Histol Histopathol (2016) 31: 547-555

http://www.hh.um.es

Histology and Histopathology

From Cell Biology to Tissue Engineering

Fucosyltransferase 8 expression in breast cancer patients: A high throughput tissue microarray analysis

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Summary. The aim of this study was to compare the expression of fucosyltransferase 8 (FUT8) in breast cancer tissue and to investigate the relationship between this marker with tumor progression and its applicability to differential diagnosis. An immunohistochemical study was performed for FUT8 using the tissue microarray technique. In addition, the mRNA and protein levels of FUT8 in the tissue were also tested by real-time PCR and Western blot. There was a significant difference in cytoplasmic expression of FUT8 between breast cancer tissue and matched normal tissue ($p<0.001$). The percent of FUT8 staining in breast cancer tissues ranging from negative, weak positive, positive and strong positive were 2.7%, 40.2%, 54% and 3.2%, respectively. High FUT8 protein expression correlated with lymphatic metastasis ($p=0.008$) and with stage status ($p=0.039$). We detected that reduced FUT8 expression correlated with disease-free survival ($p=0.02$) and overall survival ($p=0.04$) of breast cancer patients. Expression of FUT8 can stratify breast cancer tissue and may be considered a prognostic marker for breast cancer patients.

Key words: FUT8; Prognosis, Breast cancer, Tissue microarray

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DOI: 10.14670/HH-11-693

Introduction

Breast cancer is the most common cause of cancer death among women and the most frequently diagnosed cancer in 140 of 184 countries worldwide (Jemal et al., 2011). The incidence of breast cancer has increased steadily at an alarming rate over the past two decades (from 29.9/100,000 in 1989-1993 to 50.1/100,000 in 2004-2008 in Chinese urban areas, and from 6.5/100,000 to 17.3/100,000 in Chinese rural areas), making breast cancer the most common and fifth most common cancer for Chinese urban and rural females, respectively (Chen et al., 2012). Hence, given the considerable public health importance of breast cancer, it is crucial to quickly identify new biomarkers with the potential to predict patient prognosis, drug resistance development and treatment choice (Dos Anjos Pultz et al., 2014).

The fucosyltransferase (FUT) family of enzymes are involved in the synthesis of cell-surface antigens through catalyzing the transfer of GDP-fucose to the N-acetylglucosamines residue of glycoproteins (Yang et al., 2013). Numerous alterations in the composition and structure of cell-surface glycoproteins are always detected when neoplastic transformation happens (Hakomori, 1989). Emerging data indicate that many of these alterations are mediated by the production of extensive fucosylated sugar chains (Yamashiki et al., 1999). FUT8 catalyzes the transfer of a fucose from GDP-fucose to the innermost GlcNAc residue of hybrid and complex N-linked oligosaccharides in glycoproteins via $\alpha(1,6)$-linkage to form core fucosylation in mammals.

FUT8 has marked functions on signal transduction

(Wang et al., 2005), cell adhesion (Zhao et al., 2006) and intracellular signaling (Li et al., 2012). Actually, the levels of α(1,6)FT (FUT8) activity and expression have been described in a variety of human tumors and are normally associated with an advanced disease stage (Hutchinson et al., 1991; Miyoshi et al., 1999; Muinelo-Romay et al., 2008). In support of this, several studies have demonstrated that the up-regulation of FUT8 mRNA, protein, and activity has been observed in several malignant tumors including colorectal cancers, leukemia, liver and lung cancer (Muinelo-Romay et al., 2008; Chen et al., 2013; Ji et al., 2013; Sasaki et al., 2013). In addition, several signal routes are involved in the FUT8 mediated tumor metastasis or tumor multidrug resistance (MDR). For example, FUT8 expression markedly modulated the activity of the phosphoinositide 3 kinase (PI3K)/Akt signaling pathway and MDR-related protein 1 (MRP1) expression (Cheng et al., 2013). In FUT8 knock-out ($Fut8^{-/-}$) mice, the lungs of the surviving adult mice showed emphysematous changes, which can be partly attributed to a lack of α1,6-fucosylation of the TGF-β1 receptor, resulting in the dysregulation of TGF-β1 receptor activation and signaling (Gao et al., 2012).

