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ORIGINAL ARTICLE

Upregulation of Fucosyltransferase 3, 8 and protein O-Fucosyltransferase 1, 2 genes in esophageal cancer stem-like cells (CSLCs)

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Abstract

Recently, studies have shown that Fucosylation plays an important role in the invasion and metastatic process of CSLCs. Understanding the expression pattern of fucosyltransferase (FUT) genes may help to suggest better-targeted therapy strategies for esophageal squamous cell carcinoma (ESCC). The study aimed to address the expression pattern of FUT gene variants in esophageal CSLCs and parental adherent cells. Sphere formation method was used to enrich CSLCs. Expression of FUT genes was examined in tumor sphere and parental adherent cells using the RT-PCR method and then relative expression of detected variants was performed by the Real-Time PCR method in both groups. The detected FUTs, also, were assessed in fresh ESCC tumors and the matched healthy controls. Analysis of The cell surface carbohydrate Lewis x (LeX, CD15) was performed by flow cytometry. Molecular analysis showed that the expression of FUT 3, 8 and POFUT1, 2 genes in tumorsphere were significantly higher than parental adherent cells. Analysis of fresh ESCC tumor tissues and the matched healthy controls showed that FUT8 and POFUT1, 2 genes in contrast to FUT 3 have higher expression in tumor tissues than controls. Flow cytometric analyses revealed that tumorsphere and their parent cells do not differ significantly in Lewis x surface marker. The present study showed that FUT 3, 8 and POFUT1, 2 genes upregulated in esophageal CSLCs in comparison to adherent cells. Understanding the expression pattern of FUT gene variants may help to suggest better-targeted therapy strategies for ESCC.

Keywords ESCC cancer · Fucosyltransferase genes · Lewis x · Cancer stem-like cells (CSLCs)

Introduction

Esophageal cancer (EC) is the eighth common cancer and the sixth world's leading cause of death among cancers [2]. Histologically, there are two main types of esophageal cancers: esophageal squamous cell carcinoma (ESCC) and adenocarcinoma (ADC) [16]. ESCC is much more common in the so-called Asian EC belt, which begins from the center and north of China, extending from Central Asia and North India to Golestan province in Iran [10]. Reducing ESCC mortality requires early prevention through reduced risk factors and secondary prevention through early detection and effective targeted therapy. Molecular changes in the esophageal tissue may be a suitable target for early diagnosis and treatment [35].

Post-translational modifications (PTMs), such as phosphorylation, acetylation, and glycosylation, adjust many cellular functions [41]. Recently, studies have shown that Fucosylation, as one of the most important PTMs, associated with cancer pathogenesis [45]. Fucosyltransferases (FUTs) are enzymes have been involved in the synthesis of glycoconjugates, such as glycolipids and glycoproteins, and up to date, 13 of these enzymes have been identified in human genome including FUT1–11 and protein-o-fucosyltransferase 1–2 (pofut1–2) [6, 20]. In mammals, fucosylated glycans play important roles in many biological processes such as immune responses and metastatic behavior of Cancer stem-like cells (CSLCs) [3, 8]. CSLCs are a minority population of cancer cells that have self-renewal and tumor initiation, progression, and metastasis in the patients [7]. Several methods have been developed for enrichment of CSLCs including the sphere

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formation assay, side population (SP) isolation, aldehyde dehydrogenase activity, drug resistance and fluorescence-activated cell sorting (FACS) [31, 37].

It has been shown that inhibition of fucosylation reduces the formation of tumorosphere, induces smaller spheres and also affects the invasion ability of CSLCs [8]. Abnormal fucosylation resulting from the shortage or increase in the expression of FUTs is associated with a variety of diseases including cystic fibrosis, type 2 leukocyte connective defects and cancers [24]. Fucosyltransferase enzymes have been studied in a number of cancers, including lung [17], pancreatic [22], prostate [40], liver [9], colorectal [26], ovarian [5] and breast cancer [15], but there is little information about the association of these enzymes with esophageal cancer and also the expression pattern of FUT gene variants of CSLCs is unknown. This study aimed to address the expression pattern of fucosyltransferases in esophageal CSLCs and adherent cells. Understanding the expression pattern of different FUT genes may help to suggest better-targeted therapy strategies.

Materials and methods

The study was approved by the Ethics Committee of Golestan University of Medical Sciences (IR. GOUMS.REC.1396.317) and written informed consent was obtained from all patients.

