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Altered expression of TGF-β1 and TGF-βR2 in tissue samples compared to blood is associated with food habits and survival in esophageal squamous cell carcinoma

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ABSTRACT

In the transforming growth factor β (TGF-β) signaling pathway, TGF-β1 and TGF-β receptor 2 (TGF-βR2) are essential regulatory components which play an important role in different type of cancer. Expressions of TGF-β1 and TGF-βR2 were done by real-time qPCR in both biopsy and blood samples collected from esophageal squamous cell carcinoma (ESCC) patients (n = 76). The expression profiles were correlated with different lifestyle factors and clinicopathological parameters. Kaplan-Meier survival analysis and Cox regression analysis were performed to estimate survival and hazard outcomes of different parameters. TGF-β1 showed upregulation in 91% tissue samples (2.84 $\pm$ 1.34*) and 55% blood samples (2.43 $\pm$ 1.24*) whereas expression of TGF-βR2 showed downregulation in 89% tissue samples (0.27 $\pm$ 0.23*) and 75% blood samples (0.30 $\pm$ 0.26*). Among all the parameters, TGF-β1 expression is significant with histopathology grade, consumption of betel nut and smoked food whereas TGF-βR2 expression is significant only with dysphagia grade in both blood and tissue samples and while analyzing both male and female patients separately. Consuming alcohol and hot food, difference in tumor stage and metastasis were found to have statistically significant ($P < 0.05$) impact on survival and mortality of male patients while consuming hot food, tobacco, metastasis and TGF-βR2 expression in tissue level were found to associate with survival and mortality of

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female patients. Expression of both TGF-β1 and TGF-βR2 in tissue samples may be prospective biomarkers for screening of ESCC among the Northeast population. Survival outcomes and hazard analysis supports the importance of some clinicopathological and lifestyle factors on ESCC development, whereas expression study depicts association of change in expression of the studied genes in ESCC patients.
*Mean fold change.

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Introduction

Esophageal cancer possesses one of the poor prognoses and it is one of the most lethal cancers with an overall 5 year survival rate of less than 20%.[1,2] There are 2 types of esophageal cancer, adenocarcinoma and squamous cell carcinoma.[3] With the emergence of new technologies, esophageal cancer biomarkers play an interesting role in cancer research and their association with different clinicopathologic characteristics can provide some promising targets for the diagnosis and treatment of cancer.[4,5]

The role of transforming growth factor-β (TGF-β) signaling in esophageal squamous cell carcinoma has been studied recently in which the isoform Transforming growth factor β1 (TGF-β1) plays a key role in various biological processes like immunity regulation, cell proliferation, immune surveillance etc.[6-8] The biological effects of TGF-β are mediated by 2 independent receptors TGF-β receptor 1 (TGF-βR1) and TGF-β receptor 2 (TGF-βR2). Downregulation or loss of TGF-βR2 has been observed in many human cancers. It can regulate the TGF-β pathway by negative-feedback mechanisms, but very little is known about the underlying mechanism.[9] Deregulation of its expression and activity has been observed in the pathogenesis of numerous diseases including cancer.[10] Both TGF-β1 and TGF-βR2 play key role in regulating the TGF β signaling pathway which plays a dual role in carcinogenesis as a tumor promoter and tumor suppressor.[11-13] Many tumors expressing overexpression of TGF-β and other TGF-β signaling genes have been noted which associate with different clinicopathological parameters.[14-17]

This study focuses on the expression profile of TGF-β1 and TGF-βR2 gene with risk of ESCC and expression profile is compared in blood and tissue samples. We also analyzed the association of their expression profile with different lifestyle factors and clinicopathological parameters and risk of ESCC development. Moreover, survival analysis and hazard outcomes were also checked for the studied parameters.

Materials and methods

Sample collection

A total of 76 ESCC patients (49 males and 27 females) and an equal number of age- and sex-matched healthy individuals were enrolled in the study with informed consent. The diagnosis of esophageal cancer was done by upper gastrointestinal endoscopy and by pathologic evaluation of tumor biopsy samples. Standard venipuncture was used to collect blood samples. Tumor tissue and adjacent normal tissue were taken by biopsy from all the study participants. All samples of esophageal cancer patients were collected from Guwahati Medical College Hospital, Guwahati and North East Cancer Hospital, Jorabat with approval from the Ethics Committee.

The duration of the study was from December 2016 to December 2019. Patients were followed up during this period and detailed information are collected from all the study

participants on diet, physical activity, medical history, regular use of alcohol, tobacco, betel nuts, etc. Patients were divided into 2 or more groups for each studied clinicopathological and lifestyle factors. Dysphasia grade was categorized according to the modified O'Rourke grading system. Histopathology grade, tumor stage, node stage, and metastasis were determined using the American Joint Committee on Cancer staging manual. Patients were divided into 5 age groups: 30-40 years, 40-50 years, 50-60 years, 60-70 years, and 70-80 years. Location of tumor was categorized into upper, middle, and lower part of esophagus. Nonconsumer, nonchewers, and nonsmokers are those patients who do not have any history of exposure; on the other hand, consumers, chewers, and smokers are those patients who have regularly (weekly or more) or occasionally (monthly or biweekly) exposure to the targeted lifestyle factors. For alcohol, patients were divided to nonalcoholic (who do not consume) and alcoholic (who consume regularly or occasionally). Type of tea consumption was divided to consumption of red tea, consumption of milk tea, and consumption of both red and milk tea. Amount of tea consumption was divided to low (1-2 times/d), medium (3-4 times/d), and high (5 or more times/d) amount. Amount of khar consumption was divided to no (who do not consume), low (who consume 1-2 times/mo), medium (who consume 3-4 times/mo), and high (who consume 5 or more times/mo) amount.

RNA isolation and complementary DNA (cDNA) preparation

The total RNA was isolated manually from blood and homogenized tissue samples using TRIzol Reagent (Invitrogen). The cDNA was prepared using iScript Reverse Transcription Reagents (Bio-Rad Laboratories, Inc.) and maintained at −20°C.

mRNA expression analysis by real-time PCR

mRNA expression analysis was performed in a Rotor-Gene Q real-time PCR detection system (Qiagen) for both blood and tissue samples. ß-actin, a house keeping gene, was used as a reference gene for normalization. The primer sequences for TGF-β1, TGF-βR2, and ß-actin genes were: Forward (F): 5′-TCGCCAGAGTGGTTATCTT-3′, Reverse (R): 5′-TAGTGAACCCGTTGATGTCC-3′; F: 5′-TGTGGCTGTATGGAGAAAGAAT-3′, R: 5′-ACAAGTCAGGATTGCTGGTG-3′; and F: 5′-AGATGTGGATCAGCAAGCAG-3′, R: 5′-GCGCAAGTTAGGTTTTGTCA-3′, respectively. PCR amplification was performed using the SYBR Green method according to the supplier's instructions. The formula Comparative Ct ($2^{-\Delta\Delta Ct}$) method was used for manual estimation of the level of expression of the studied genes.