However, the role of FUT8, as well as any potential prognostic value in breast cancer has not been fully examined. Here, we performed a tissue microarray study to examine FUT8 expression in breast tumors and matched normal tissues. In addition, we explored the prognosis potential of FUT8 expression by looking into the relationship between FUT8 expression and clinico-pathological parameters among breast cancer patients.

Materials and methods

Patients and tissue samples

The collection of tissue specimens was conducted under an approved protocol from the Institutional Review Board and met the standards of the Declaration of Helsinki in its revised version of 1975 and its amendments of 1983, 1989, and 1996. We received consent from all patients. Tissue specimens included 189 breast cancer cases and 9 adjacent normal cases which were obtained from patients undergoing breast surgery at the Third Affiliated Hospital of Qiqihaer Medical University performed between March 2008 and December 2012. Seven cases of distal normal tissue were also enrolled. Normal tissue calculation was included, both adjacent normal tissue and distal normal tissue. Tumors were graded based on Silverberg's grading system (Shimizu et al., 1998) and staged following the FIGO staging system. All tumors were reviewed by one pathologist to confirm tumor type, histologic grade and stage. Disease-free survival (DFS) and overall survival (OS) were estimated as the time from diagnosis to recurrence or death, respectively. Follow-up period ranged from 8 to 72 months (median 50±18.58 months). Recurrence and death occurred in 42% and 30% of patients, respectively. Due to that most of the patients were not local patients and could not be followed up, only 50 patients had the follow up data and were included in the following survival analysis.

Tissue microarray and immunohistochemistry

The tissue microarray was established as described previously by Kononen et al. (1998). In short, tissue microarray blocks were obtained with 1.0 millimetre diameter of representative regions of each case (3 cores of different regions of the tumor from a single tissue block). The cores were carefully selected on hematoxyline-eosin (HE) stained sections and inserted into new paraffin blocks through a tissue microarray workstation (Minicore; ALPHELYS, Plaisir, France). As for the immunohistochemistry determination, we picked up randomly 3 samples for invasive ductal carcinoma and invasive lobular carcinoma. The sections with 5 micron thickness were deparaffinized with xylene, followed by washing with ethanol. Then, the sections were incubated with 0.3% hydrogen peroxide for 15 minutes to inhibit endogenous peroxidase activity, followed by heating in 0.01 mol/L citrate buffer (pH=6.0) in a microwave oven for 5 minutes at 100°C for antigen retrieval. Sections were incubated with FUT8 antibody (polyclonal; 1:100, Santa Cruz) over night at 4°C, followed by incubation with biotin labeled goat anti-mouse IgG and HRP-conjugated streptavidin. Negative controls were performed using PBS instead of primary antibody. Semiquantitative evaluation for the immunohistochemistry was performed by 2 pathologists. The slides were scored according to staining intensity, - (negative), + (weak positive), ++ (positive), +++ (strong positive). In the analysis of prognostic factors, DFS and OS, the total of negative (-) and weak positive (+) were defined as low expression of FUT8, the total of positive (++) and strong positive (+++) were defined as high expression of FUT8.

Western blot

Five tissues in each group were randomly selected and homogenized in an ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 100 mmol/L NaCl, and 1% Triton X-100) containing protease inhibitors (aprotinin, leupeptin, phenylmethylsulfonyl fluoride, and pepstatin). For each sample, 2 μg of total protein was separated by standard SDS gel (12.5%) electrophoresis and transferred to polyvinylidene difluoride membranes. Blots were incubated with the primary FUT8 antibody (Santa Cruz Biotechnology, USA; 1:200) overnight at 4°C, followed by the incubation of the corresponding HRP-conjugated secondary antibody (Santa Cruz Biotechnology, USA; 1:2000). GAPDH was used as the internal control. Signal was detected using an enhanced luminescence kit (Millipore, Germany).