Cell culture and sphere formation

The ESCC-cell line YM-1 is available in our Lab. (Department of Molecular Medicine, Faculty of Advanced Medical Technologies, Golestan University of Medical Sciences, Gorgan, Iran) [1]. YM-1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (glutamax +1 g/l d-glucose Cat.No. BI 1027) supplemented with 10% foetal bovine serum (FBS Cat.No. gibco 10,270–106) and antibiotics (100 U penicillin/0.1 mg/ml streptomycin Cat.No. BI 1036) and then Incubated at 37 °C in a humidified air atmosphere containing 5% CO_2.

To enrich CSLCs, YM-1 cells were cultured in DMEM/F12 medium containing B27 supplement (50x, Invitrogen), human recombinant EGF (10 ng/ml Cat.No. Rp-1102-25) and bFGF; (10 ng/ml Cat.No. Rp-1101-60) without serum at a density of $1x10^5$ cells/well in low attachment 6 cm culture dishes. After three passages (about 10 days) tumorspheres was provided. For passage of tumorspheres, first, the consumed SFM medium was transferred to a 15 ml sterile falcon to settle the floating tumorspheres. Second, 2 ml of PBS was added and washed out after a few seconds. This was repeated two or three times. 0.5 ml of 0.05% trypsin was added and incubated for 3 min in a 37 °C incubator. Then, trypsin was removed by three-times washing with PBS and single cells were transferred to a new low attachment culture dishes containing the SFM medium.

Patient samples

In the present study, five ESCC tumor tissues and five adjacent normal tissues were collected during 5 months (May to September 2018) from Sayyad Shirazi Hospital (Gorgan, Iran). Written informed consent was obtained from all patients. All of these specimens were confirmed by the pathology laboratory of the hospital.

Real-time PCR assay (qRT-PCR)

Total RNA was extracted by Trizol (Invitrogen Cat.No.15596–026) and cDNA synthesis was performed using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific# k1621) according to the manufacturer's instructions. First of all, RT-PCR was performed for all fucosyltransferase genes to screen which one has an expression in our samples. Regarding the RT-PCR gel electrophoresis, four fucosyltransferases were selected (Fut 3, Fut 8, POFut 1 and POFut 2) for quantitative gene expression assessment, with different gene expression in tumorsphere comparing to the parental adherent cells. Expression of SOX2 and Nanog genes were also measured as stem cell markers.

Some sequences of primers were obtained from published papers and others were designed and purchased from Denazist Co. The list of Primers is shown in Table 1.

Real-time PCR was carried out by qPCR kit (Ampliqon A325402–25 2x qPCR Master mix green-High Rox) using Roche RT-PCR Detection System (LightCycler ® 96 System, Switzerland). The thermal cycling condition was optimized for FUT 1 with 95° C for 5 min, 34 cycles of 95° C for 20 s, 61° C for 20 s and 72° C for 20 s and one step of final extension at 72° C for 2 min. For FUT2, FUT4, FUT5, FUT7, FUT10, FUT11, POFut 1 and POFut2 same thermal condition program was used with 38 cycles of amplification with 40 s of extension time. For other genes (FUT 3, FUT 6, FUT 8, FUT 9) the same thermal program was used with 38 cycles of amplification with 30 s of annealing at 59° C and 40 s of extension at 72° C. For gene expression normalization, the GAPDH was used as housekeeping gene using the 2^{-dct} or 2^{-ddct} formula.

Flow cytometry

Esophageal cancer cells (adherent and tumorsphere) were harvested using trypsinization and then processed for staining immediately. For functional assessment, one of the main fucosyltransferase substrates, CD15 (Lewis X) cell surface marker was stained using fluorescein isothiocyanate (FITC)–labeled mouse anti-human IgM (BD-332778, EMD

