Investigation of mRNA Expression Levels of *TGIFLX* and *OCT1* Homeobox Genes in Colorectal Cancer

Narges Sadeghipour[1], Farnaz Lotfiyani[1], Reza Raoofian[2], Mohammad-Reza Noori-Daloii[2], Cyrus Azimi[3], Mansour Heidari[4]

[1] *Department of Biology, Science and Research Branch, Islamic Azad University, Tehran, Iran*

[2] *Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

[3] *Genetics Group, Cancer Research Center, Cancer Institute of Iran, Tehran University of Medical Sciences, Tehran, Iran*

[4] *Experimental Research Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran*

Received: 05 Jul. 2018; Accepted: 10 Sep. 2018

Abstract- The OCT1 and TGIFLX transcription factors are members of homeodomains whose expressions have been implicated in normal and abnormal development. However, the expression of *TGIFLX* and *OCT1* in colorectal cancer is unknown. This study aimed to detect the expression of *OCT1* and *TGIFLX* in clinical samples of colorectal cancer. Twenty-six pairs of colorectal cancer tissue and adjacent non-tumoral tissue were obtained at the time of surgery from patients with colorectal cancer. The expression of *TGIFLX* and *OCT1* was detected by real time reverse transcriptase polymerase chain reaction (RT-PCR). *OCT1* was down-regulated in colorectal carcinoma samples in both males (58.33%) and females (57.14%). By contrast, TGIFLX was mainly (41.63%) expressed in colorectal tumors of males' samples but not in para-neoplastic normal tissues. *OCT1* expression was not significantly associated with the gender and site of primary tumor ($P>0.05$), but the expression of *TGIFLX* was associated with male patients ($P<0.05$). In conclusion, dysregulation of *OCT1* and *TGIFLX* genes might be novel prognostic biomarkers for patients with colorectal cancer.

Acta Med Iran 2018;56(10):653-659.

Keywords: Colorectal cancer; *TGIFLX*; *OCT1*; Real time RT-PCR

Introduction

Colorectal cancer is (CRC) a widespread public health problem and many new cases are diagnosed every year (1). In spite of the fact that development of new strategies in diagnosis and therapeutic methods, colorectal cancer in patients show unsatisfactory and poor prognosis (2). So far, the identification and characterization of novel prognostic and predictive biomarkers for CRC have been focused on many studies,and several candidate biomarkers have been reported (3-7). Therefore, recognition of genes whose expressions are implicated in abnormal growth and the development of colorectal cancer are important.

Homeobox genes consist of a large gene family involved in development and differentiation (8-9) which can be subdivided into the clustered and non-clustered homeobox genes (10). The characteristic of homeobox genes is a conserved 180 base pair long motif which encodes a DNA- binding homeodomain with helix-turn-helix super-secondary structure (11). According to the NMR and X-ray crystallography studies, classic homeodomain is a 60 amino acid structure in which three conserved α helices surround a hydrophobic center (12). HOX genes are implicated in normal and tumorigenesis.

The OCT1 is a member of POU-domain containing family (13). POU-homeodomain genes are expressed in early developmental processes as transcriptional regulators. *OCT1* belongs to the POU class II and identifies its target sequences by the octamer-binding site (14). Different genes such as *pit1*, histone genes, some interleukins,and immunoglobulins are influenced by OCT1 transcription factor (15). The probable role of *OCT1* in the procedures of malignancy has been indicated,and the inhibition of oncogenesis in mouse embryonic stem cells and mice with the deficiency of p53 observed in the absence of *OCT1*(16). *OCT1* may play an important role in the abnormal expression of cyclinD1 and down-regulation of cyclinD1 has been

Corresponding Author: M. Heidari
Experimental Research Center, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran
Tel: +98 21 88953005, Fax: +98 21 88953005, E-mail address: mheidari@sina.tums.ac.ir

detected in the *OCT1* knockdown CRC cells. Therefore, colorectal cancer cell spreading may be influenced by OCT1 transcription factor (15,17). Modulating complex functions of POU protein OCT1 has been proposed in the expression of *CDX2* gene (18).