Real-time PCR

In each group, total RNA from 5 randomly selected samples was extracted from tissue and 2 μL total RNA was utilized for reverse transcription (RT) to generate cDNA using a cDNA synthesis kit (Fermentas). The cDNA was subjected to real time quantitative PCR with defined primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The PCR primers for FUT8 were 5'- CCATTTCAGGT TTGTTTGGTAG -3'and 5'- ATTGGTCCCGCTTCTC ACTT-3'. The PCR primers for β-actin which used as endogenous control were 5'-CTGGGACGACATGGAG AAAA-3'and 5'- AAGGAAGGCTGGAAGAGTGC-3'. The data were analyzed using the ABI 7300 system SDS software (Applied Biosystems, Foster City, CA, USA). All the relative expression levels of these mRNAs were determined by the $2^{-\Delta\Delta Ct}$ method.

Statistical analysis

SPSS 16.0 software package was used for statistical analysis. Differences between groups were examined by chi-square test, Fisher's test, and Spearman correlation. A p-value of <0.05 was considered to be significant. The Kaplan-Meier method with censoring was used to estimate survival probability including overall survival (OS) and disease-free survival (DFS) rates, with the curve differences assessed by log-rank test. Comparison between groups used one-way ANOVA followed by Tukey's post hoc test. P<0.05 was considered significant.

Results

Study population and demographic features of the patients

The patients' ages ranged from 27 to 82 years, with the median age 56.39±12.12 years. 74.1% of the cases were categorized as invasive ductal carcinoma (IDC). Other presentations included invasive lobular carcinoma (ILC 23.8%), medullary carcinoma (MA 0.5%), mucinous carcinoma (1%), invasive papillary carcinoma (0.5%). Most patients (78.8%) were histologically graduated as grade 2. In addition, 8% of the cases were graduated as grade 3, the other 13.2% cases were graduated as grade 1. T1 for tumors up to 2 cm in diameter; T2 for tumors from 2 to 5 cm; T3 for tumors greater than 5 cm, and T4 for tumors that have spread into surrounding tissue (Schifano et al., 2006). According to the above criterion, we identified 13.8% of cases as T1, 70.4% of cases as T2, 9% of cases as T3, and 6.9% of cases as T4. Axillary nodal metastases were identified in 19.2% of cases.

FUT8 expression in breast cancer tissues

As evidenced by immunohistochemical brown staining in Fig. 1A, FUT8 predominently expressed in the cytoplasm of breast cancer tissues categorized as invasive ductal carcinoma and invasive lobular carcinoma. In contrast, no positive staining of FUT8 existed in the normal tissue, indicating that FUT8 expression in normal tissues was markedly lower. Table 1 showed that the intensity of staining was weak in 76 (40.2%) cases, moderate in 102 (54%) cases, and strong in 6 (3.2%) cases. Negative FUT8 staining was observed in 2.6% of cancer compared to 87.5% of normal. All differences were statistically significant (p<0.001).

FUT8 mRNA and protein expressions in tumor and normal tissue

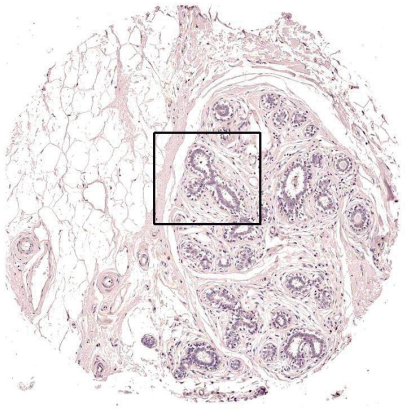
Both mRNA and protein levels of FUT8 were investigated for the individual tissue. As the data show, levels of FUT8 of malignant tissue in both mRNA (Fig. 2C) and protein (Fig. 2A,B) were up-regulated 3.63-fold (p<0.001) and 5.82-fold (p<0.01) respectively compared with the normal tissue. There existed no statistical difference between adjacent and normal tissue in both mRNA level and protein level. The change of mRNA was consistent with that of protein which indicated that the FUT8 change was mediated by transcription mechanism.