Table 1 Primers used in the Real-Time PCR and RT-PCR reactions

Gene	Accession Number	Forward primer	Reverse primer	Amplicon size (bp)	Ref
FUT 1	NM_001329877.1	AGGTATAAACACACCCTCTGTGCTT	GAGTTCAGGGACAGACAGTGGTT	80	[28]
FUT 2	NM_000511.5	CTCGCTACAGCTCCCTCATCTT	CGTGGGAGGTGTCAATGTTCT	78	designed
FUT 3	NM_000149.3	TCCCTTTTCGTCACACTCAGG	ACCGAACTGGTCTAAGCCTTG	71	designed
FUT 4	NM_002033.3	AATTGGGCTCCTGCACAC	CCAGGTGCTGCGAGTTCT	68	[28]
FUT 5	NM_002034.2	TATGGCAGTGGAACCTGTCA	CGTCCACAGCAGGATCAGTA	100	designed
FUT 6	NM_000150.2	GCGTGTGTCTCAAGACGATCC	GGAAGCGGGACCCATTAGG	50	designed
FUT 7	NM_004479.3	TCCGCGTGCGACTGTTC	GTGTGGGTAGCGGTCACAGA	65	designed
FUT 8	NM_004480.4	TTCAAGTGGTCGAGCTTCCC	GTACAAGTCGATCTGCGAGGT	96	designed
FUT 9	NM_006581.3	CAAGGATTACATCACGGAAAAGC	TGGTCCCAGAACAACAGGTACA	70	[28]
FUT 10	NM_032664.3	GCTTACCTCTGCCTCGGAAAG	GCAGTGTAGTTGAACAAGGTGAT	115	designed
FUT 11	NM_173540.2	TTCTACGGCACAGACTTCCG	AGTGGCGACTGAAGGTGGA	160	designed
POFUT1	NM_015352.1	AAGCCTCCTTTCACCAACCTC	TGATGACCCGATGGTAAGCCT	82	designed
POFUT2	NM_015227.5	GGCAGAGTCCTGACATCCAC	ACTGCTCATACTCGATGACGG	99	designed
SOX2	NM_003106.4	TACAGCATGTCCTACTCGCAG	GAGGAAGAGGTAACCACAGGG	110	[43]
Nanog	NM_024865.4	ATTCAGCACAGCCCTGATTCTTC	TTTTTGCGACACTCTTCTCTGC	76	[43]
GapDH	NM_001256799.2	CATCATCCCTGCCTCTACTG	GCCTGCTTCACCACCTTC	180	[43]

Biosciences). The mouse IgG1 (BD-345815) was used as the isotype control.

The appropriate volume of CD15 was added to cells and vortex gently and incubated for 30 min in the dark at room temperature then 2 mL of 1X BD FACS lysing solution was added and vortex gently and incubate for 10 min in the dark at room temperature, after that was centrifuged at 300 g for 5 min, the supernatant was removed and 2 mL of wash buffer (phosphate-buffered saline with 0.1% sodium azide) was added and centrifuged at 200 g for 5 min, the supernatant was removed. The stained cells were analyzed with the BD Accuri ™ C6 flow cytometer (NJ, USA) and data analysis were performed by BD Accuri ™ C6 version 1.0.264.21 software (BD, USA).

Statistical analysis

SPSS (version 16) was used for statistical analysis. A student's t test was used to analyze the statistical significance of the data. A chi-square test was used for analyzing the association between different FUT genes and CSLCs. A p value of less than 0.05 was considered to be statistically significant.

Results

YM1 tumor spheres over express the stemness genes

The ability to grow in suspension in serum-free medium was investigated with a tumor-initiating cell-selection method. The cell used in the current study was derived from a squamous esophageal carcinoma tumor primary culture [1] which is characterized by adherent epithelial-like phenotype (Fig. 1a). Following the non-adherent cell culture condition, the YM-1 tumorspheres were observed already after 72 h in serum-free medium (Fig. 1b). The size of tumorspheres became larger on the fifth day (Fig. 1c). After about 1 week, when the diameter of the tumorspheres reached about 60 μm, they were passaged before differentiation. After 3 passages the morphology of these tumorspheres was closer to the CSC-LCs than the previous tumorspheres and was circular (Fig.1d).

To characterize the stemness of the tumorsphere, the genes expression level of SOX2 and Nanog were assessed in comparison to the corresponding adherent cells of the YM-1 cell line. qRTPCR results showed that esophagus tumorspheres highly overexpress the SOX2 and Nanog stemness genes significantly in comparison to the adherent cells. ($P < 0.05$) (Fig. 2).

Esophagus cancer cells express all of the FUT genes excepting the FUT4

The expression of FUT genes (1–13) was assessed in CSLCs and adherents YM-1 cells by the RT-PCR method. Following the gel electrophoresis (2% agarose) we found that YM-1 express all of the FUT genes, however, no expression of the FUT4 was detected (Fig. 3a). To investigate if the same expression pattern exists in the tumor and normal tissues, the expression of all FUT genes, also, was assessed in pooled

