# IDENTIFYING THE SNP (C258T) POLYMORPHISMS OF FOXO3 GENE AND ITS ASSOCIATION WITH PRODUCTIVE PERFORMANCE OF AWASSI SHEEP

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## ABSTRACT

**This study was carried out to separate the DNA genetic material, determining the genotypes of the Foxo3 gene, and studying the effect of the genetic conformation of the mutation (C258T) rs 160226273, and to study the effect of genetic polymorphism of this mutation with the productive performance of Awassi sheep. A total of forty-five Awassi ewes of 2-4 years old were used currently as a source for blood sampling. These ewes produced 54 births, and their ages and weights were followed up from birth until the sixth month of age for the period from5/1 2022 to 25/6 2022.at the AL-Fayha Station for sheep Breeding. The mutation found within the studied segment (365 bp) of exon 2 of the Foxo3 gene, produced three genotypes (CC, CT, and TT), and their distribution rates were 36.36, 52.27, 11.36% respectively. The variation among these ratios was significant ($p \le 0.01$), and the allelic frequency was 0.625 and 0.375 for both the C and T alleles. The results showed that the effect of the genotypes of the Foxo3 gene was significant ($p \le 0.05$), on some traits represented by the ratio of solids non-fat for milk components, and some growth characteristics of lambs such as weaned weight, weight gain between birth and weaning weight, weight at six months of age, weight gain between weaning weight, weight at six months, and weights of lambs at three months of age.**

**Keywords: sheep, fork head gene, nucleotide sequencing technology, production, genotype.**

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## تحديد المظاهر الوراثية للطفرة (C258) rs160226273 لجين Foxo3 وعلاقتها بالأداء الإنتاجي للأغنام العواسي

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**باحث استاذ**

## المستخلص

**أجريت هذه الدراسة بهدف فصل المادة الوراثية (DNA) وتحديد التراكيب الوراثية لجين Foxo3 ودراسة تأثير التشكل الوراثي للطفرة . rs 160226273 (C258T)و تأثيره في الصفات المدروسة الإنتاجية المدروسة. اشتملت عينات التجربة للدراسة الحالية على 45 نعجة غنم سلالة العواسي المحلي بأعمار تراوحت بين 2–4 سنوات. انتجت هذه النعاج 54 مولود وتمت متابعة اعمارها واوزانها من الولادة ولغاية الشهر السادس من العمر. في محطة أغنام الفيحاء للفترة من 2022/1/5 ولغاية 2022/6/25. انتجت هذه الطفرة التي وجدت ضمن القطعة المدروسة وبحجم (365bp) للاكسون رقم 2 من الجين Foxo3 ثلاث تراكيب وراثية (CC وCT وTT) وبلغت نسب توزيعها 36.36 ،52.27، 11.36%تتابعا وكان التباين بين هذه النسب عالي المعنوية ($p \le 0.01$) وبلغ التكرار الاليلي (0.625 و0.375) لكل من الاليل C وT.تبين من النتائج ان تأثير التراكيب الوراثية لجين Foxo3 كان معنويا($p \le 0.05$) في بعض الصفات المدروسة المتمثلة بنسبة المواد الصلبة غير الدهنية لمكونات الحليب وبعض صفات النمو للحملان كوزن الفطام ومعدل الزيادة الوزنية بين وزني الميلاد والفطام والوزن لعمر ستة اشهر ومعدل الزيادة الوزنية بين وزن الفطام والوزن لستة اشهر واوزان الحملان للأشهر الثلاثة من العمر.**