TGIFLX (TG-interacting factor (TGIF)-like X) belongs to the transforming growth-interacting factors group (TGIFs), a member of three amino acid loop extension (TALE) superclass (19-20). In contrast with the classic homeobox genes, TALE superclass encodes a homeodomain with three extra amino acids between helix 1 and 2 (19). DNA binding site is mainly identified by highly conserved helix 3 of TALE homeodomain (19). These proteins function as transcription factors in various important embryonic developmental processes (21-22). The human *TGIFLX* gene includes 2 exons and located in Xq21.3 locus. *TGIFLX* resulted from the retrotransposition of homeobox *TGIF2* gene to the X-chromosome and its specific expression in the adult testis has been indicated (20,23).

Materials and Methods

Patients and tumor samples

Matched cancer and normal specimens were obtained from 26 cases of stage I and II of CRC patients. All specimens of CRC were collected and confirmed by histopathological examination at Iran Tumor Bank, Cancer Institute of Tehran University, Imam Khomeini Hospital. Samples were carried in liquid nitrogen and stored at -70 ºC.

Total RNA extraction and cDNA synthesis

Total RNA was extracted using Tripure Isolation Reagent (Roche, Mannheim, Germany) according to the manufacturer's guidelines and stored at -70 ºC for further tests. Total RNA was treated with RNase-free DNase I Enzyme (Fermentas). Quantitative control of RNA was performed by Spectrophotometry. The cDNA was synthesized using 1 µg of confirmed total RNA with PrimeScript™ 1st Strand cDNA Synthesis Kit (Takara Bio Inc, Shiga, Japan). The cDNA samples with acceptable quality level via GAPDH RT-PCR were stored at -70°C for following investigations.

Real-Time RT-PCR

To investigate the expression of *OCT1*and *TGIFLX* genes in cancer tissues and adjacent normal tissues real time RT-PCR was performed using specific primers, OCT1 F 5´- TTCCTCTCGCCGTGTTGTG-3´ and OCT1 R 5´- GTTCCGTCTCCATCCTTTCTTTC-3´ and TGIFLX primers was ordered from Qiagen company. Every real time RT-PCR reaction was contained 1µl of each primer (5pmoles/µl), 1µl of cDNA and 10 µl of SYBR Premix Ex Taq II (Takara Bio Inc, Shiga, Japan). Primer blast and single peak melting curves confirmed the specificity of primers. In this study,*GAPDH1* housekeeping gene used as an internal control and reference gene. The GAPDH primers including GAPF 5'- CACCAGGGCTGCTTTTAAC-3´ and GAPR 5´- ATCTCGCTCCTGGAAGAT-3 designed using Primer3 software.

Statistical analysis

REST-RG-Version 3 was used for gene expression alterations analysis. Associations between gene expression, gender,and tumor location were analyzed with Statistical Package for Social Science -Version 15 (SPSS) by Chi-Square test.

Results

The demographic characteristics of the colorectal tumors including age, gender, tumor location, grade of malignancy and histological type are shown in table1.

Table 1. Demographic characteristics of the colorectal cancer samples

		Number	Percentage
Gender	**Male**	12	46.15%
	Female	14	53.85%
Age	**50<**	2	7.69%
	50≥	24	92.31%
Tumor location	**Colon**	17	65.38%
	Rectum	9	34.62%
Grade of Malignancy	**I**	6	23.08%
	II	20	76.92%
Histological Type	**Adenocarcinoma**	22	81.62%
	Mucinous (colloid)	4	15.38%

mRNA expressions of *OCT1* and *TGIFLX* genes in CRC using Real Time RT-PCR

In order to evaluate the expression of *OCT1* and *TGIFLX* genes at the mRNA levels, the quantity and purity of extracted RNA samples were verified by Spectrophotometer. The mean concentration and optical density 280/260 nm were 667 ng/µl and 1.88, respectively. The results of qualitative control of RNA were examined via GAPDH RT-PCR (data not shown).

Also, single band of real time RT-PCR product of each primer and the expected length of amplified segments according to the primers on an agarose gel electrophoresis verified the specificity of reactions (data not shown).