Statistical analysis

All the statistical analyses were performed on Statistical Package for Social Sciences version 18.0. All the data were calculated as mean ± standard deviation. All the tests were 2-tailed and considered significant when the *P*value <0.05. The nonparametric Mann-Whitney U test or Kruskal-Wallis H test was selected for the association study with different lifestyle factors and clinicopathologic parameters. Kaplan-Meier survival analysis was carried out using the log-rank test and univariate analysis was constructed using Cox's regression model.

Results

Expression of TGF-β1 and TGF-βR2 gene in ESCC

Out of 76 ESCC cases, 69 cases (91%) showed upregulation of TGF-β1 with mean fold change 2.84 ± 1.34, whereas 7 cases (9%) showed downregulation (0.49 ± 0.23) for tissue samples. For

blood samples, 42 cases (55%) showed upregulation of TGF-β1 (2.43 $\pm$ 1.24), whereas 34 cases (45%) showed downregulation (0.29 $\pm$ 0.26). While analyzing TGF-βR2 expression, 68 cases (89%) showed downregulation (0.27 $\pm$ 0.23) and 8 cases (11%) showed upregulation (1.59 $\pm$ 0.64) in tissue samples. For blood samples, 57 cases (75%) showed downregulation (0.30 $\pm$ 0.26) and 19 cases (25%) showed upregulation (2.62 $\pm$ 1.47). Separate expression profile of TGF-β1 and TGF-βR2 gene in male and female patients is listed in Table 1.

Association of expression of TGF-β1 and TGF-βR2 gene and clinicopathological parameters in ESCC

We analyzed the association of the expression profile of TGF-β1 and TGF-βR2 with 8 different clinicopathologic parameters. Differences in histopathology grade and location of the tumor showed significant difference ($P < 0.05$) with the change in the expression of TGF-β1 in tissue samples among male cases and female cases showed significant difference only in histopathology grade. While analyzing blood samples, both male and female cases showed significant difference only in histopathology grade. For TGF-βR2 gene, male cases showed significant difference in dysphasia grade and age group while females showed significant association in dysphasia grade, age group, and histopathology grade in tissue samples. In blood samples, both male and female patients showed significant association only in dysphasia grade. Association study of TGF-β1 and TGF-βR2 gene expression with different clinicopathological parameters in male and female patients is listed in Table 2.

Association of expression of TGF-β1 and TGF-βR2 gene and different lifestyle factors in ESCC

The study also targeted the association of 16 different lifestyle factors (some food habits) with the expression study. In TGF-β1 expression study, significant associations ($P < 0.05$) were found in the consumption of betel nut, tobacco, spices, smoked food, hot food and difference in amount of tea taken in tissue samples of male patients. Among females, habits of consumption of betel nut, smoked food, hot food and fast food were noted with significant association in tissue samples. While analyzing blood samples, consumption of betel nut, tobacco, smoked food, and difference in types of tea taken showed significant difference among male patients while consumption of betel nut, hot food, smoked food, difference in types of tea taken, and differences in amount of khar consumed showed significant difference among female patients. For TGF-βR2 gene, male cases showed significant difference in smoking, consumption of spices, fast food, and alcohol while females showed significant association with consumption of spices, tobacco, khar, difference in the amount of khar consumption and difference in amount of tea taken in tissue samples. In blood samples, males showed significant association with consumption of khar, pickles, and smoking while females showed significant association with consumption of khar and difference in the amount of khar consumption. Association study of TGF-β1 and TGF-βR2 gene expression with different lifestyle factors in male and female patients is listed in Table 3.

Relationship between TGF-β1 and TGF-βR2 expression in ESCC

No significant association was found between TGF-β1 and TGF-βR2 expression in both blood and tissue samples. But significant correlation in TGF-β1 expression between blood and tissue samples has seen with $P = 0.008$ and Pearson coefficient $= 0.303$. Again, no association has been observed in TGF-βR2 expression in blood and tissue samples. Association study of TGF-β1 and TGF-βR2 gene expression is listed in Table 4.

Table 1
Expression profile of TGF-β1 and TGF-βR2 gene in blood and tissue of ESCC patients as mean ± standard deviation.

	TGF-β1 tissue		TGF-β1 blood		TGF-βR2 tissue		TGF-βR2 blood	
	Upregulation	Downregulation	Upregulation	Downregulation	Upregulation	Downregulation	Upregulation	Downregulation
Total (N = 76)	2.84 ± 1.34 (91%)	0.49 ± 0.23 (9%)	2.43 ± 1.24 (55%)	0.29 ± 0.26 (45%)	1.59 ± 0.64 (11%)	0.27 ± 0.23 (89%)	2.62 ± 1.47 (25%)	0.30 ± 0.26 (75%)
Male (N = 49)	3.05 ± 1.50 (92%)	0.51 ± 0.30 (8%)	2.83 ± 1.25 (57%)	0.30 ± 0.24 (43%)	1.59 ± 0.59 (10%)	0.28 ± 0.20 (90%)	2.50 ± 1.58 (29%)	0.23 ± 0.23 (71%)
Female (N = 27)	2.44 ± 0.88 (89%)	0.46 ± 0.15 (11%)	1.65 ± 0.81 (52%)	0.26 ± 0.28 (48%)	1.60 ± 0.86 (11%)	0.25 ± 0.27 (89%)	2.97 ± 1.19 (19%)	0.42 ± 0.28 (81%)

N = Number of patients.

Table 2
Association of TGF-β1 and TGF-βR2 gene expression with different clinicopathologic parameters in ESCC.