Correlation analysis between FUT8 expression and clinico-pathological features

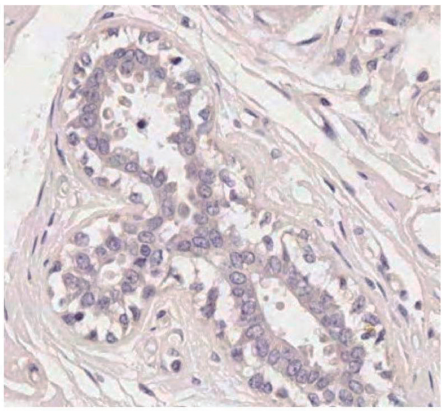
As shown in Table 2, the correlation analysis revealed that elevated expression of FUT8 was significantly associated with lymphatic metastasis and stage, with p values of 0.008 and 0.011, respectively. There existed a slight correlation between FUT8 and tumor size but the p value was not statistically significant (p=0.079). No significant associations were observed between the expression level of FUT8 with patient age, pathological type, tumor size and distant

Table 1. The expression levels of FUT8 in breast cancers and adjacent normal breast tissues detected by tissue microarray.

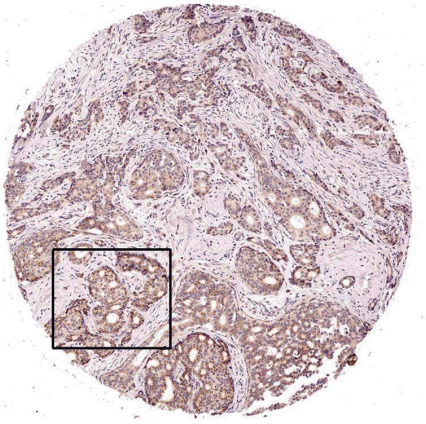
	n	FUT8 expression n (%)				p value
		(-)	(+)	(++)	(+++)	
Breast cancer tissue	189	5(2.7%)	76(40.2%)	102(54%)	6(3.2%)	p=0
Normal tissue (Adjacent and distal normal tissue)	16	14(87.5%)	1(6.3%)	1(6.3%)	0	χ^2=126.32



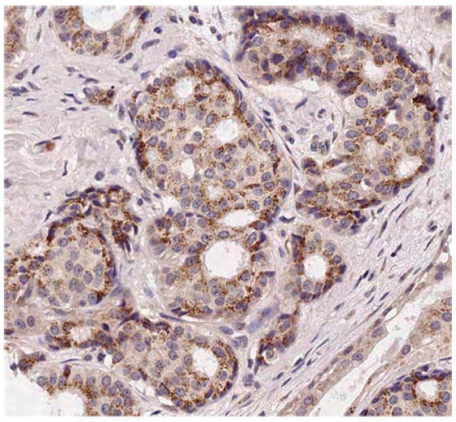
Normal tissue (40×)



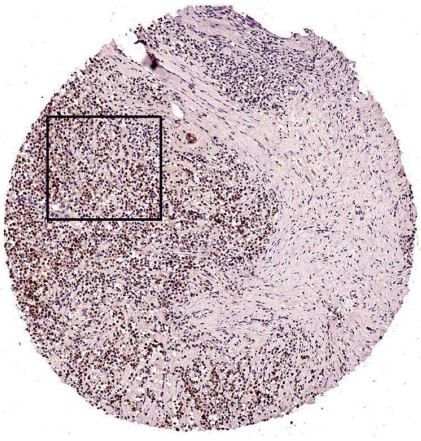
Normal tissue (200×)



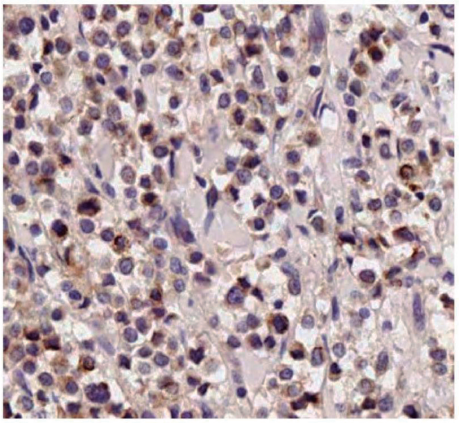
Invasive ductal carcinoma of breast cancer tissue (40×)