***OCT1* gene expression in colorectal cancer**

The mRNA expression of OCT1 was evaluated in both cancerous and non-tumoral adjacent tissues of 24 patients. Real time RT-PCR revealed the *OCT1* down-regulation (Gene Fold: -1.545, *P*: 0.0165) in colorectal carcinoma samples in both males (58.33%) and females (57.14%) (Table 2). But two patients expressed *OCT1* only in nontumoral samples. It may be due to mix-up with tumoral adjacent tissues.

Table 2. *OCT1* mRNA expression status in CRC

	Males	Females	Total patients
Down-regulation	58.33%	57.14%	57.69%
Upregulation	16.66%	14.28%	15.38%
Total Significant Dysregulation	75%	71.42%	73.07%

CRC: Colorectal cancer

Furthermore, the associations between *OCT1* gene expression at the mRNA level, gender and tumor location were analyzed with SPSS-Version 15. We found that *OCT1* expression was not significantly associated with the gender as well as with site of primary tumor ($P>0.05$).

***TGIFLX* gene expression in colorectal cancer**

In order to analyze the expression of TGIFLX in colorectal cancer and non-tumoral samples in males and females with different tumor locations, we conducted real time RT-PCR as explained in the materials and methods. Interestingly, in spite of the mainly (41.63%) expressed in colorectal tumors of males, we could not detect *TGIFLX* mRNA expression in the non-tumoral tissues. Also, as shown in table 3 the expression of TGIFLX was not associated with tumor location while its expression was significantly associated with male patients ($P<0.05$) (Table 4).

Table 3. Association between *TGIFLX* gene expression and tumor location

		Section		Total
		Colonic	Rectal	
TGIFLX Expression Status	**Positive Count**	3	2	5
	%within	60.0%	40.0%	100.0%
	TGIFLX Expression Status %within section	27.3%	25.0%	26.3%
	Negative Count	8	6	14
	%within	57.1%	42.9%	100.0%
	TGIFLX Expression Status %within section	72.7%	75.0%	73.7%
Total Count		11	8	19
%within		57.9%	42.1%	100.0%
TGIFLX Expression Status %within section		100.0%	100.0%	100.0%

Chi-Square Test

	Value	df	Asymp.Sig. (2-sided)	Exact.Sig. (2-sided)	Exact.Sig. (1-sided)
Pearson Chi-Square	012[b]	1	.912		
Continuity correction*	.000	1	1.000		
Likelihood Ratio	.012	1	.911	1.000	.664
Fisher Exact Test					
Linear-by-Linear Association	.012	1	.914		
N of Valid cases	19				

Table 4. Significant association between *TGIFLX* gene expression and genderCrosstab

		Sex		Total
		Female	Male	
TGIFLX Expression Status	**Positive Count**	0	5	5
	%within	.0%	100.0%	100.0%
	TGIFLX Expression Status %within sex	.0%	45.5%	23.8%
	negative Count	10	6	16
	%within	62.5%	37.5%	100.0%
	TGIFLX Expression Status %within sex	100.0%	54.5%	76.2%
Total Count		10	11	21
%within		47.6%	52.4%	100.0%
TGIFLX Expression Status %within sex		100.0%	100.0%	100.0%

Chi-Square Test

	Value	df	Asymp.Sig. (2-sided)	Exact.Sig. (2-sided)	Exact.Sig. (1-sided)
Pearson Chi-Square	5.966[b]	1	.015		
Continuity correction*	3.723	1	.054		
Likelihood Ratio	7.895	1	.005	.035	.023
Fisher Exact Test					
Linear-by-Linear Association	5.682	1	.017		
N of Valid cases	21				

Discussion

CRC is common in both men and women. Recently the prevalence of CRC has increased and is one of the most common reasons for death from cancer in western and developing regions (1). Some investigations verified the homeobox genes functions in gastrointestinal evolvement and cancer (24). These genes are involved in the developmental and differential process as transcription factors by activation or repression of the target genes expression (25-26). Although various reports indicating about homeobox genes pathological roles in cancer, unfortunately, are not complete and comprehensive. In addition, it has been shown that they act as tumor suppressor genes at least in some cases whereas, in other cases play oncogenic roles in human tumors.