**الكلمات المفتاحية: اغنام، جين راس الشوكة. التتابع النيوكلوتيدي، الإنتاج، التركيب الوراثي**

***جزء من أطروحة دكتوراه للباحث الأول.**

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## INTRODUCTION

The breed of Awassi sheep is one of the most important breeds in the country, which constitutes 58.2% of the local breeds (20). Among the prominent characteristics of this strain, is its tolerance to high temperatures and living in the poor lands of grasses (15). Improve the genetic characteristics of Awassi sheep is done by studying genes that are related and have an impact on productive and reproductive characteristics, and conducting a comparison between the genetic makeup of this breed with others in the world .The study of (1) have revealed developing the productive, and reproductive performance of Awassi breed, and the effect of the genetic polymorphism of the GDF-9 gene, and the resulting mutations in the studied regions, and the overlap between them. They obtained significant results for the milk yield, growth traits of lambs, and resistance traits. (23) showed the effect of gene (BMP15) polymorphism on some traits of Awassi Sheep, and they obtained significant results of prolificacy, milk yield and growth traits for their lambs. The Foxo3 gene is one of the members of the Fox family (class O3) and has wide expression in cell nuclei of various tissues and contributes to regulating the vital functions of other genes through many post-translational mechanisms (PTMS). These genes are related to the pathways of insulin and insulin-like growth factor (IGF) (19), cell differentiation (22), muscles hypertrophy (17, 12). ferther more, DNA damage repair (7), oxidative stress protection (22). Excluding data of (5, 6), other studies on Foxo3 did not undertaken on sheep. The current study was carried out to detect the effect variant of Foxo3 gene of the studded traits, during the mutation (C258T) rs160226273 of second exon of Foxo3 gene.

## MATERIALS AND METHODS

Study was carried out at Al-Fayhaa Sheep Farm, located at 55 kilometers south of Baghdad. A sample of 45 local Awassi ewes, 2-4 years old, with their lambs, were used. The genetic analyzes were done at the laboratories of the Advancement Scientific Company for molecular genetics, from 5/1/2022 to 1/6/2022. Blood was collected via jugular veni puncture (5 ml). Three ml of blood were drown into a plastic test container on the anticoagulant (K2EDTA). The samples were preserved at -20 Celsius for 3-5 days for the purpose of DNA extraction .The milk sample was taken once during the month, and the collection process takes place in the morning, for a period of three months period per each ewes.. The daily milk yield is calculated through the following equation: (16)

Daily milk yield = (one milk yield /day* 2 * NO of milking ewe)

1. **Body weight measurements:**

All born lambs were weighed immediately after birth using a field scale before lactation. They were also weighed for the first three months, at weaning and for the sixth months old to measure the weight gain between weights. Body for the mentioned lambs.

2. **Measurements of the body dimensions of the ewes (3).**

The body dimensions were taken as follows.

A-Chest circumference: The chest circumference area was measured by wrapping the tape around the chest area (just behind the front legs).

B- Body length: The body length was measured using a tape measure graduated in centimeters to measure the distance from the side of the back, starting from the cervical vertebra to the coccygeal vertebra.

C - Abdominal circumference: The abdominal area was measured by wrapping the tape around the abdomen area directly in front of the hind legs).

D- The height of the front: the distance was measured from the highest point in the back (shoulder bone) to the end of the front legs.

**DNA extraction:** The process of DNA extracting from blood samples was carried out for the purpose of conducting a molecular examination of the Foxo3 gene, according to the instructions of the kit (KIT) prepared by PR omega.

**Primers preparation**: The primers, which were prepared in the form of lyophilized powder, were supplied by the Korean company Macrogen. This powder was dissolved by cooling by adding 300 microliters of deionized water in order to obtain the required final concentration of 100 pmo/µl as a stock solution. By adding 10µl of buffer solution (stored at -20°C) to 90µl of deionized water to reach a final concentration of an active working solution of 10pmol/µl.

**Identification of primers**:

Primers have been described in the above by (5). As shown F5`-AACTCTATCCGGCACAACC-3`

R: 5`-GCCAGGATGGGGGACAG -3`) was used to amplify the studied fragment with a size of (365 bp) in exon. 2 of the Foxo3 gene of the studied sheep sample and according to the PCR reaction technique program).

**Accession number of Foxo3 gene**

Protein details for Foxo3 gene (UniprotKB/Swiss-prot).

Protein symbol; 043524-Foxo3-HUMAN; ecommended name: Fork head box proteinO3

**Statistical analysis:** The current data were analyzed statistically using the program SAS (21), to study the effect of the genotypes of the Foxo3 gene (C258T C/T CC, CT, and TT. By applying the general linear model (GLM)) and the significant differences between the averages were compared using a Least Squares test (9).The statistical model.