Invasive ductal carcinoma of breast cancer tissue (200×)



Invasive lobular carcinoma of breast cancer tissue (40×)



Invasive lobular carcinoma of breast cancer tissue (200×)

Fig. 1. The expressions of FUT8 protein in breast cancer tissues with different pathological types and normal tissues. In breast cancer tissue, the cytoplasmic staining of FUT8 was categorized as invasive ductal carcinoma and invasive lobular carcinoma. In normal tissue, the cytoplasmic staining of FUT8 was much slighter.

metastasis. As for the breast cancer molecular subtype, we additionally analyzed the relationship between FUT8 and ER expression and prognosis. As shown in Table 4, in patients with low FUT8 expression, the five-year survival rate of ER-positive was 92.8%, with a significant increase compared to that of ER-negative. In patients of high FUT8 expression, the five-year survival rate of ER-positive and ER-negative had no remarkable difference (P>0.05).

Table 2. Association between FUT8 expression level and clinico-pathological parameters.

Factor	Group	case	FUT8 expression level		p value
			Low-E (-)(+)	High-E (++)(+++)	
Age	≤35	12	5	7	p=0.931
	>35	177	76	101	χ^2=0.007
Type	IDC	140	53	87	p=0.066
	ILC	45	27	18	χ^2=8.371
	IPC	1		1	
	CM	2	1	1	
	MA	1		1	
Size	T1	26	14	12	p=0.079
	T2	133	60	73	χ^2=6.82
	T3	17	5	12	
	T4	13	2	11	
Lymph node	Negative	151	72	79	p=0.008**
	Positive	38	9	29	χ^2=7.139
Stage	I	25	14	11	p=0.011*
	IIa	109	53	56	χ^2=13.086
	IIb	40	13	27	
	IIIb	12	1	11	
	IV	3		3	
Distant metastasis	M0	188	81	107	p=0.385
	M1	1		1	χ^2=0.754

Low-E, Low expression of FUT8; High-E, High expression of FUT8.

Survival analysis by Kaplan-Meier method

In our presented research, the median follow-up time was 50 ± 18.58 months (range 8-72 months). During the follow-up period, 21 of the 50 patients suffered from recurrence (42%), and 15 of 50 patients died (30%). We detected that the overall survival (OS) was 55.74±2.18 months (95% CI 51.46-60.00) in the patients with FUT8 low-expression (negative and slight expression) and 45.19±4.33 months (95% CI 37.97-52.42) in the patients with FUT8 high-expression (moderate and strong expression). In addition, the mean disease-free survival (DFS) was found to be 51.71±3.53 months (95% CI 45.57-57.84) in the patients with FUT8 low-expression and 38.35±2.89 months (95% CI 30.46-46.25) in the

Table 3. Log-rank analysis of FUT8 protein expression in breast cancer with OS and DFS.

Characteristics	$\bar{x}$±s	95% CI		Log-rank	P value
	(month)	Lower	Upper	value	
Overall Survival					
low	55.74±2.18	51.46	60	4.22	0.04
high	45.19±4.33	37.97	52.42		
Disease-free survival					
low	51.71±3.53	45.57	57.84	5.31	0.02
high	38.35±2.89	30.46	46.25		