*OCT1*gene expression in colorectal cancer

In the present study, *OCT1* mRNA was evaluated in both cancerous and non-tumoral adjacent tissues of 24 patients. Our findings revealed a significant down-regulation of *OCT1* expression with *P*: 0.0165 was generally observed in cancerous samples. But two patients expressed *OCT1* only in non-tumoral samples. This may be due to mix-up of tumoral with non-tumoral tissues. The results of different studies suggest two opposite protumorigenic and tumor suppressive

functions for *OCT1* gene in cancers.

***OCT1* as a protumorigenic gene**

Some examinations propose protumorigenic role of *OCT1* gene. The expression of OCT1 target genes has been shown to upregulate in lung cancer (27). Also, there is an interaction between OCT1 and some metabolic enzymes in a glycolytic complex (28). Metabolism often switches from oxidative reactions to the glycolysis pathway for adjustment to hypoxia in primary and metastatic tumor cells (29). From the other point of view,*OCT1*-negative cells produce their energy through the oxidative pathway. The absence of *OCT1* alters the expression of some metabolic regulatory genes and preventstumorigenesis by interference with glycolytic metabolic shifts. So a control point may be active in the metabolism of stimulated cells by oncogenic signals (16). Also,*OCT1*upregulation and its *CDX2* promoter-binding ability have been recognized in an investigation on intestinal gastric cancer and metaplasia. But *OCT1* role in the induction of *CDX2* expression and IM initiation remained unclear (30). According to several studies Cyclin D1 overexpression has been observed in CRC (31-32). *OCT1* may influence colorectal cancer cell propagation by a probable role in the abnormal cyclin D1 expression which controls cell cycle (15,17).

***OCT1*as a tumor suppressor gene**

It should be noted that some studies suppose *OCT1*tumor suppressive roles. Findings show that *OCT1* and BRCA-1 tumor suppressor gene association stops growth and actives *GADD45* gene transcription (33-34). So the probable tumor suppressive function of *OCT1*in response to DNA damage can be concluded (35). On the other hand, irregular responses to radiation and dysregulation of several cellular stress-associated genes have been observed in *OCT1* -negative fibroblasts. They have suggested that *OCT1* transcription factor acts as a stress sensor and regulates target genes expression which results in a proper response (36).

According to the studies and considering two aforesaid opposite hypotheses our results can represent the lack of *OCT1* functional independence as a new hypothesis. This hypothesis is, in fact, originated from the nature of the homeobox genes. Some studies have shown these proteins in tumor suppressor mode,but sometimes they have played oncogenic role in other investigations. The *OCT1* performance is not clear exactly,andmeta-analysis suggests that this gene has a dual dependent function in different tissues. From the evidence, it seems that *OCT1* as an indicator which involved in stem cell differentiation has a very important and vital role so that the absence of this gene in the mouse fetus can cause death from lack of cell differentiation (37). Our studies are in agreement with this hypothesis, because even though we observed the diversity of expression at the level of *OCT1* mRNA in cancerous tissues, but considerably and significantly *OCT1*expression had been reduced in comparison with normal tissues. Also, this conclusion is in full agreement with the results of Shahriar *et al*., (Unpublished data). Our results have been obtained from the comparison of *OCT1* expression in cancerous and normal adjacent colorectal samples,and more studies are needed about the *OCT1* function and its expression pattern in more and different tissues.

mRNA expression of *TGIFLX* in CRC

Also in this study,*TGIFLX* gene was not expressed in both males and females' adjacent non-tumoral tissues. While aberrant TGIFLX expression was observed in 41.63% of colorectal tumors of males' samples.

The function of TGIFLX has not been clearly known,but some studies have revealed its testis-specific expression in adult males (20). The probable role of *TGIFLX* gene in differentiation and spermatogenesis (38) and also the association of *TGIFLX* expression with prostate cancer (39) progression have been suggested. According to Aarabi's study, the pattern of *TGIFLX* gene expression can be influenced by gender. On the other hand, investigations revealed that *TGIFLX* normally expressed in adults' testis (20). Its abnormal expression in 41.63% of our cancerous male samples reconfirms probable gender-influenced *TGIFLX* expression pattern. The *TGIFLX* gene had expressed only in the male cancerous samples,and its mRNA wasn't detected in the female colorectal tumors and paraneoplastic normal tissues in our investigation. According to our results and the investigations of Blanco-Arias (2002)، Ousati Ashtiani (2009) and Aarabi(2008) the role of aberrant *TGIFLX* expression in cancer development is proposed. X chromosome defects can result in aberrant *TGIFLX* expression in males if gender influences *TGIFLX* expression pattern. So the frequency of aberrant expression in studied male samples compared to female tissues is justifiable. As well as the results suggest that *TGIFLX* may play a role as a tumor suppressor gene and its aberrant expression can be caused by abnormal activity of gene regulatory regions.