$Y_{iJK}=\mu+G_i+e_{iJK}$

$Y_{iJK}$: the observed value of genotype

µ: the overall mean of the trait

$G_i$: effect of the genotype of Foxo3 gene

$e_{iJK}$: error term $(^2\delta)$

Chi-square ($\chi^2$) test was used to compare the percentages of distribution of genotypes of Foxo3 gene in the studied sheep sample. The following law was applied to calculate the allelic frequency according to the Hardy-Weinberg rule (11)

$$P_A = \frac{2*No\ .of\ homozygous+1*No\ .of\ Heterozygous}{2*Totalnumber\ of\ samples}$$

$p_A$: Frequency of first allele  $p + q = 1$  $q_B = 1-p_A$ Frequency of the second allele

## RESULTS AND DISCUSSION

**Polymerase chain reaction (PCR) of the Foxo3 gene** Molecular detection of the Foxo3 gene was carried out using polymerase chain reaction (PCR) by the instructions provided in the personal kit **Go** Taq Green Master Mix with a volume of 25 microliters. These tubes were transferred the polymerase reaction device only. The reaction conditions of the duplicated genetic segments, and then the product of the reaction was transferred after the completion of the reaction in the electrophoresis device to ensure that the required piece of DNA was doubled, Table (1).

**Table 1.The program used in the molecular detection using the (PCR) technique**

| Steps | C° | m:s | Cycle |
|---|---|---|---|
| **Initial Denaturation** | **95** | **05:00** | **1** |
| **Denaturation** | **95** | **00:30** | |
| **Annealing** | **60** | **00:30** | **30** |
| **Extension** | **72** | **00:30** | |
| **Final extension** | **72** | **07:00** | **1** |

**Identification of nucleotide sequences**

The PCR output was sent to Macrogen-Corporation-Korea, the sequences of the nitrogenous bases of the DNA. The output was obtained using Genious software.

**Identification the phenotypes of the Foxo3 gene:** The genetic morphology of the C258T mutation (rs160226273) resulting from the Foxo3 gene was determined for the studied piece, which has a size of 365 nitrogenous bases (Figure1), and By reading the sequences of the nitrogenous bases, it was revealed that there was a genetic variation resulting in the change of the nitrogenous base thymine to cytosine. Three genotypes were produced for the studied Awassi ewe's sample, which are CC / CT / TT respectively (Figure 2).