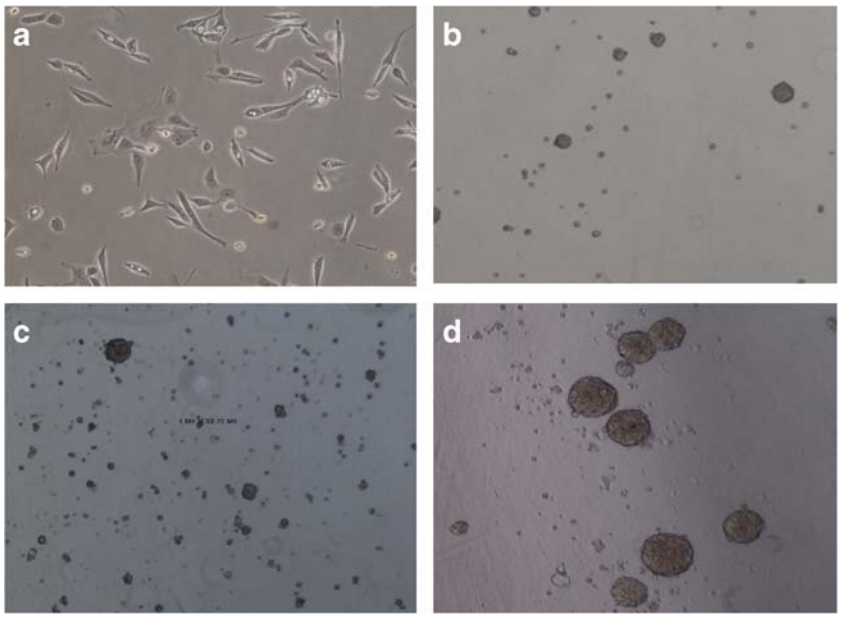

Fig. 1 YM-1 Tumorsphere formation process – **a** Adherent YM-1 cells in complete medium supplemented with FBS 5% (magnification: 100x), **b** five day following tumorsphere enrichment (magnification: 40X), **c** Tumorspheres after three days in passage number 3 (magnification: 40X), **d** Tumorspheres after five days in passage number 3 (magnification: 40X)

($N = 5$) ESCC and normal tissues by RT-PCR method. As it is shown in Figs. 3b and 3c, it was observed that both FUT 4 and 5 have no expression in tissues and the FUT10 also lacks expression in esophagus normal tissue. The profiling results have been summarized in Table 2. Our result showed that YM-1 CSLCs (tumorspheres) do not express FUT 1, 2, 4, 5 and FUT 10 genes (Fig. 3d). The confirmed FUT genes (FUT 3, 8, POFUT1 and POFUT2) were selected for quantitative real-time PCR analysis.

FUT genes are highly expressed in esophagus tumor tissues and tumorspheres.

The transcription levels of four Futs (FUT3, FUT8, PoFut 1 and PoFut 2) were examined using qRT-PCR in tumorsphere and parental cells. All of them displayed with higher mRNA expression levels in tumorsphere compared with the parental cells (Fig. 4a). Particularly, FUT 3 was the most overexpressed with more than 8 folds up-regulation in YM-1 CSLCs (P value = 0.037) and the FUT 8 showed the least up-regulation with approximate 2 times in CSLCs (P value = 0.031).

Quantitative gene expression assessment in ESCC tissues ($N = 5$) and the matched Normal esophageal tissues ($N = 5$) has been shown in Fig. 4b. Our result showed that three of FUTs (FUT8, PoFut 1 and PoFut 2) have higher expression in tumors, however, only the upregulation of the POFut2 was statistically significant (p value = 0.01). Interestingly we found that ESCC tumor tissues express a lower level of FUT3 when compared to the matched Normal tissues (P value = 0.005) which were different from the tumorsphere pattern. (Fig. 4b).

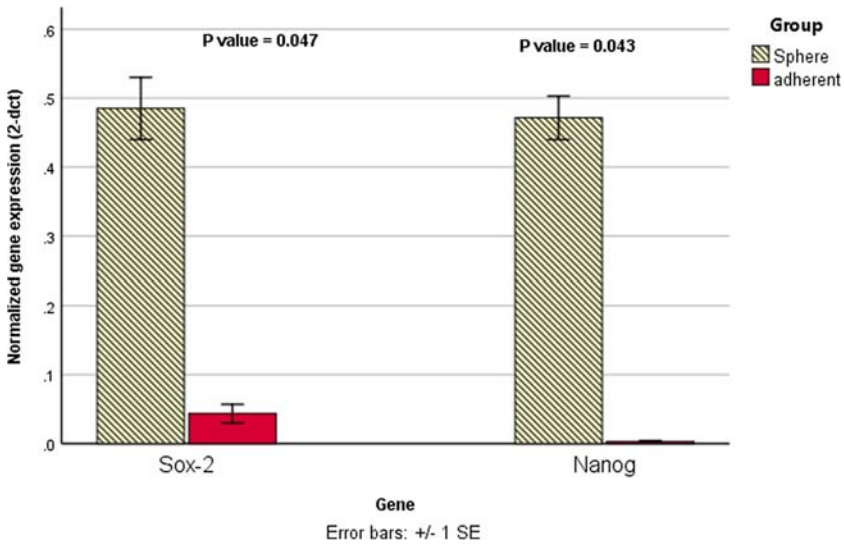

Fig. 2 Expression of SOX2 and Nanog genes by qRT-PCR. The expression of SOX2 and Nanog were measured by qRT-PCR in adherent and tumorsphere cells that both of them were increased significantly in tumorsphere in comparison to adherent cells. The housekeeping gene, GAPDH was used as the control gene