So finding the exact role of *TGIFLX* gene in the

pathway of cancer and even use of it as a probable biomarker can be the aims of future studies.

Acknowledgments

This work was supported with Tehran university of medical sciences.

References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics. CA Cancer J Clin 2016;66:7-30.
2. Tonini G, Imperatori M, Vincenzi B, Frezza AM, Santini D. Rechallenge therapy and treatment holiday: different strategies in management of metastatic colorectal cancer. J Exp Clin Cancer Res 2013;32:92.
3. Xi RC, Biao WS, Gang ZZ. Significant elevation of survivin and livin expression in human colorectal cancer: inverse correlation between expression and overall survival. Onkologie 2011;34:428-32.
4. Li XD, Miao SY, Wang GL, Yang L, Shu YQ, Yin YM. Amphiregulin and epiregulin expression in colorectal carcinoma and the correlation with clinicopathological characteristics. Onkologie 2010;33:353-8.
5. Wang F, Zhang P, Ma Y, Yang J, Moyer MP, Shi C,et al. NIRF is frequently upregulated in colorectal cancer and its oncogenicity can be suppressed by let-7a microRNA. Cancer Lett 2012;314:223-31.
6. Liang QL, Li ZY, Chen GQ, Lai ZN, Wang BR, Huang J. Prognostic value of serum soluble Fas in patients with locally advanced unresectable rectal cancer receiving concurrent chemoradiotherapy. J Zhejiang Univ Sci B 2010;11:912-7.
7. Liang QL, Wang BR, Li GH. DcR3 and survivin are highly expressed in colorectal carcinoma and closely correlated to its clinicopathologic parameters. J Zhejiang Univ Sci B 2009;10:675-82.
8. Mark M, Rijli FM, Chambon P. Homeobox genes in embryogenesis and pathogenesis. Pediatr Res 1997;42:421-9.
9. Gehring WJ, Hiromi Y. Homeotic genes and the homeobox. Annu Rev Genet 1986;20:147-73.
10. Holland PW, Booth HA, Bruford EA. Classification and nomenclature of all human homeobox genes. BMC Biol 2007;5:47.
11. Gehring, W.J., M. Affolter, and T. Burglin, Homeodomain proteins. Annu Rev Biochem 1994;63:487-526.
12. Gehring WJ, Qian YQ, Billeter M, Furukubo-Tokunaga K, Schier AF, Resendez-Perez D,et al. Homeodomain-DNA recognition. Cell 1994;78:211-23.
13. Verrijzer CP, Van der Vliet PC. POU domain transcription factors. Biochim Biophys Acta 1993;1173:1-21.
14. Ryan AK, Rosenfeld MG. POU domain family values: flexibility, partnerships, and developmental codes. Genes Dev 1997;11:1207-25.
15. Wang YP, Song GH, Chen J, Xiao C, Li C, Zhong L, et al. Elevated OCT1 participates in colon tumorigenesis and independently predicts poor prognoses of colorectal cancer patients. Tumour Biol 2016;37:3247-55.
16. Shakya A, Cooksey R, Cox JE, Wang V, McClain DA, Tantin D. OCT1 loss of function induces a coordinate metabolic shift that opposes tumorigenicity. Nat Cell Biol 2009;11:320-7.
17. Boulon S, Dantonel JC, Binet V, Vié A, Blanchard JM, Hipskind RA, Philips A. Oct-1 potentiates CREB-driven cyclin D1 promoter activation via a phospho-CREB- and CREB binding protein-independent mechanism. Mol Cell Biol 2002;22:7769-79.
18. Jin T, Li H. Pou homeodomain protein OCT1 is implicated in the expression of the caudal-related homeobox gene Cdx-2. J Biol Chem 2001;276:14752-8.
19. Bertolino E, Reimund B, Wildt-Perinic D, Clerc RG. A novel homeobox protein which recognizes a TGT core and functionally interferes with a retinoid-responsive motif. J Biol Chem 1995;270:31178-88.
20. Blanco-Arias P,Sargent CA, Affara NA. The human-specific Yp11.2/Xq21.3 homology block encodes a potentially functional testis-specific TGIF-like retroposon. Mamm Genome 2002;13:463-8.
21. Melhuish TA, Gallo CM, Wotton D. TGIF2 interacts with histone deacetylase 1 and represses transcription. J Biol Chem 2001;276:32109-14.
22. Powers SE, Taniguchi K, Yen W, Melhuish TA, Shen J, Walsh CA, et al. Tgif1 and Tgif2 regulate Nodal signaling and are required for gastrulation. Development 2010;137:249-59.
23. Lai YL, Li H, Chiang HS, Hsieh-Li HM. Expression of a novel TGIF subclass homeobox gene, Tex1, in the spermatids of mouse testis during spermatogenesis. Mech Dev 2002;113:185-7.
24. Yu YY, Pan YS, Zhu ZG. Homeobox genes and their functions on development and neoplasm in gastrointestinal tract. Eur J Surg Oncol 2007;33:129-32.
25. Cillo C, Cantile M, Faiella A, Boncinelli E. Homeobox genes in normal and malignant cells. J Cell Physiol 2001;188:161-9.
26. Chen H, Sukumar S. Role of homeobox genes in normal mammary gland development and breast tumorigenesis. J Mammary Gland Biol Neoplasia 200;8:159-75.
27. Reymann S, Borlak J. Transcription profiling of lung