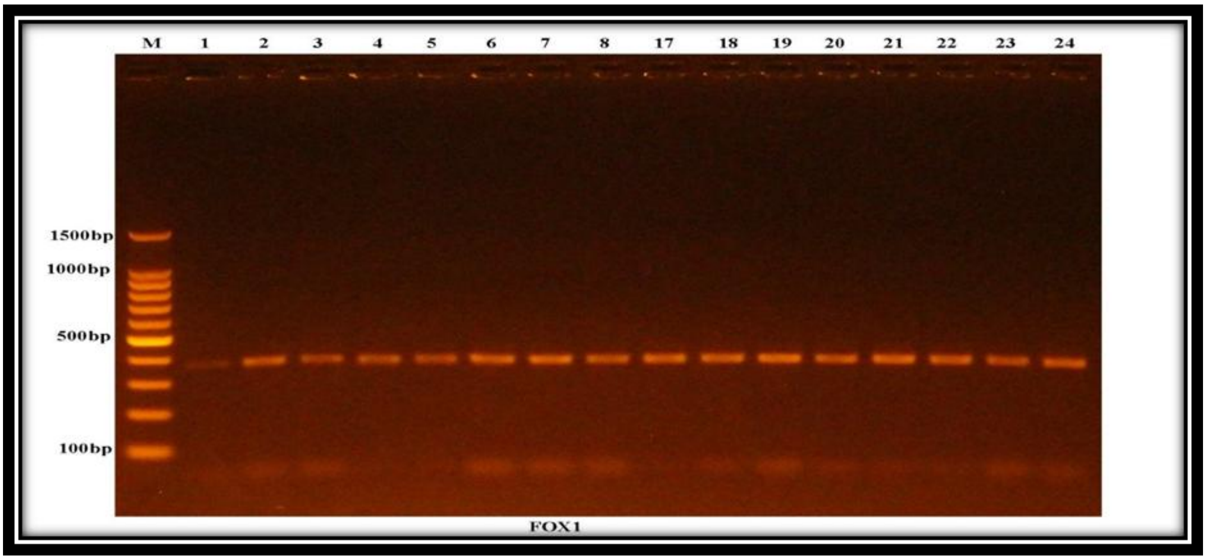

**Figure 1. Electrophoresis of PCR product for detection of Foxo3 gene using 2% agarose gel (60 min, 10 V/cm) Pathway M: DNA molecular scale of 100 base pairs, Pathway 1-24: Foxo3 gene Digestion products the PCR.**

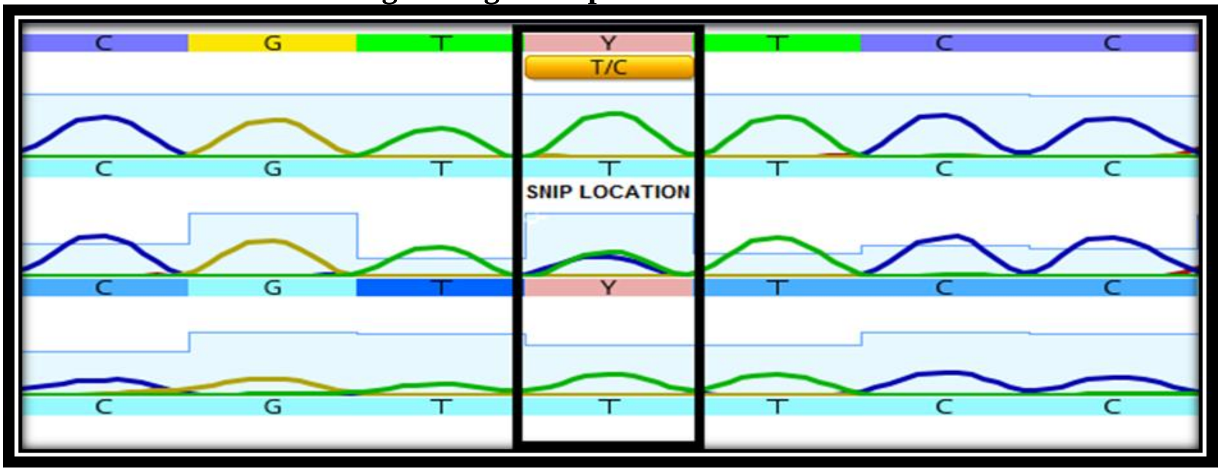

**Figure 2. Genetic mutation (T258C) rs1602262273 of Foxo3 gene in Awassi ewes studied using Sanger sequencing, which shows the sequence of the nitrogenous bases thymine T and cytosine C**

**Genotype ratios of the C258T mutation of the Foxo3 gene:** Table (2) shows the number of genotypes, their ratios, and the allelic frequency of the Foxo3 gene, showing highly significant differences ($p \leq 0.01$), among the distribution ratios of these different genotypes (36.36, 52.27, 11.36%) for both CC, CT and TT respectively. The proportion of individuals present in the herd that had the C allele were heterozygous or that they were homozygous. These individuals outperformed the individuals carrying the wild genetic makeup TT. Note that the current study indicated a high percentage of the hybrid composition. In addition, these percentages differ between studies conducted by researchers on other sheep breeds, as it appeared in (5). during their study on New Zealand sheep, there were three alleles for Foxo3 gene A, B, C, and five genotypes (AA, AB, AC, BC, CC), and it also appeared in (13). Three alleles for the same gene with four genotypes (AA, AB, AC, CC) and the Absence of the BC genotype. It appeared in (14). The genotype AA only when it was studied on another genes on the Iranian breed of sheep (Sang sari sheep), and in a study conducted by (10). On Egyptian and Saudi breeds (Najdi, Sadie, Baricy and Hari) it was found that the distribution rate of individuals carrying the hybrid genotype AC was higher. from the CC genotype of these studied strains. The BB genotype and its rate was {100%}. The differences that appears in the distribution ratios among studies may be due to the existence of differences among strains as well as related to the nutrition factors, or it may be due to other genetic factors such as the effect caused by modified genes (8). The frequency of the C allele of local Awassi sheep was {0.625}, while the frequency of the T allele was (0.375). This result expresses the prevalence of the C allele of the Foxo3 gene in that sample