Clinicopathological parameters	No of cases (%)		TGF-β1 tissue				TGF-β1 blood				TGF-βR2 tissue				TGF-βR2 blood			
	Male	Female	Male		Female		Male		Female		Male		Female		Male		Female	
			mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value
Age group				0.870		0.099		0.382		0.856		0.028*		0.710		0.274		0.398
30-40 y	04(08.16)	05(18.51)	3.17 ± 1.43		2.96 ± 0.69		2.45 ± 1.99		0.74 ± 0.53		0.83 ± 0.41		0.29 ± 0.30		1.91 ± 3.01		2.15 ± 2.00	
40-50 y	09(18.36)	05(18.51)	2.62 ± 1.22		1.63 ± 1.20		2.90 ± 2.05		1.53 ± 1.65		0.58 ± 0.52		0.35 ± 0.38		0.64 ± 0.67		1.11 ± 1.13	
50-60 y	17(34.69)	12(44.44)	3.19 ± 1.74		1.95 ± 0.94		1.44 ± 1.45		0.97 ± 0.75		0.27 ± 0.20		0.41 ± 0.75		0.95 ± 1.28		0.40 ± 0.30	
60-70 y	15(30.61)	04(14.81)	2.84 ± 1.77		3.02 ± 0.92		1.32 ± 1.15		0.48 ± 0.73		0.26 ± 0.28		0.64 ± 0.45		0.87 ± 1.22		0.63 ± 0.24	
70-80 y	04(08.16)	01(03.70)	3.48 ± 1.79		1.43 ± 0.00		1.31 ± 0.76		1.04 ± 0.00		0.81 ± 1.16		0.19 ± 0.00		0.06 ± 0.03		0.65 ± 0.00	
Gender				0.146				0.056				0.413				0.285		
Male	49(64.47)		2.84 ± 1.60				1.75 ± 1.58				0.41 ± 0.47				0.81 ± 1.33			
Female	27(35.52)		2.22 ± 1.04				0.98 ± 0.93				0.40 ± 0.55				0.90 ± 1.13			
Location of tumor				0.018*		0.747		0.315		0.664		0.908		0.366		0.966		0.875
Upper esophagus	12(24.48)	12(44.44)	3.23 ± 1.90		2.29 ± 1.04		1.97 ± 1.51		0.92 ± 1.09		0.37 ± 0.27		0.61 ± 0.74		0.99 ± 1.46		0.91 ± 1.24	
Middle esophagus	18(36.73)	09(33.33)	1.94 ± 0.82		2.30 ± 0.99		1.36 ± 1.41		1.19 ± 0.92		0.38 ± 0.34		0.27 ± 0.30		0.99 ± 1.68		1.16 ± 1.35	
Lower esophagus	19(38.77)	06(22.22)	3.45 ± 1.64		1.95 ± 1.27		1.97 ± 1.77		0.79 ± 0.59		0.48 ± 0.66		0.20 ± 0.23		0.70 ± 0.85		0.47 ± 0.29	
Histopathology				0.024*		0.004*		0.047*		0.006*		0.968		0.033*		0.752		0.249
grade	18(36.73)	08(29.62)	2.10 ± 1.34		1.23 ± 0.89		1.16 ± 1.53		0.27 ± 0.36		0.41 ± 0.57		0.24 ± 0.37		0.78 ± 1.25		0.37 ± 0.26	
Grade1:well	27(35.52)	16(59.25)	3.13 ± 1.61		2.50 ± 0.81		1.98 ± 1.49		1.16 ± 0.78		0.40 ± 0.40		0.56 ± 0.63		1.00 ± 1.48		0.99 ± 1.14	
differentiated	04(08.16)	03(11.11)	4.25 ± 1.22		3.33 ± 0.39		2.81 ± 1.82		1.92 ± 1.59		0.49 ± 0.55		0.02 ± .0.03		0.46 ± 0.41		1.78 ± 2.12	
Grade2: moderately differentiated																		
Grade3:poorly differentiated																		
Dysphagia grade				0.763		0.543		0.758		0.730		0.024*		0.041*		0.029*		0.010*
Grade 0:	00(00.00)	01(03.70)	2.59 ± 1.47		3.78 ± 0.00		1.94 ± 1.88		0.82 ± 0.00		0.62 ± 0.61		0.00 ± 0.00		1.06 ± 1.55		4.22 ± 0.00	
Asymptomatic	23(46.93)	11(40.74)	3.21 ± 2.22		2.18 ± 1.04		2.15 ± 1.31		0.83 ± 0.81		0.38 ± 0.24		0.57 ± 0.35		0.02 ± 0.02		1.01 ± 0.78	
Grade1: Solids with	03(06.12)	03(11.11)	3.09 ± 1.57		2.39 ± 1.83		1.53 ± 1.20		0.72 ± 1.16		0.23 ± 0.13		0.25 ± 0.38		0.79 ± 1.25		0.15 ± 0.14	
some dysphagia	19(38.77)	11(40.74)	2.86 ± 2.43		2.04 ± 0.88		1.38 ± 1.78		1.28 ± 1.06		0.11 ± 0.09		0.35 ± 0.75		0.85 ± 0.57		0.38 ± 0.21	
Grade2: Soft or	04(08.16)	01(03.70)			2.53 ± 0.00				0.34 ± 0.00				0.00 ± 0.00				4.25 ± 0.00	
pureed food only																		
Grade3:Liquids only																		
Grade 4: No swallowing at all																		
Tumor stage				0.944		0.363		0.993		0.086		0.326		0.089		0.353		0.853
Stage1	00(00.00)	02(07.40)	3.00 ± 1.85		1.83 ± 1.78		1.64 ± 1.57		0.11 ± 0.06		0.42 ± 0.65		0.69 ± 0.64		1.20 ± 1.32		0.53 ± 0.08	
Stage2	14(28.57)	07(25.92)	2.72 ± 1.57		1.62 ± 1.18		1.75 ± 1.45		0.64 ± 0.59		0.44 ± 0.36		0.53 ± 0.95		0.72 ± 1.42		1.11 ± 1.64	
Stage3	22(44.89)	13(26.53)	2.87 ± 1.46		2.54 ± 0.95		1.86 ± 1.58		0.99 ± 0.79		0.36 ± 0.44		0.41 ± 0.33		0.79 ± 1.22		0.73 ± 0.59	
Stage4	13(26.53)	05(18.51)			2.37 ± 0.65				1.80 ± 1.32				0.09 ± 0.11				1.18 ± 1.72	

(*continued on next page*)

Table 2 (*continued*)