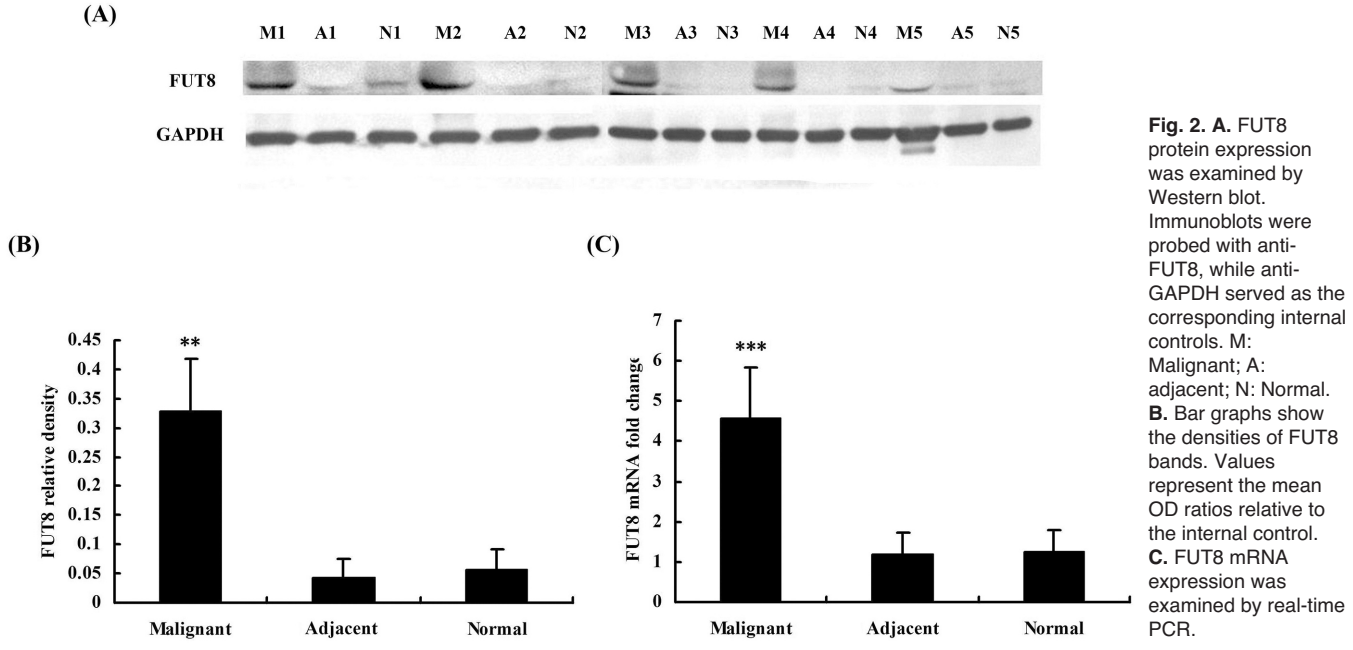


Fig. 2. A. FUT8 protein expression was examined by Western blot. Immunoblots were probed with anti-FUT8, while anti-GAPDH served as the corresponding internal controls. M: Malignant; A: adjacent; N: Normal. **B.** Bar graphs show the densities of FUT8 bands. Values represent the mean OD ratios relative to the internal control. **C.** FUT8 mRNA expression was examined by real-time PCR.

patients with FUT8 low-expression. Moreover, the difference of both OS and DFS were statistically significant, with the p value of 0.04 and 0.02, respectively (Table 3, Fig. 3).

Discussion

Nowadays, new tumour biomarkers for diagnosis, prognosis and follow-up of the disease, as well as on the development of new therapeutic targets focus on the glycoproteins (Hammarstrom, 1999). In particular, the study of the α(1,6)fucosylated proteins expressed in tumour cells has achieved high importance (Muinelo-Romay et al., 2011). Several researchers have reported the specific alteration of FUT8 activity and expression in malignant processes (Miyoshi et al., 1999; Takahashi et al., 2000). Unlike other FUTs, which are functionally redundant, FUT8 is the only enzyme responsible for the α1,6-linked (core) fucosylation by adding fucose to the innermost GlcNAc residue of an N-linked glycan (Chen et al., 2013). This catalytic activity constitutes a form of posttranslational modification, because it modulates the steric conformational properties of the core-fucosylated glucide antenna and, consequently, the action of other fucosyltransferases on carbohydrate side chains and the biological function of resulting glycoproteins (Stubbs et al., 1996).

Several studies have demonstrated the association between the FUT8 of different cell adhesion molecules during tumor development and the capacity of dissemination of these transformed cells (Kojima et al., 1996; Geng et al., 2004). Ji et al. (2013) reported that higher FUT8 expression level in tumor tissues than

Table 4. Relationship between FUT8 and ER expression and prognosis.