**Table 2. The genotypes, percentages and allelic frequency of the Foxo3 gene in the studied ewes of Awassi ewes**

| Percentage(%) | Number of ewes | Genotype |
|---|---|---|
| 36.36 | 16 | CC |
| 52.27 | 23 | CT |
| 11.36 | 5 | TT |
| 100 % | 44 | Total |
| 28.500•• | --- | Chi($\chi^2$) |
| | | .($p\leq0.01$) ** |
| Frequency | | Allele |
| 0.625 | | T |
| 0.375 | | C |

**Relationship of the C258T mutation to daily milk yield and milk components:** Results in Table (3) shows differences in the daily milk yield rate. This agrees to what was presented by the study in terms of the non-significant effect of the genotypes of the Foxo3 gene on daily milk yield.and contrary to what was reported by (3). It was shown, that the average daily milk yield was 724.72 ± 56.16 g which is less than the average daily milk yield of the current study which amounted to 1004.65 ±16.08g .Milk for Awassi ewes or for other breeds, may be related to the difference among those breeds, or to factors related to the quality of nutrition, the number of the herd the methods of measurement used, and other factors (4). It is also clear, that the differences in the percentage of sold non-fat for the components of milk according to the different genotypes of the Foxo3 gene was significant ($p\leq0.05$), reached a maximum of 10.77 ± 0.13% in the CC genotype, and it was the lowest 10.03 ± 0.30% with the TT genotype. It is clear that the differences in the percentage of milk protein according to the genotype of the Foxo3 gene were not significant, and their values of 5.03 ± 0.12, 4.97 ± 0.13 and 4.53 ± 0.30%. There is no variation in the protein percentage according to the genotype of Foxo3 gene in ewes. The effect of mutation (C258T) in variation for solds non-fat materials components of milk is important because of its relation with the manufacturing state of milk minerals, and cheese formation and the results shows the distinguishing for the CC genotype of this trait contrasting with the CT and TT genotypes , including the observation of the effect of the Foxo3 gene in comparison with other components like fat and lactose**.**

**Table 3. Relationship of Foxo3 genotypes with daily milk yield and milk components of Awassi ewes (Means±SE)**

| Mean ± standard error | | | | | Number of ewe | Genotype |
|---|---|---|---|---|---|---|
| Sold non-fat sold% | Protein% | Lactose% | Fat % | Daily Milk Kg | | |
| a 0.17±10.72 | a 0.13±4.97 | a 0.03±4.91 | a 0.37±5.37 | a 17.37±990.57 | 23 | CT |
| a 0.13±10.77 | a 0.12±5.03 | a 0.03±4.95 | a 0.37±5.40 | a 30.51±1043 | 16 | CC |
| b 0.30±10.03 | a 0.30±4.53 | a 0.07±4.83 | a 0.63±6.39 | a 50.52±953.33 | 5 | TT |
| * | N.S | N.S | N.S | N.S | TotaL44 | N.S |

Means with different letters within the same column are significant ($P\leq0.05$), N.S: Non-significant

**Relationship of the C258T mutation of the Foxo3 gene to maternal body dimensions**: It is clear from Table (4), that the body dimensions of the ewes were not significantly affected by the different genotypes of the Foxo3 gene. The average body length was 54.04 ± 0.66, 53.37 ± 0.79, and 52.60 ± 1.07 cm respectively in CT, CC and TT genotypes. The results did not reveal the significant differences in the circumference of the chest, and the dimensions respectively, 96.30 ± 0.87, 96.87 ± 1.15, and 94.60 ± 0.50 cm. The difference in the genotypes of the Foxo3 gene was not significant in the body dimensions of ewes, represented by the abdominal circumference and wither height (97.26 ±1.08 ,97.62± 1.17 ,98.80±3.18),and (66.34±0.74,65.81±1.32,64.40±1.86cm) for each one of the genotypes CT,CC and TT respectively.