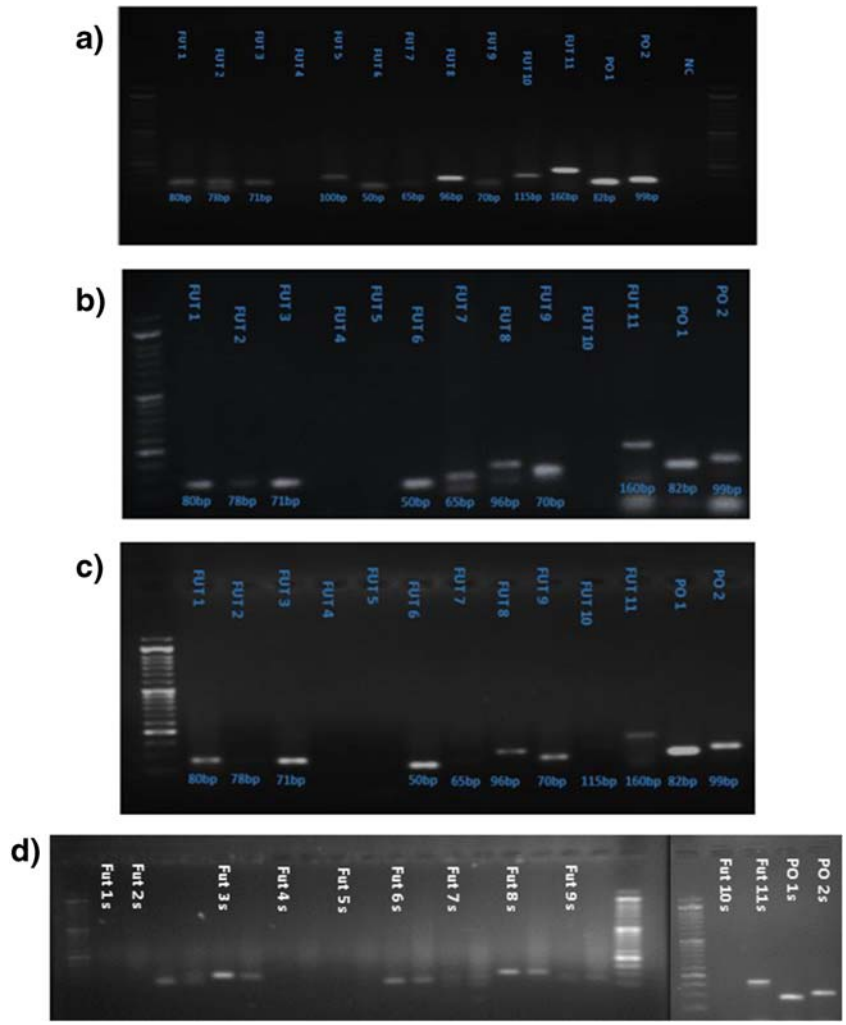

Fig. 3 Agarose gel electrophoresis of FUTs gene expression measured by RT-PCR. YM-1 cell line (**a**) The pooled five normal esophageal tissues (**b**) The pooled five ESCC tumor tissues (**c**) and YM-1 Tumorsphere (**d**). The 50 bp DNA Ladder was used in the first or last lanes. Each lane has been labeled with the corresponding gene. Unlabeled lanes in D are not related to tumorspheres. As shown in Table 1, the amplicon size of Fut1 to poFut2 are 80,78,71,68,100,50,65,96,70, 115,160,82, and 99 bp respectively. NC: negative control, FUT: Fucosyltransferase, PO: POFut

Esophagus tumorspheres express same Lewis X to the adherent cancer cells.

Regarding the differential FUT gene expression at the mRNA level, we investigated the availability of the CD15 lewis X antigen as the surface marker of FUT activity. Regardless to more FUTs (Fut3, FUT8, PouFut1, and PouFut2) expression in tumorspheres, our results revealed that the esophageal tumorspheres and their adherent counterpart do not differ significantly (P value = 0.15) in Lewis X levels, however, surface expression of Lewis X was decreased in CSLCs (Fig. 5).