FUT8 expression	ER (+)	Five-year survival rate	ER (-)	Five-year survival rate	P value
Low-E(-)(+)	14	92.8%	7	57.1%	P =0.049*
High-E (++)(+++)	19	57.9%	10	50%	P=0.684

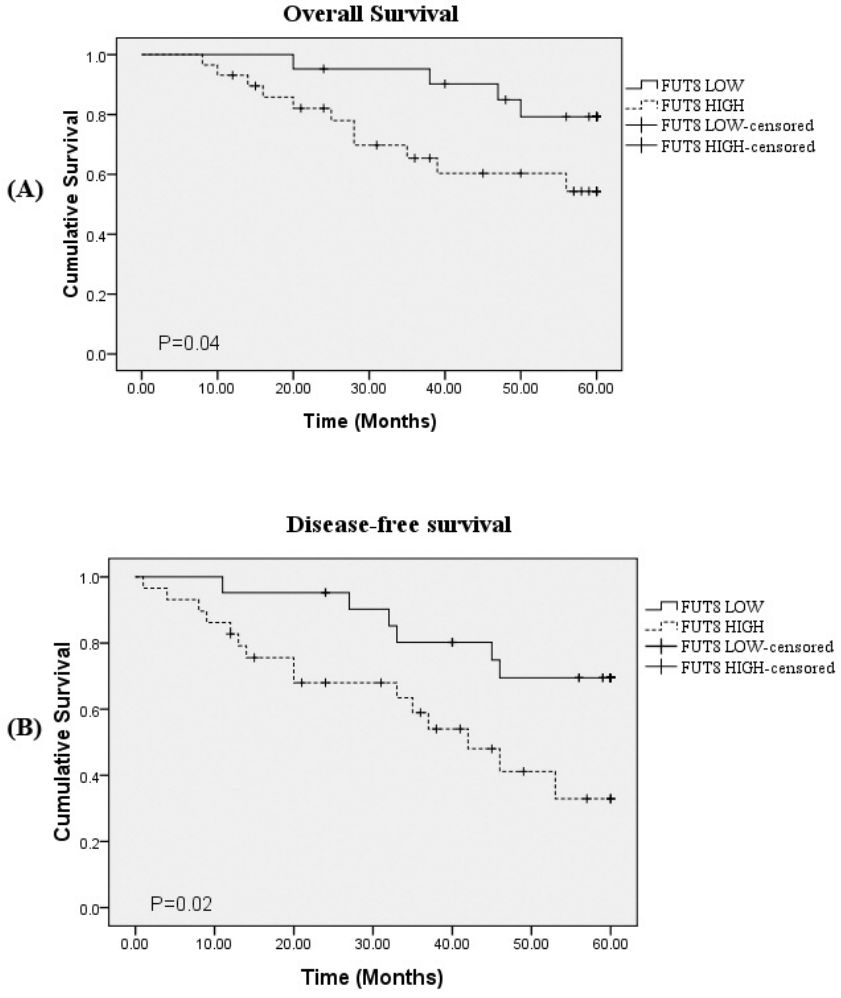


Fig. 3. Kaplan-Meier survival curve comparison of FUT8 with OS **(A)** and DFS **(B).** There were significant differences in DFS and OS between patients with high FUT8 expression and patients with low expression of FUT8.

adjacent noncancerous liver tissues, and FUT8 knockdown suppressed the tumor proliferation, migration and invasion of MHCC97-H cells, a kind of hepatoma cell line. By using PC3 and LNCaP cells which are prostate cancer cell (PCa) lines, Wang et al. (Wang et al., 2014) found that FUT8 overexpression in LNCaP cells increased PCa cell migration, while loss of FUT8 in PC3 cells decreased cell motility. In breast cancer, expression level of FUT8 gave adjusted p-values <0.05 for association with breast cancer mortality. Increased expression of FUT8 also correlated with a decreased risk of breast cancer recurrence (Andres et al., 2013). Compared with this study, our results from western blot and tissue microarray analysis displayed the same trend, that FUT8 expression was predominant in malignant cancer tissues localized mainly in the cytoplasm. Our microarray analyses indicate that core fucosylation could regulate the expression of genes involved in a wide range of cellular functions, which could explain the higher malignancy in breast cancer tissue with FUT8 over-expression.