**Table 4. The relationship of Foxo3 Genotypes and dimensions of the Awassi ewes.**

| Mean ±Standard Error (cm) | | | | | |
|---|---|---|---|---|---|
| **Wither height** | **Abdominal girth** | **Body length** | **Heart girth** | **Number of ewes** | **Genotype** |
| **66.34±0.74 a** | **97.26±1.08 a** | **54.04±0.66 a** | **96.30±0.87 a** | **23** | **CT** |
| **65.81±1.22 a** | **97.62±1.17 a** | **53.37±0.79 a** | **96.87±1.15 a** | **16** | **CC** |
| **64.40±1.86 a** | **98.80±3.18 a** | **52.60±1.07 a** | **94.60±0.50 a** | **5** | **TT** |
| **N.S** | **N.S** | **N.S** | **N.S** | **Total 44** | **Level Significant** |

**N.S: Non-significant**

**Relationship of the genetic mutation C258T of the foxo3 gene with the growth characteristics Awassi lamb.:** The results of Table (5) indicated that differences in genotypes of gene Foxo3 for the birth weight and gain of birth weight and live weight for dams lacked significance. The weight rates at weaning were significant (p≤0.05)being 32.25 ± 0.73, 31.73 ± 0.40 and 28.80 ± 0.86 kg, and the weight gain rates significant (p≤0.05) among them were 27.90 ± 0.65, 27.57 ± 0.35 and 24.75 ± 0.90 kg for the genotypes CC, CT and TT, respectively. These results indicated that lambs born from ewes with genotype CC are superior to other genotypes, and ewes with genotype CT are superior to their counterparts with genotype TT. Explained that there is a highly significant variation in growth traits, including body weights at birth and at weaning for Awassi lambs, due to the different genetic factors of their mothers and of the offspring themselves, as well as a difference in the genotypes (strain) of the mothers. (24) found significant differences in the weights at weaning for his study on the Turkish Awassi sheep, and this result is in agreement with the current study regarding the weight of the lambs at weaning.

**Table 5. The relationship of the genotypes of the Foxo3 gene to the growth characteristics of Awassi lambs.(Means±SE)**

| Mean ± Standard Error | | | | | Number of ewes | Genotype |
|---|---|---|---|---|---|---|
| **Weight live** | **Growth Rate** | **Gain** | **Weaning weight** | **Birth weight** | | |
| **42.19±0.66 a** | **0.86±0.002 A** | **27.57±0.35 a** | **31.73±0.40 a** | **4.16±0.09 a** | **23** | **CT** |
| **43.53±0.72 a** | **0.86±0.002 A** | **27.90±0.65 a** | **32.25±0.73 a** | **4.34±0.11 a** | **16** | **CC** |
| **43.80±2.29 a** | **0.85±0.002 A** | **24.75±0.90 b** | **28.80±0.86 b** | **40.05±0.12 a** | **5** | **TT** |
| **N.S** | **N.S** | ***** | ***** | **N.S** | **Total 44** | **Level Significant** |