adenocarcinomas of c-myc-transgenic mice: identification of the c-myc regulatory gene network. BMC Syst Biol 2008;2:46.

28. Zheng L, Roeder RG, Luo Y. S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component. Cell 2003;114:255-66.
29. Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 2006;66:8927-30.
30. Almeida R, Almeida J, Shoshkes M, Mendes N, Mesquita P, Silva E,et al. OCT-1 is over-expressed in intestinal metaplasia and intestinal gastric carcinomas and binds to, but does not transactivate, CDX2 in gastric cells. J Pathol 2005;207:396-401.
31. Arber N, Hibshoosh H, Moss SF, Sutter T, Zhang Y, Begg M,et al. Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis. Gastroenterology 1996;110:669-74.
32. Bartkova J, Lukas J, Strauss M, Bartek J. The PRAD-1/cyclin D1 oncogene product accumulates aberrantly in a subset of colorectal carcinomas. Int J Cancer 1994;58:568-73.
33. Fan W, Jin S, Tong T, Zhao H, Fan F, Antinore MJ, Rajasekaran B,et al. BRCA1 regulates GADD45 through its interactions with the OCT-1 and CAAT motifs. J Biol Chem 2002;277:8061-7.
34. Jin S, Fan F, Fan W, Zhao H, Tong T, Blanck P, et al. Transcription factors Oct-1 and NF-YA regulate the p53-independent induction of the GADD45 following DNA damage. Oncogene 2001;20:2683-90.
35. Wysocka J, Herr W. The herpes simplex virus VP16-induced complex: the makings of a regulatory switch. Trends Biochem Sci 2003;28:294-304.
36. Tantin D, Schild-Poulter C, Wang V, Haché RJ, Sharp PA. The octamer binding transcription factor Oct-1 is a stress sensor. Cancer Res 2005;65:10750-8.
37. Sebastiano V, Dalvai M, Gentile L, Schubart K, Sutter J, Wu GM, et al. OCT1 regulates trophoblast development during early mouse embryogenesis. Development 2010;137:3551-60.
38. Aarabi M, Ousati-Ashtiani Z, Nazarian A, Modarressi MH, Heidari M. Association of TGIFLX/Y mRNA expression with azoospermia in infertile men. Mol Reprod Dev 2008;75:1761-6.
39. Ousati Ashtiani Z, Ayati M, Modarresi MH, Raoofian R, Sabah Goulian B, Greene WK,et al. Association of TGIFLX/Y mRNA expression with prostate cancer. Med Oncol 2009;26:73-7.