Discussion

Recent studies have shown that aberrant fucosylation occur in CSLCs and this mechanism has a great importance in the invasion and metastatic behavior of CSLCs [18, 23]. Therefore, understanding the molecular mechanisms of fucosylation, especially fucosyltransferase enzymes, may help to improve effective therapeutic strategies that target metastatic CSLCs.

Our findings showed that all the FUT genes excepting the FUT4 are expressed at the mRNA level in Esophagus tissue (tumors, normal and YM1 cells). Previous reports indicate the

Table 2 the profiling status of FUT expression at mRNA level

	FUT1	FUT2	FUT3	FUT4	FUT5	FUT6	FUT7	FUT8	FUT9	FUT10	FUT11	POFUT1	POFUT2
YM-1 cells	+	+	+	–	+	+	+	+	+	+	+	+	+
Tumorsphere	–	–	+	–	–	+	+	+	+	–	+	+	+
ESCC tissue	+	+	+	–	–	+	+	+	+	+	+	+	+
Normal tissue	+	+	+	–	–	+	+	+	+	–	+	+	+

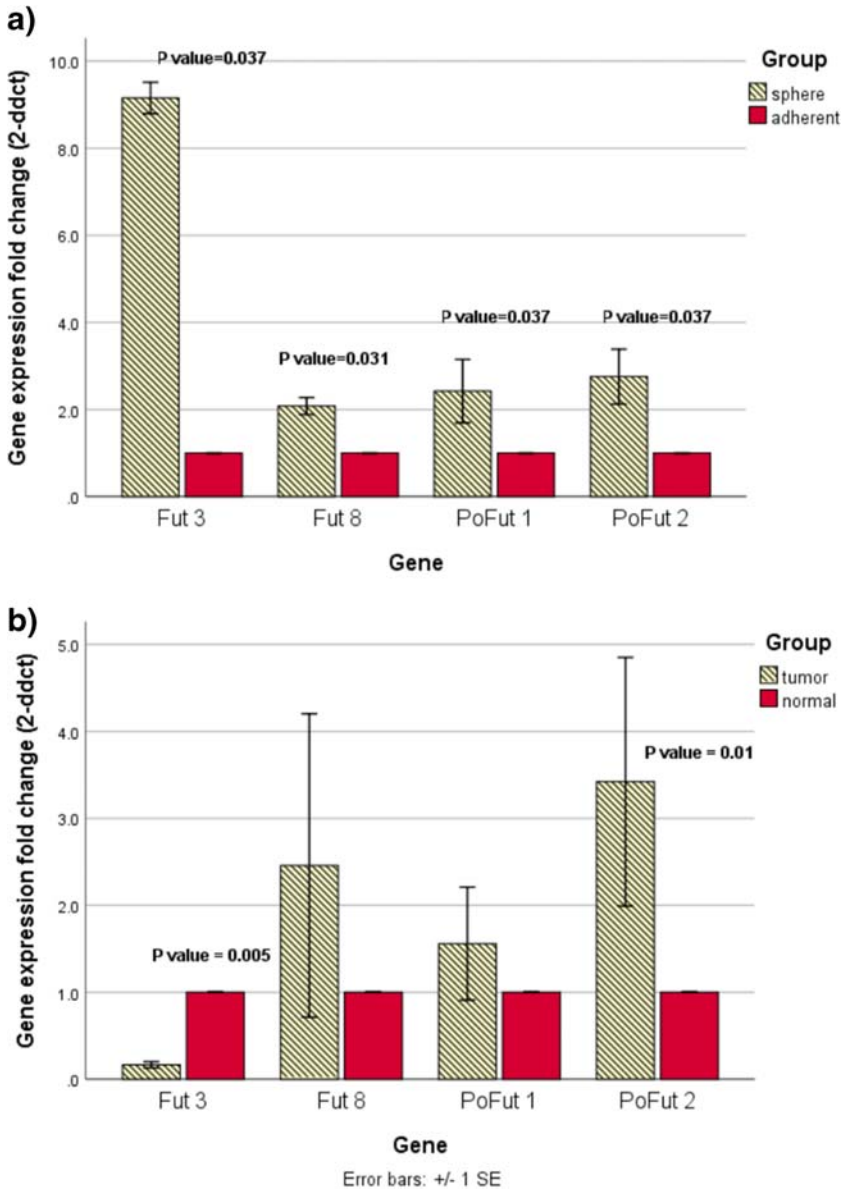

Fig. 4 Quantitative gene expression (qRT-PCR) of the FUT genes. The expression of FUT3, 8 and PoFut1, 2 were statistically significant in tumorspheres comparing to the parental adherent cells (**a**). Also, the expression of FUT8 and PoFut1, 2 were statistically significant in matched ESCC comparing to the normal tissues (**b**). Interestingly, the expression of FUT3 in normal samples was significantly higher than tumor tissues. The housekeeping gene, GAPDH was used as the control gene. FUT: Fucosyltransferase