Clinicopathological parameters	No of cases (%)		TGF-β1 tissue				TGF-β1 blood				TGF-βR2 tissue				TGF-βR2 blood			
	Male	Female	Male		Female		Male		Female		Male		Female		Male		Female	
			mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value
Node stage				0.543		0.236		0.237		0.071		0.957		0.679		0.963		0.569
Stage 0	13(26.53)	07(25.92)	2.60 ± 1.85		1.55 ± 1.10		1.08 ± 1.30		0.36 ± 0.50		0.46 ± 0.65		0.31 ± 0.40		1.09 ± 1.45		0.58 ± 0.54	
Stage1	23(46.93)	09(33.33)	2.67 ± 1.48		2.28 ± 1.02		1.73 ± 1.54		1.65 ± 1.23		0.35 ± 0.32		0.28 ± 0.33		0.71 ± 0.97		0.74 ± 0.93	
Stage2	10(20.40)	08(29.62)	3.33 ± 1.63		2.61 ± 0.93		2.19 ± 1.38		0.73 ± 0.47		0.41 ± 0.38		0.70 ± 0.85		1.21 ± 2.05		1.48 ± 1.72	
Stage3	03(06.12)	03(11.11)	3.58 ± 1.50		2.55 ± 0.97		3.00 ± 2.44		1.10 ± 0.07		0.68 ± 0.93		0.20 ± 0.14		0.39 ± 0.31		0.56 ± 0.24	
Metastasis				0.259		0.142		0.493		0.803		0.493		0.190		0.344		0.574
Absent	42(85.71)	22(81.48)	2.73 ± 1.58		2.09 ± 1.03		1.63 ± 1.49		1.02 ± 1.01		0.41 ± 0.46		0.39 ± 0.60		0.95 ± 1.41		0.99 ± 1.24	
Present	07(14.28)	05(18.51)	3.50 ± 1.64		2.79 ± 1.00		2.43 ± 2.03		0.81 ± 0.41		0.41 ± 0.60		0.45 ± 0.25		0.50 ± 0.77		0.48 ± 0.32	

P value <0.05 were considered to be statistically significant and are indicated as * in the table.

Table 3
Association of TGF-β1 and TGF-βR2 gene expression with different lifestyle factors in ESCC.

Lifestyle factors(food habits)	No of cases (%)		TGF-β1 tissue				TGF-β1 blood				TGF-βR2 tissue				TGF-βR2 blood			
	Male	Female	Male		Female		Male		Female		Male		Female		Male		Female	
			mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value
Betel nut				0.029*		0.031*		0.043*		0.031*		0.208		0.537		0.180		0.217
Chewers	38(77.55)	24(88.88)	3.12 ± 1.61		2.37 ± 0.98		2.01 ± 1.64		1.09 ± 0.92		0.46 ± 0.52		0.41 ± 0.56		0.72 ± 1.20		0.97 ± 1.18	
Nonchewers	11(22.44)	03(11.11)	1.90 ± 1.21		0.98 ± 0.67		0.83 ± 0.87		0.09 ± 0.10		0.25 ± 0.21		0.35 ± 0.59		1.41 ± 1.66		0.34 ± 0.54	
Tobacco				0.010*		0.200		0.047*		0.841		0.052		0.035*		0.281		0.688
Chewers	38(77.55)	17(62.96)	3.16 ± 1.62		2.01 ± 1.17		1.94 ± 1.55		1.04 ± 1.06		0.48 ± 0.51		0.22 ± 0.26		1.03 ± 1.47		0.81 ± 1.04	
Nonchewers	11(22.44)	10(37.03)	1.76 ± 0.98		2.57 ± 0.70		1.08 ± 1.57		0.88 ± 0.67		0.20 ± 0.17		0.72 ± 0.77		0.33 ± 0.37		1.05 ± 1.32	
Alcohol				0.602		0.441		0.521		0.441		0.020*		0.158		0.302		0.304
Alcoholic	23(46.93)	01(03.70)	2.76 ± 1.75		1.63 ± 0.00		2.00 ± 1.82		0.20 ± 0.00		0.28 ± 0.36		1.04 ± 0.00		0.55 ± 0.74		0.97 ± 0.00	
Nonalcoholic	26(53.06)	26(96.29)	2.92 ± 1.49		2.24 ± 1.06		1.52 ± 1.33		1.01 ± 0.93		0.53 ± 0.53		0.38 ± 0.55		1.16 ± 1.66		0.89 ± 1.16	
Smoking				0.469		0.199		0.717		0.369		0.015*		0.441		0.021*		0.608
Smokers	27(55.10)	01(03.70)	2.69 ± 1.61		3.48 ± 0.00		1.77 ± 1.48		1.33 ± 0.00		0.25 ± 0.20		0.64 ± 0.00		0.69 ± 1.27		0.67 ± 0.00	
Nonsmokers	22(44.89)	26(96.29)	3.04 ± 1.61		2.17 ± 1.03		1.72 ± 1.72		0.97 ± 0.94		0.62 ± 0.62		0.39 ± 0.56		1.10 ± 1.41		0.90 ± 1.16	
Meat				0.157		0.521		0.777		0.521		0.358		0.095		0.138		0.898
Consumers	48(97.95)	26(96.29)	2.79 ± 1.58		2.24 ± 1.06		1.76 ± 1.59		0.97 ± 0.94		0.41 ± 0.48		0.32 ± 0.34		0.89 ± 1.34		0.91 ± 1.16	
Nonconsumers	01(02.04)	01(03.70)	5.20 ± 0.00		1.68 ± 0.00		082 ± 0.00		1.27 ± 0.00		0.53 ± 0.00		2.60 ± 0.00		0.02 ± 0.00		0.56 ± 0.00	
Fish				0.884		0.711		0.228		0.165		0.243		0.459		0.841		0.643
Consumers	45(91.83)	25(92.59)	2.82 ± 1.51		2.19 ± 1.05		1.84 ± 1.61		0.93 ± 0.93		0.40 ± 0.47		0.33 ± 0.35		0.91 ± 1.38		0.93 ± 1.17	
Nonconsumers	04(08.16)	02(07.40)	3.08 ± 2.75		2.55 ± 1.23		0.64 ± 0.47		1.67 ± 0.56		0.60 ± 0.47		1.32 ± 1.80		0.54 ± 0.69		0.43 ± 0.17	
Egg				0.211		0.165		0.802		0.247		0.835		0.877		0.786		0.190
Consumers	46(93.87)	24(88.88)	2.77 ± 1.60		2.31 ± 1.07		1.77 ± 1.59		1.05 ± 0.94		0.42 ± 0.48		0.34 ± 0.35		0.90 ± 1.37		0.97 ± 1.18	
Nonconsumers	03(06.12)	03(11.11)	3.93 ± 1.32		1.47 ± 0.38		1.36 ± 1.55		0.47 ± 0.69		0.29 ± 0.21		0.88 ± 1.48		0.54 ± 0.59		0.30 ± 0.27	
Hot food				0.044*		0.045*		0.928		0.034*		0.821		0.763		0.726		0.160
Consumers	36(73.46)	17(62.96)	3.08 ± 1.44		2.53 ± 0.94		1.73 ± 1.53		1.28 ± 1.01		0.36 ± 0.33		0.40 ± 0.62		0.85 ± 1.20		1.08 ± 1.29	
Nonconsumers	13(26.53)	10(37.03)	2.18 ± 1.88		1.68 ± 1.03		1.79 ± 1.77		0.55 ± 0.59		0.57 ± 0.73		0.40 ± 0.45		0.94 ± 1.71		0.58 ± 0.76	
Smoked food				0.024*		0.021*		0.002*		0.007*		0.579		0.802		0.651		0.088
Consumers	43(87.75)	17(62.96)	3.01 ± 1.49		2.57 ± 0.91		1.96 ± 1.56		1.30 ± 0.98		0.40 ± 0.47		0.44 ± 0.62		0.84 ± 1.15		1.19 ± 1.34	
Nonconsumers	06(12.24)	10(37.03)	1.67 ± 1.98		1.62 ± 1.03		0.19 ± 0.17		0.44 ± 0.51		0.49 ± 0.49		0.35 ± 0.43		1.03 ± 2.04		0.39 ± 0.32	
Fast food				0.452		0.031*		0.528		0.145		0.044*		0.225		0.677		0.286
Consumers	29(59.18)	14(51.85)	2.94 ± 1.37		2.65 ± 0.77		1.84 ± 1.49		1.28 ± 1.12		0.27 ± 0.22		0.30 ± 0.38		0.89 ± 1.28		1.29 ± 1.46	
Nonconsumers	20(40.81)	13(48.14)	2.70 ± 1.91		1.76 ± 1.13		1.61 ± 1.72		0.66 ± 0.54		0.62 ± 0.65		0.51 ± 0.69		0.85 ± 1.44		0.47 ± 0.32	
Spices				0.018*		0.641		0.868		0.448		0.042*		0.036*		0.820		0.641
Consumers	30(61.22)	21(77.77)	3.22 ± 1.45		2.27 ± 1.04		1.68 ± 1.41		1.07 ± 0.99		0.27 ± 0.21		0.25 ± 0.31		0.91 ± 1.28		0.87 ± 1.20	
Nonconsumers	19(38.77)	06(22.22)	2.25 ± 1.68		2.03 ± 1.12		1.86 ± 1.87		0.67 ± 0.64		0.65 ± 0.67		0.93 ± 0.88		0.82 ± 1.46		0.99 ± 0.98	
Pickle				0.064		0.811		0.915		0.453		0.714		0.891		0.022*		0.056
Consumers	44(89.79)	23(85.18)	2.71 ± 1.57		2.25 ± 0.99		1.76 ± 1.63		1.05 ± 0.96		0.41 ± 0.48		0.43 ± 0.59		0.63 ± 0.90		1.01 ± 1.19	
Nonconsumers	05(10.20	04(14.81)	4.06 ± 1.45		2.05 ± 1.48		1.66 ± 1.29		0.62 ± 0.67		0.46 ± 0.47		0.27 ± 0.28		2.64 ± 2.44		0.25 ± 0.31	