The results of microarray were further verified by real-time PCR. The mRNA level of FUT8 was consistent with the protein level which indicated that the difference of FUT8 in normal and malignant tissue was mediated by transcription mechanism. A study displayed that the expression level of ceramide, an inducer of cell apoptosis, was increased in the lungs of Fut8(-/-) mice which indicated that FUT8 inhibited apoptosis in cells (Wang et al., 2009). Thus, it is possible that FUT8 is a driver of breast carcinogenesis. The data support that over-expression of FUT8 may be a strong risk factor for breast cancer.

Clinically, a high level of FUT8 may contribute to therapeutic resistance and decreased survival in several malignancies. FUT8 expression correlated with advanced-stage and poor prognosis in studies of lung cancer and breast cancer (Geng et al., 2004; Andres et al., 2013). We also observed a correlation between the staining score of FUT8 and clinico-pathological features. Our study showed a significant correlation between FUT8 and lymphatic metastasis, and stage as well. In addition, a trend correlation was observed between FUT8 and tumor size, and type as well. In breast cancer tissue, Coordinate expression of FUT8 with either estrogen receptor (ESR1) or progesterone receptor (PGR) was detected (Andres and Wittliff, 2012). Consistent with this, we also found that expression of FUT8 correlated with ER expression status. The estrogen receptor proteins, encoded by the genes ESR1, are established clinical biomarkers in breast cancer specimens that are utilized for therapy selection and as prognostic factors (Hammond et al., 2010). About 75% of all incident breast cancers occur in post-menopausal women, and approximately 80% of those breast cancers express the estrogen receptor (that is, they are ER-positive) (Anderson et al., 2002). Recurrence and death from ER-positive breast cancer can be effectively reduced through estrogen suppression or antagonism (Djalalov et al., 2015). When FUT8 expression was low, patients with ER-positive had a better prognosis than that with ER-negative. In contrast, when FUT8 expressed highly, the subtype of ER had no effect on survival rate. According to this result, the possible mechanism we speculated is that when FUT8 is over-expressed, some pathways mediated by ER may be stimulated, thus making ER not sensitive to drug treatment, which results in a low survival rate.

The relationship between this parameter may be considered for a new combined therapeutic approach for the treatment of breast cancer in the near future. In this study, FUT8 significantly correlated with patients' shortened overall survival. The survival curve also showed that the patients with FUT8 high expression had a significantly shorter disease free survival when compared to the patients with FUT8 low expression. Thus, it is interesting that the poor prognosis related to FUT8 expression reflects a pathological process in breast cancer. This observation was in agreement with previous findings, which showed that FUT8 expression was found to be inversely correlated to OS and DFS in non-small cell lung cancer patients (Honma et al., 2015).

In conclusion, this presented paper has firstly demonstrated that FUT8 expression may be a good prognostic factor for predicting breast cancer progression after surgery. Our findings suggest that FUT8 could be a promising tumor marker and therapeutic target for this pathology. However, further multicentric studies are needed to validate the use of FUT8 in the therapeutic management of breast cancer.

Acknowledgements. This work was supported by the National Natural Science Foundation of China (No. 81202084), Ministry of Education key project of China (No. 212047), Key Program from Qigihar Medical University of China (No. QY2013ZD-02) and Qigihar Municipal Science and Technology Project (No. SFGG-201201).

Disclosure of potential conflicts of interest. This paper has never been published and is not under simultaneous review by another journal. All authors have read and approved to submit it to your journal. There is no conflict of interest of any authors in relation to the submission.

Research involving Human Participants and/or Animals. This study was involved Human Participants which approved by the Institutional Review Board of Qiqihar Medical University.

Informed consent. Delayed, written informed consent was obtained from all enrollees after they were clinically stabilized.

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Accepted November 24, 2015