importance function of the FUT 4 in epithelial-mesenchymal transition (EMT). The FUT4 can regulate EMT by facilitating access to mesenchymal phenotype [42]. In current study, YM-1 cell line was used which has the epithelial/ epithelial like phenotype. Also the tissue samples had the ESCC histology which is epithelial originated. Regarding the FUT 4 function in mesenchymal commitment, the lack of expression of FUT4 in our study can be attributed to the epithelial essence of the used samples.

Quantitative gene expression analysis showed that FUT8 and POFUT1, 2 genes have higher expression in tumor tissues than controls. Over-expression of FUT genes have been reported in different human carcinomas. Leng *et al.* in 2018 measured the mRNA expression of all 13 fucosyltransferases in plasma of 64 patients with lung cancer and 32 healthy subjects using Droplet digital PCR. They also examined 48 lung cancer tissues and 48 normal lung tissues using RT-PCR. They founded that the expression of FUT7, 8 and POFut1 genes in tumor tissues are higher than the normal tissues, while the Fut-4 gene variant exhibits low level of mRNA expression in the lung cancer tissue than normal tissues. In plasma samples, Fut8 and Pofut1 had high levels of plasma expression in patients with lung cancer compared to controls. [17]. We observed significant up-regulation of the FUT 3, 8, POFUT1 and 2 genes in tumorsphere (CSLCs) comparing to the parental adherent cells ($P < 0.05$). Previous studies in orospheres reported significant up-regulation of the FUT3, FUT6 isoforms and Sialyl Lewis x (CD15s) indicating their function in the metastatic behavior of orospheres [8]. The Over expression of FUT enzymes in CSLCs population highlights the