(*continued on next page*)

Table 3 (*continued*)

Lifestyle factors(food habits)	No of cases (%)		TGF-β1 tissue				TGF-β1 blood				TGF-βR2 tissue				TGF-βR2 blood			
	Male	Female	Male		Female		Male		Female		Male		Female		Male		Female	
			mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value	mRNA expression	p value
Type of tea				0.169		0.194		0.047*		0.024*		0.764		0.865		0.521		0.080
Red tea	13(26.53)	05(18.51)	2.24 ± 1.74		1.39 ± 1.16		1.12 ± 1.65		0.24 ± 0.44		0.37 ± 0.35		0.30 ± 0.48		0.70 ± 0.82		0.22 ± 0.27	
Milk tea	16(32.65)	10(37.03)	2.84 ± 1.41		2.39 ± 0.77		2.16 ± 1.70		1.23 ± 0.95		0.37 ± 0.44		0.40 ± 0.39		1.14 ± 1.52		1.28 ± 1.36	
Both	20(40.81)	12(44.44)	3.24 ± 1.59		2.42 ± 1.10		1.82 ± 1.37		1.09 ± 0.95		0.48 ± 0.57		0.45 ± 0.71		0.78 ± 1.47		0.85 ± 1.07	
Amount of tea Low				0.025*		0.904		0.859		0.784		0.345		0.040*		0.973		0.504
Medium	16(32.65)	17(62.96)	1.95 ± 1.26		2.22 ± 1.06		1.64 ± 1.66		1.02 ± 1.02		0.32 ± 0.35		0.32 ± 0.65		0.95 ± 1.58		0.91 ± 1.31	
High	22(44.89)	08(16.32)	3.24 ± 1.54		2.26 ± 1.21		1.87 ± 1.65		0.86 ± 0.88		0.50 ± 0.58		0.61 ± 0.32		0.90 ± 1.34		1.00 ± 0.88	
	11(22.44)	02(07.40)	3.36 ± 1.73		1.99 ± 0.08		1.65 ± 1.42		1.17 ± 0.19		0.37 ± 0.39		0.27 ± 0.02		0.71 ± 0.99		0.40 ± 0.05	
Khar				0.503		0.293		0.814		0.580		0.598		0.011*		0.027*		0.003*
Consumers	39(79.59)	20(74.07)	2.76 ± 1.51		2.08 ± 1.12		1.80 ± 1.63		0.94 ± 0.96		0.43 ± 0.38		0.22 ± 0.27		0.61 ± 0.93		0.57 ± 0.89	
Nonconsumers	10(20.40)	07(25.92)	3.16 ± 1.98		2.60 ± 0.73		1.53 ± 1.41		1.10 ± 0.87		0.41 ± 0.50		0.92 ± 0.81		1.91 ± 2.08		1.81 ± 1.32	
Amount of khar				0.404		0.445		0.680		0.036*		0.462		0.005*		0.101		0.029*
No	10(20.40)	07(25.92)	3.16 ± 1.98		2.60 ± 0.73		1.53 ± 1.41		1.10 ± 0.87		0.43 ± 0.38		0.92 ± 0.81		1.91 ± 2.08		1.81 ± 1.32	
Low	23(46.93)	08(16.32)	2.46 ± 1.43		1.80 ± 1.08		1.65 ± 1.62		0.36 ± 0.43		0.36 ± 0.29		0.37 ± 0.33		0.43 ± 0.59		0.41 ± 0.31	
Medium	06(12.24)	06(22.22)	3.46 ± 1.22		2.57 ± 0.86		1.56 ± 1.21		1.56 ± 0.55		0.50 ± 0.99		0.20 ± 0.23		0.94 ± 1.34		0.48 ± 0.24	
High	10(20.40)	06(22.22)	3.02 ± 1.78		1.98 ± 1.40		2.29 ± 1.91		1.10 ± 1.39		0.47 ± 0.53		0.04 ± 0.09		0.83 ± 1.27		0.89 ± 1.64	

P value <0.05 were considered to be statistically significant and are indicated as * in the table.

