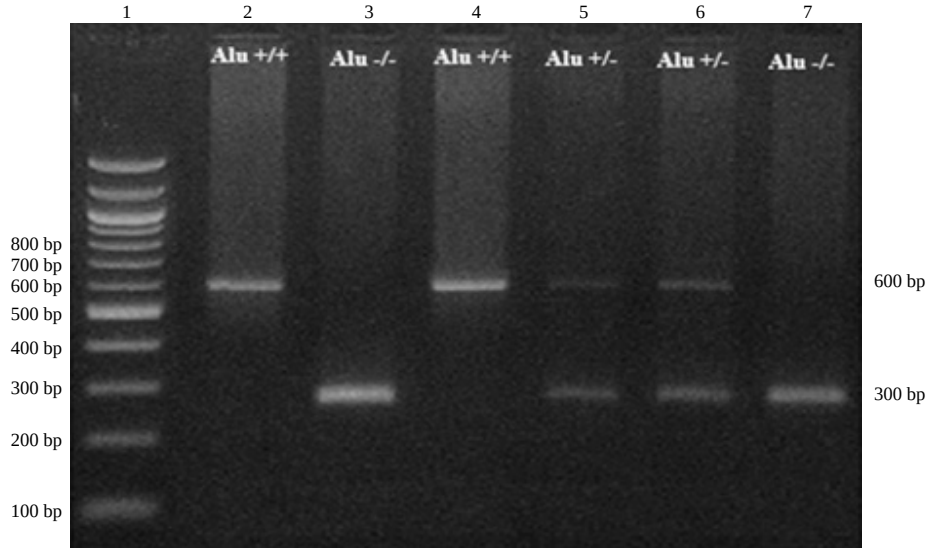

Fig. 1: Representative amplified *Alu* segment of the *tPA* gene showing insertion ($Alu^{+/+}$) and/or deletion ($Alu^{-/-}$) of the segment. Lane 1:100 bp Ladder, Lanes 2 and 4: *Alu* insertion ($Alu^{+/+}$) at 600 bp, Lanes 3 and 7: *Alu* deletion ($Alu^{-/-}$) at 300 bp, Lanes 5 and 6: *Alu* insertion and deletion ($Alu^{+/-}$) at 300 bp and 600 bp

carrying the insert (*Alu*$^{+/+}$) in both of the study groups (p>0.05). Furthermore, the results presented in Table 2 show that the allelic frequencies of both the insert allele (*Alu*$^{+}$) and deletion allele (*Alu*$^{-}$) between normal and breast cancer individuals differ significantly (p<0.05). Compared to the *Alu*$^{+}$ allele, the *Alu*$^{-}$ allele was the dominant allele in the normal individuals, while both alleles were equally distributed among the breast cancer individuals.

Assuming the recessive model (Table 2), the results demonstrate a significant protective effect of *tPA Alu*$^{-/-}$ genotype against breast cancer. The *Alu*$^{-/-}$ genotype was 2.8 times more frequent in the control population than in the breast cancer patients (odds ratio of 12.25, p<0.05).

DISCUSSION

In the current study, statistical analysis of *tPA* genotypic percentages of both *Alu*$^{-/-}$ and *Alu*$^{+/-}$ between the normal individuals and the breast cancer patients (Table 2), has shown a significant difference (p<0.05) with the *Alu*$^{-}$ allele being the dominant allele in the normal individuals. The predominance of the *Alu*$^{-/-}$ genotype in the normal group represents a protective deletion with respect to breast cancer.

Alu repeats are short, approximately 300 bp, interspersed DNA elements (SINEs) that are ubiquitously distributed in the human genome[20,30]. They multiply by retroposition, a process by which mobile elements replicate via an RNA intermediate[31,32]. These elements have been proposed to have a number of functions in the human genome such as genomic duplications, genomic conversion as well as genomic deletions. These genomic changes could affect gene expression and lead to abnormal proteins resulting in genetic diseases[11,33].

The plasminogen activator system is composed of plasminogen, plasmin, tissue-plasminogen activator (tPA), urokinase-plasminogen activator (uPA) and inhibitors [plasminogen activator inhibitor-1 (PAI-1) and PAI-2][7]. The role of urokinase plasminogen activator (uPA) during cancer metastasis has long been established[8-11]. However, the involvement of Tissue Plasminogen Activator (tPA) in invasion and metastasis is less understood. The tPA, as a constituent of the fibrinolysis system, converts plasminogen into plasmin which in turn dissolves intravascular blood clots (thrombolysis)[12,13].

It was found that the *Alu* polymorphism in intron 8 of the *tPA* gene is involved in tPA plasma levels[25]. However, others did not find an association between genetic variations at the *tPA* locus and plasma levels of tPA indicating the involvement of other genetic factors[26,34]. Nevertheless, a study by Jern *et al.*[27] reported an association between *tPA* gene polymorphism and the forearm vascular release rate of tPA *in vivo.* Subjects homozygous for the insertion (*Alu*$^{+/+}$) had a significantly higher release than both heterozygotes (*Alu*$^{+/-}$) or subjects homozygous for deletion (*Alu*$^{-/-}$).

The concentration of free circulating tPA depends on its secretion, clearance, complex formation with PAI-1 and release rate[35-37]. However, it was found that tPA release rate and not its concentration determines the thrombolytic potential[27].

Studies reporting on the role of tPA in cancer cell invasion and metastasis are contradictory. On one hand, it was reported that high intratumoral plasma concentration of tPA is associated with a better prognosis in breast cancer[17-19]. On the other hand, a poor prognosis in colorectal cancer was associated with increased secretion of tPA[15]. In addition, it was reported that the increased concentration of tPA could degrade fibrin and thus prevents metastatic cancer cells from implantation[38]. However, other studies have associated hyperfibrinolysis with metastatic breast cancer[28]. Present results support the involvement of tPA and hyperfibrinolysis in breast cancer[28]. The predominance of the *Alu*$^{-/-}$ genotype in the normal group (Table 1, 2) indicates a lower release rate of tPA and thus reduced concentration of free circulating tPA[27].

Hyperfibrinolysis can result in the development and progression of vascular diseases leading to endothelial dysfunction and vascular injury[39], this in turn would lead to metastatic cancer cells moving out of the vasculature (extravasate) and invading surrounding tissues[5]. Plasmin activation by tPA might also activate a number of matrix metalloproteinases such as collagenase, which could destruct the extracellular matrix of the endothelial surfaces leading to cancer cell invasion[5,40]. This suggests that tPA may play an important role during the different steps of malignancy such as cell migration and invasion.

The current study found an association between *Alu* polymorphism of the *tPA* gene and breast cancer. The presence of the *Alu*$^{-/-}$ genotype could exhibit a protective effect against developing breast cancer. However, the major limitations of the study were the small sample size and sample selection bias since the study was a case-control study. Researchers think that with a larger sample size, the *Alu*$^{+/+}$ genotype could be used as genetic biomarker for breast cancer. Extensive biological studies to understand the mechanism by which the *Alu* polymorphism in intron 8 of *tPA* gene affects tPA function and release rate are needed. In addition, further research is required to relate the invasiveness and aggressiveness of breast cancer and *Alu* polymorphism.

CONCLUSION

The present study demonstrates an association between Tissue Plasminogen Activator (*tPA)* gene polymorphism ($Alu^{-/-}$ and $Alu^{+/-}$) with breast cancer. The predominance of the $Alu^{-/-}$ genotype of *tPA* gene within the normal group represents a protective deletion with respect to breast cancer.

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