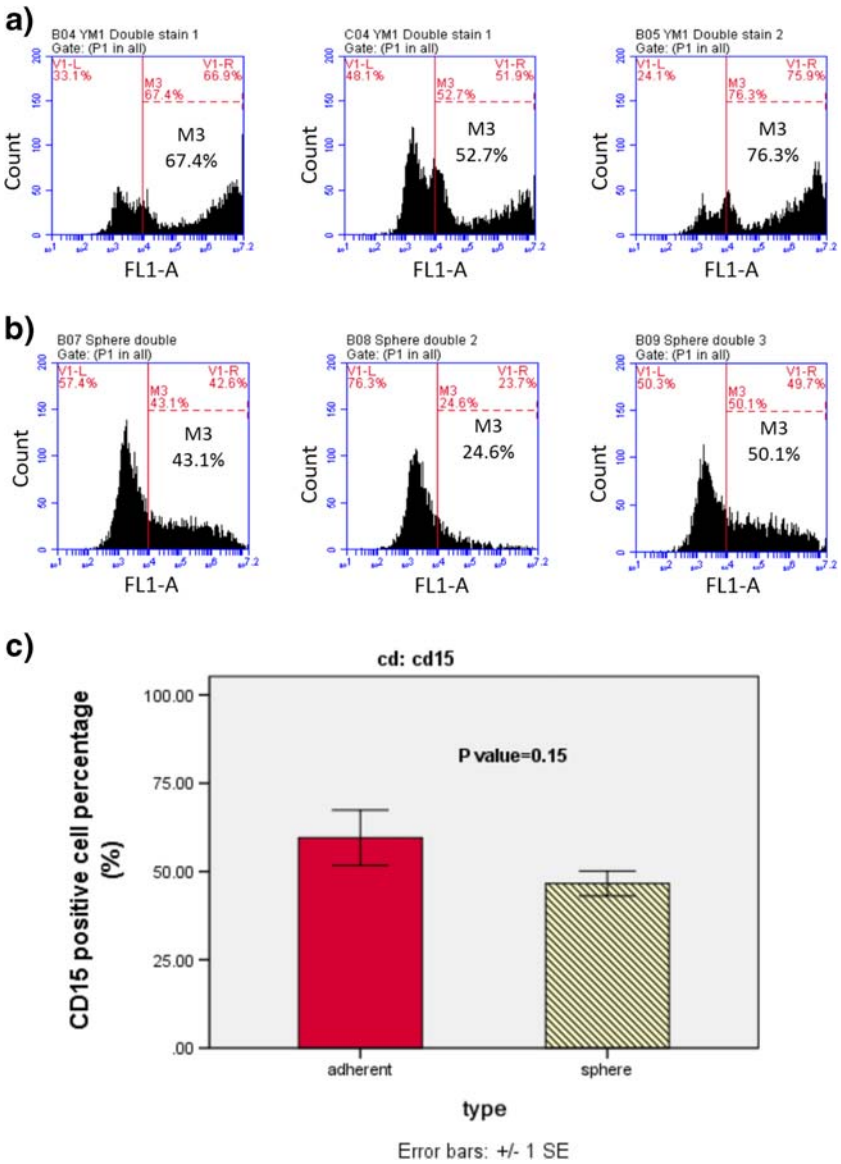

Fig. 5 The surface expression of Lewis X antigen (CD15) in YM-1 adherent cells and tumorspheres. The histogram of CD15 positive cells of three experiments has been shown for YM-1 adherent cells **(a)** and YM-1 tumorspheres **(b)**. The average of positive cell percentages has been summarized in the graph (**c**). The gate has been placed according to the background of isotype control (not shown here)

importance of fucosylation in self-renewal and neoplastic capabilities of CSLCs [4].

One mechanism could be the post translational modification and fucosylation of the extracellular domain of Notch receptor which can be activated by Pofut1 [29]. We found over expression of the POFUT1 and 2 in ESCC tumors and CSLCs. Concordantly Ma *et al.* in 2016 reported that Pofut1 is overexpressed in HCC cell lines and clinical HCC tissues, and Pofut1 overexpression clinically correlated with the undesirable survival and high disease recurrence in HCC [21]. The most over-expressed Fut gene in CSLCs in our experiments was FUT3. FUT 3 can contribute in fucosylation of the TβR I (TGF-β receptor) and the TβR II mediated [30]. It has been reported that FUT 3 plays fundamental role in TGF-β signaling and the EMT process [11], which is associated with the CSLCs [34].

The other FUT gene variant we found over expressed is FUT8 gene, which is essential for the EGF receptor mediated biological functions via the PI3K/Akt signaling pathway [39]. By binding to its ligand, EGFR formed homo- and heterodimers, which activated distinct downstream signaling such as the PI3K/Akt pathway [36]. Excessive expression of FUT8 has been observed in several malignant tumors, which is associated with the severity of these cancers [12, 13].

In humans, there are six α 1, 3 fucosyltransferase (FUT3 to FUT9) that fucosylate terminal lactose aminyl glycans and make Lewis X (LeX or CD15) or Sialyl Lewis X (sLeX or CD15s) structures that play a key role in cell migration, development and immune system [33]. FUT3 exhibits the greatest specificity synthesizing all Lewis antigens, Le a, Le b, sLe a, LeX, LeY, and sLeX [27]. Mondal *et al.* In 2018, showed that CD15s

biosynthesis is mainly carried out by FUT6 and FUT7; however FUT4 and FUT9 contributed mainly in CD15 production [25]. Although a decrease in LeX expression was observed in tumorspheres compared to adherent cells, this decrease was not statistically significant. Given the role of FUT4 and FUT9 in LeX synthesis and the other hand, the underexpression of these genes in our cell line, so the decrease of LeX expression was not unexpected. However, further studies are needed. It is important to note that LeX is a tetrasaccharide carbohydrate which is usually attached to O-glycans on the surface of cells [19]. Aberrant sialylation has important roles in the disturbance of cell-cell recognition, cell adhesion, antigenicity, protein targeting and invasion [44]. Studies of malignant cells have revealed changes in cell surfaces and membranes in terms of the sialic acid content of glycoproteins and glycolipids; accordingly, alteration in sialylation is one of the main characteristics of malignant transformation [32]. Also, aberrant glycosylation alter oncogenic behaviors in colorectal cancer by impairing expression or stability in the intestinal mucosa [14]. Hence, altered glycosylation is linked to tumor initiation, progression and metastasis [38].Therefore, the change in the surface of sphere cells may have caused a slight change in LeX content. Our findings suggest to assess the CD15s changes in ESCC tumor tissues and CSLCs with esophageal cancer origins. Also, functional studies may address better the role of FUT4 and FUT9 in LeX synthesis.

Conclusions

Our findings showed that FUT 3, 8 and POFUT1, 2 genes upregulated in esophageal CSLCs comparison to adherent cells. Also, analysis of fresh ESCC tissues and the matched healthy controls showed that FUT8 and POFUT1, 2 genes in contrast to FUT 3 have higher expression in tumor tissues than controls. Understanding the expression pattern of FUT genes may help to suggest the better targeted therapy strategies for ESCC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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