Table 4
Association of TGF-β1 and TGF-βR2 gene expression in blood and tissue level.

	P value	Pearson correlation
TGF-β1 tissue and TGF-β1 blood	0.008*	0.303
TGF-βR2 tissue and TGF-βR2 blood	0.876	0.018
TGF-β1 tissue and TGF-βR2 tissue	0.978	0.003
TGF-βR2 blood and TGF-βR2 blood	0.835	-0.024

P value <0.05 were considered to be statistically significant and are indicated as * in the table.

Survival analysis

In this study, ESCC patients were followed up until death, and the Kaplan-Meier survival analysis was carried with different factors to check their role in ESCC. The mean survival time for nonalcoholic and alcoholic patients were 21 months and 15 months ($P = 0.030$), respectively, among males. Males having hot food in their diet were observed to have lower survival time of 16 months compared to the others who do not take it having survival of 25 months ($P = 0.021$). Males having tumor stage 2 with a mean survival time of approximately 23 months were noted better survival than stage 3 and stage 4 having survival time of approximately 18 and 12 months, respectively ($P = 0.032$). Similarly, node stages 0, 1, 2, and 3 were noted with different mean survival time of approximately 20, 21, 12, and 10 months, respectively ($P = 0.029$). Nonmetastasis male patients were seen to be having a better survival of approximately 20 months than metastasis male patients of having survival of approximately 9 months ($P = 0.000$).

Similarly, the mean survival time for tobacco chewers and nonchewers patients were 13 months and 22 months ($P = 0.034$) respectively among females. Female patients having hot food and smoked food in their diet were seen to have lower significant survival time ($P = 0.002$ and $P = 0.046$, respectively). Females having tumor stage 1 with survival time of approximately 31 months were noted better survival among all the other stages, and stages 2, 3, and 4 were noted lower survival time of approximately 15, 11, 12 months, respectively ($P = 0.032$). Females having metastasis were seen to be having a lower survival of approximately 6 months than nonmetastasis females having survival of approximately 19 months ($P = 0.000$). Statistically significant survival difference ($P = 0.004$) was also seen in TGF-βR2 expression in tissue samples of female patients. Survival analysis in males and females is listed in Table 5.

Cox regression analyses and hazard outcomes

According to the univariate model of Cox regression, the hazard ratio (HR) represents the ratio of the hazard outcomes corresponding to the conditions represented by 2 groups of a variable. A HR of 1 represents that there is no survival difference between the 2 groups and a HR of less than or greater than 1 represents that one group possesses better survival than the other. According to this model, the hazard (mortality) ratio for an alcoholic male (group 1) patient is 1.853 times higher that of a nonalcoholic male (group 2) patient ($P = 0.043$). Similarly, the hazard rate is 2.080 times higher for males taking hot food ($P = 0.034$) and is 2.224 times higher for males having a tumor in the lower part of the esophagus compared to males having it in the upper part ($P = 0.047$). The HR for male having tumor stage 4 is 2.632 times compared to tumor stage 2 ($P = 0.017$). Again, the HR for a metastatic male patient is 5.539 times that of a nonmetaststic patient ($P = 0.000$) indicating a very high mortality rate in metastatic patients.

Similarly, the hazard rate is 2.456 times higher for tobacco chewers compared to nonchewers among females ($P = 0.044$) and is 3.713 times higher for females taking hot food ($P = 0.005$). Hazard rate is also 5.345 times higher in females having histopathology grade 3 compared to one having histopathology grade1 ($P = 0.026$). Significant difference of hazard rate was also seen between node grade 0 and node grade 4 ($P = 0.025$) among females. The HR for a metastatic female patient is 5.726 times that of a nonmetastatic female ($P = 0.002$). Moreover, the hazard

Table 5
Survival analysis of ESCC patients.

Parameters	Grouping	Male					Female				
		Mean estimate	Standard error	95% confidence interval		*P* value	Mean estimate	Standard error	95% confidence interval		*P* value
				Lower bound	Upper bound				Lower bound	Upper bound	
Age group	30-40 y	14.500	4.406	5.863	23.137		10.000	1.897	6.281	13.719	
						0.232					0.155
	40-50 y	13.889	2.728	8.542	19.236		20.200	5.181	10.046	30.354	
	50-60 y	16.176	2.002	12.252	20.101		18.667	2.945	12.895	24.438	
	60-70 y	23.600	2.921	17.875	29.325		17.250	7.543	2.466	32.034	
	70-80 y	21.750	4.366	13.193	30.307		14.000	0.000	14.000	14.000	
Gender	Male	18.551	1.478	15.654	21.448	0.491					
	Female	16.963	1.988	13.066	20.860						
Location of tumor	Upper part	22.833	3.401	16.167	29.499	0.083	19.250	3.436	12.516	25.984	0.294
	Middle part	19.389	2.659	14.178	24.600		13.556	2.724	8.217	18.894	
	Lower part	14.947	1.444	12.117	17.777		17.500	4.137	9.391	25.609	
	Grade1	20.222	2.628	15.072	25.372	0.333	22.250	3.222	15.934	28.566	0.052
Histopathology grade											
	Grade2	18.148	1.925	14.375	21.921		15.938	2.653	10.738	21.137	
	Grade3	13.250	1.887	9.551	16.949		8.333	2.603	3.231	13.436	
Dysphagia grade	Grade 0	-	-	-	-	0.079	13.000	0.000	13.000	13.000	0.605
	Grade1	19.000	1.687	15.693	22.307		18.545	3.713	11.267	25.823	
	Grade2	25.667	6.771	12.395	38.939		20.333	6.960	6.691	33.975	
	Grade3	14.316	2.082	10.236	18.396		15.273	2.754	9.875	20.670	
	Grade4	27.750	5.249	17.463	38.037		12.000	0.000	12.000	12.000	
Tumor stage	Stage1	-	-	-	-	0.032*	31.000	7.000	17.280	44.720	0.032*
	Stage2	23.286	2.237	18.901	27.670		25.286	2.398	20.586	29.985	
	Stage3	18.818	2.023	14.853	22.784		11.923	2.408	7.202	16.644	
	Stage4	12.769	2.866	7.152	18.387		12.800	3.426	6.084	19.516	
Node stage	Stage0	20.077	2.625	14.932	25.222	0.029*	22.000	3.773	14.604	29.396	0.116
	Stage1	21.304	2.344	16.710	25.899		17.333	2.995	11.462	23.204	
	Stage2	12.600	2.202	8.284	16.916		15.750	4.216	7.486	24.014	
	Stage3	10.667	3.667	3.480	17.853		7.333	3.333	0.800	13.867	