**Means with different letters are significant differences (p≤0.05*), N.S: Non-significant**

**Relationship of the genetic mutation C258T of the Foxo3 gene with the growth characteristics of Awassi lambs.**

The results of Table (6) shows that the multiple effect of the Foxo3 gene for the above genotype on weaning weight, and weight at six months of age and the weight gain rate among them was significant (p≤0.05), and the weight rates at weaning were respectively 32.25 ± 0.73, 31.73 ± 0.40 and 28.80 ± 1.28 kg, while the weight for six months was 41.75 ± 0.83, 41.04 ± 0.49 and 38.20 ± 0.86 kg respectively .The weight ranged between 37.40 ± 0.74, 36.88 ± 0.43, and 34.15 ± 1.29 kg for the genotypes CC, CT, and TT, respectively. The results show that lambs born from mothers with the genotype CC are superior to other genotypes, and the ewes with the genotype CT outperform ewes from TT genotype. It was shown that there was a variation in the growth characteristics of the lambs, as shown in Table (6). It was confirmed that there is a highly significant variation in the studied growth characteristics of Iraqi mountain goat kids, represented by birth weights, weaning weights, and weight at the age of six months, resulting from the phenotypic and genetic correlations between each pair of these traits. These were consistent with the results of our current study.

**Table 6. The relationship of the genotypes of the Foxo3 gene to the growth characteristics of Awassi lambs.**

| Mean ± Standard Error | | | | Number of ewes | Genotype |
|---|---|---|---|---|---|
| **Type of Birth** | **Gain Rate** | **Weight of sixth month** | **Weaning weight** | | |
| 1.13±0.07 a | 36.88±0.43 a | 41.04±0.49 a | 31.73±0.4 a | 23 | CT |
| 1.12±0.08 a | 37.40±0.74 a | 41.75±0.83a | 32.25±0.7 a | 16 | CC |
| 1.20±0.20 N.S | 34.15±1.29 b | 38.20±0.86 b | 28.80±1.2b | 5 | TT |
| N.S | * | * | * | 44 Total | Level Significant |

**Means with different letters within the same column are significant ($p \leq 0.05$).**

**Relationship of the C258T mutation in the growth characteristics of the first three months of Awassi sheep:** The current results in Table (7) shows the existence of a significant effect ($p \leq 0.05$)), for the mutation of the Foxo3 gene, according to the different genotypes in terms of weight for the first three months of the life of the lambs. The maximum rates were 12.31 ± 0.33, 19.12 ± 0.45 and 25.62 ± 0.64. Kg and lambs born from ewes with the CT genotype outperformed those of the TT genotype. The variation in growth traits mentioned may be attributed to the influence of that diversity and genetic factors of individuals of the same breed. It was shown that there was a highly significant variation in the growth characteristics of Awassi lambs. It was not clear that there was a significant effect of the different genotypes of the Foxo3 gene on the rate of weight gain among those weights, and the rates were close and amounted to 9.50 ± 0.32, 9.40 ±0.50 and 9.30 ±0.23 kg for the CC, TT and CT genotypes. (Table 7)**.**

**Table 7. The relationship of the genotypes of the Foxo3 gene to the growth characteristics of Awassi lambs**

| Mean ± Standard error | | | | Number of ewes | Genotype |
|---|---|---|---|---|---|
| **Gain Rate** | **Weight of three month** | **Weight of two month** | **Weight of one month** | | |
| 9.30±0.23 a | 25.60±0.39 a | 18.60±0.34 a | 11.50±0.24 a | 23 | CT |
| 9.50±0.32 a | 25.62±0.64 a | 19.12±0.45 a | 12.31±0.39 a | 16 | CC |
| 9.40± 0.50 a | 23.30±0.66 b | 16.90±0.55 b | 11.10±0.29 b | 5 | TT |
| N.S | * | * | * | 44 Total | Level Significant |

**Means with different letters within the same column are significant ($p \leq 0.05$), N.S:non-significant**

It was concluded from the current results that the genetic conformation of the above mutation had a significant effect on the studied traits, especially growth traits and some milk components of the ewes. Therefore, the CC genotype can be considered the preferred genotype for sheep, as it gave clear preference over the rest of the hybrid and wild genotypes CT and TT, and focus on individuals distinguished for this genotype and include them in genetic improvement programs through selection and cross-crossing, and this expresses the clear role of the Foxo3 gene, and its relationship to the productive and reproductive characteristics of sheep need more studies to prove the effect of the genetic diversity of the Foxo3 gene for this breed, and other local breeds using more animals.

## CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

## DECLARATION OF FUND

The authors declare that they have not received a fund.

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