(*continued on next page*)

Table 5 (*continued*)

Parameters	Grouping	Male					Female				
		Mean estimate	Standard error	95% confidence interval		*P* value	Mean estimate	Standard error	95% confidence interval		*P* value
				Lower bound	Upper bound				Lower bound	Upper bound	
Metastasis	Absent	20.143	1.588	17.029	23.256	0.000*	19.364	2.075	15.297	23.431	0.000*
	Present	9.000	1.069	6.905	11.095		6.400	2.249	1.991	10.809	
Betel nut		22.455	3.248	16.088	28.821	0.181	31.333	2.186	27.049	35.618	0.067
	Nonchewers										
	Chewers	17.395	1.601	14.257	20.533		15.167	1.927	11.390	18.943	
Tobacco		19.091	3.281	12.659	25.523	0.869	22.200	3.495	15.349	29.051	0.034*
	Nonchewers										
	Chewers	18.316	1.621	15.140	21.492		13.882	2.135	9.698	18.067	
Alcohol		21.346	2.285	16.867	25.825	0.030*	16.308	1.951	12.484	20.131	0.210
	Nonalcoholic										
	Alcoholic	15.391	1.591	12.272	18.510		34.000	0.000	34.000	34.000	
Smoking		18.773	2.264	14.334	23.211	0.799	17.115	2.060	13.078	21.153	0.536
	Nonsmokers										
	Smokers	18.296	1.911	14.550	22.043		13.000	0.000	13.000	13.000	
Meat		26.000	0.000	26.000	26.000	0.769	29.000	0.000	29.000	29.000	0.435
	Nonconsumers										
	Consumers	18.396	1.501	15.455	21.337		16.500	2.009	12.562	20.438	
Fish		27.500	5.154	17.398	37.602	0.197	24.000	5.000	14.200	33.800	0.540
	Nonconsumers										
	Consumers	17.689	1.461	14.826	20.552		16.400	2.089	12.306	20.494	
Egg		15.667	5.548	4.793	26.540	0.464	24.000	4.041	16.079	31.921	0.435
	Nonconsumers										
	Consumers	18.739	1.542	15.716	21.762		16.083	2.132	11.904	20.263	
Hot food		25.077	2.172	20.821	29.333	0.021*	25.200	2.662	19.983	30.417	0.002*
	Nonconsumers										
	Consumers	16.167	1.676	12.881	19.452		12.118	1.974	8.249	15.986	
Smoked food		18.667	4.883	9.096	28.237	0.879	21.700	3.534	14.773	28.627	0.046*
	Nonconsumers										
	Consumers	18.535	1.565	15.467	21.602		14.176	2.182	9.901	18.452	
Fast food		20.200	1.712	16.845	23.555	0.555	18.769	3.107	12.679	24.860	0.522
	Nonconsumers										
	Consumers	17.414	2.191	13.118	21.709		15.286	2.554	10.281	20.291	
Spices		22.053	2.040	18.053	26.052	0.100	24.333	4.349	15.810	32.857	0.086
	Nonconsumers										
	Consumers	16.300	1.917	12.542	20.058		14.857	2.064	10.812	18.902	

(*continued on next page*)

Table 5 (*continued*)

Parameters	Grouping	Male					Female				
		Mean estimate	Standard error	95% confidence interval		*P* value	Mean estimate	Standard error	95% confidence interval		*P* value
				Lower bound	Upper bound				Lower bound	Upper bound	
Pickle		21.200	3.541	14.259	28.141	0.619	16.750	5.170	6.617	26.883	0.678
	Nonconsumers										
	Consumers	18.250	1.600	15.115	21.385		17.000	2.202	12.684	21.316	
Type of tea	Red tea	17.692	2.161	13.456	21.929	0.855	24.800	6.020	13.001	36.599	0.142
	Milk tea	18.438	2.768	13.012	23.863		16.900	2.998	11.024	22.776	
	Both	19.100	2.471	14.257	23.943		13.750	2.478	8.894	18.606	
Amount of tea	Low	17.313	2.226	12.949	21.676	0.819	17.471	2.485	12.599	22.342	0.822
	Medium	19.636	2.236	15.254	24.018		16.750	3.886	9.134	24.366	
	High	18.000	3.504	11.131	24.869		13.500	10.500	0.000	34.080	
Khar		23.222	2.350	18.617	27.828	0.257	18.571	4.966	8.838	28.305	0.391
	Nonconsumers										
	Consumers	17.500	1.697	14.174	20.826		16.400	2.124	12.236	20.564	
Amount of khar	No	22.900	2.126	18.732	27.068	0.537	18.571	4.966	8.838	28.305	0.524
	Low	16.739	2.174	12.478	21.000		15.875	3.786	8.454	23.296	
	Medium	16.333	3.412	9.645	23.022		13.167	3.177	6.939	19.394	
	High	19.500	3.986	11.688	27.312		20.333	3.879	12.731	27.936	
TGFβ1 expression in tissue	Low	25.750	3.146	19.584	31.916	0.318	26.667	3.180	20.434	32.899	0.380
	High	17.911	1.555	14.862	20.960		15.750	2.084	11.666	19.834	
TGFβ1 expression in blood	Low	18.905	2.010	14.965	22.845	0.954	20.385	3.033	14.440	26.329	0.093
	High	18.679	2.082	14.598	22.759		14.071	2.506	9.160	18.983	
TGFβr2 expression in tissue	Low	17.864	1.476	14.970	20.757	0.218	15.042	1.846	11.424	18.660	0.004*
	High	22.600	4.618	13.548	31.652		33.667	2.603	28.564	38.769	
TGFβr2 expression in blood	Low	17.571	1.782	14.078	21.064	0.349	17.636	2.363	13.005	22.267	0.385
	High	20.929	2.467	16.093	25.764		14.800	3.426	8.084	21.516	

P value <0.05 were considered to be statistically significant and are indicated as * in the table.

rate is 0.087 times lower for females with higher level of TGF-βR2 expression in tissue samples compared to females with lower level of TGF-βR2 expression in their tissue ($P = 0.020$). Hazard analysis in males and females is listed in Table 6.

Discussion

This study targeted both blood and tissue samples of ESCC patients for expression profile analysis of the selected genes. Additionally, both male and female were separately studied for blood and tissue level gene expression analysis. Till date very less data have been reported on expression studies in tissue samples and only a few studies were conducted together in both blood and tissue samples. In India, no previous history of expression study of TGF-β signaling genes in esophageal cancer has been seen.

TGF-β1 is a cytokine that plays a key role in various biological processes like immunity regulation, cell proliferation, differentiation, apoptosis, immune surveillance, etc. Among the TGF-β super family members consisting of more than 60 proteins, it is the most well-studied isoform having various immune regulatory functions in mammals. Deregulation of its expression and activity has been observed in the pathogenesis of numerous diseases including cancer.[10] TGF-β1 plays a dual role in carcinogenesis, acting both as tumor suppressor and as tumor promoter. Generally, TGF-β1 was reported to have tumor suppressor activity in normal epithelial cells as well as early tumor developing stages. But dysregulation of TGF-β1 pathway promotes extensive signal reprogramming leading to the survival of cancer cells and allows successful spreading of cancer cells in other tissues. As TGF-β1can exert both procarcinogenic and anticarcinogenic effects during tumor progression, the exact biological conditions required for proper TGF-β1 activity are still unknown and how TGF-β1 achieves its distinct biological effects in tumor progression remains poorly understood.[18]

The biological effect of TGF-β1 is mediated by 2 independent receptors TGF-βR1and TGF-βR2 to activate its downstream signaling pathways. TGF-β ligands bind to a type II receptor and form a ligand-receptor complex at the plasma membrane which recruits and helps in phosphorylation of a type I receptor. This type I receptor then phosphorylates receptor-regulated SMADs (R-SMADS) which binds to common partner Smads (coSMADs) forming heteromeric complexes. These complexes accumulate in the nucleus where they act as transcription factors to regulate target gene expressions.[19] TGF-βR1and TGF-βR2can also mediate TGF-β1 signaling by activating non–Smad-dependent signaling pathways and these non-Smad pathways alone or in cooperation with the Smad pathway can modulate the activity of TGF-β1 signaling.[14]

TGF-βR2 also plays a vital function in the regulation of the TGF-β signaling pathway. Downregulation or loss of TGF-βR2 in many human cancers has been observed, which includes non–small cell lung cancer also. It can regulate TGF-β pathway by negative-feedback mechanisms, but very little is known about the underlying mechanism of its downregulation.[9]

In this study, the expression of TGF-β1 and TGF-βR2 gene is represented by mean fold change. The formula Comparative Ct ($2^{-\Delta\Delta Ct}$) method was used for manual estimation of the level of expression or fold change of the studied genes. If the level of expression or fold change value was calculated <1, the studied gene was considered downregulated and when the level of expression or fold change value was calculated >1, the studied gene was considered upregulated.

In this study, 91% cases showed high level of TGF-β1 expression in tissue samples, indicating its role as a prospective biomarker for screening ESCC in Northeast India. But only 55% cases showed high level of TGF-β1 expression in blood samples. The divergence in gene expression between tissue and blood level may be considered due to the diverse biological effects of TGF-β1. TGF-β1 plays distinct biological roles in cancer initiation and progression in blood and tissue level which can be predicted to be the difference in its expression level in tissue and blood level. Tissue expressed TGF-β1 induces epithelial to mesenchymal transition, promotes angiogenesis, induces evasion of immune surveillance, and thereby acts as a strong promoter of carcinogenesis, whereas blood expressed TGF-β1 could exert different effects on blood immune cells as well as epithelial cells. Part of this difference may be due to TGF-β1 being able to activate different

Table 6
Hazard analysis of ESCC patients.

Parameters	Group 1/group 2	Male				Group 1/group 2	Female			
		P value	Hazard ratio	95% confidence interval			*P* value	Hazard ratio	95% confidence interval	
				Lower	Upper				Lower	Upper
Age group	30-40 y/70-80 y	0.351	1.943	0.481	7.849	30-40 y/70-80 y	0.543	1.972	0.221	17.617
	40-50 y/70-80 y	0.362	1.751	0.525	5.840	40-50 y/70-80 y	0.515	0.477	0.051	4.430
	50-60 y/70-80 y	0.461	1.508	0.505	4.504	50-60 y/70-80 y	0.553	0.529	0.064	4.343
	60-70 y/70-80 y	0.691	0.795	0.256	2.467	60-70 y/70-80 y	0.437	0.394	0.038	4.120
Gender	Female/male	0.510	1.174	0.728	1.894					
Location of tumor	Middle/upper	0.434	1.374	0.620	3.048	Middle/upper	0.134	2.067	0.799	5.348
	Lower/upper	0.047*	2.224	1.010	4.894	Lower/upper	0.591	1.323	0.477	3.668
Histopathology grade	Grade 2/grade1	0.595	1.187	0.631	2.230	Grade 2/grade1	0.238	1.721	.699	4.234
	Grade 3/grade1	0.166	2.219	0.719	6.845	Grade 3/grade1	0.026*	5.345	1.226	23.300
Dysphagia	Grade 2/grade1	0.349	0.500	0.117	2.132	Grade 2/grade1	0.950	1.043	0.281	3.878
grade	Grade 3/grade1	0.167	1.557	0.830	2.921	Grade 3/grade1	0.285	1.642	0.661	4.078
	Grade 4/grade1	0.218	0.466	0.139	1.569	Grade 4/grade1	0.272	3.353	0.388	28.987
Tumor	Stage 3/stage 2	0.334	1.414	0.700	2.857	Stage 3/stage 2	0.064	2.511	0.947	6.657
stage	Stage 4/stage 2	0.017*	2.632	1.185	5.846	Stage 4/stage 2	0.079	2.948	0.883	9.846
Node stage	Stage 1/stage 0	0.797	0.911	0.449	1.849	Stage 1/stage 0	0.261	1.851	0.632	5.423
	Stage 2/stage 0	0.060	2.270	0.968	5.326	Stage 2/stage 0	0.346	1.668	0.575	4.838
	Stage 3/stage 0	0.099	2.961	0.815	10.759	Stage 3/stage 0	0.025*	5.435	1.232	23.976
Metastasis	Present/absent	0.000*	5.539	2.215	13.853	Present/absent	0.002*	5.726	1.873	17.500
Betel nut	Consumers/nonconsumers	0.212	1.577	0.772	3.221	Consumers/nonconsumers	0.085	3.009	0.861	10.520
Tobacco	Consumers/nonconsumers	0.877	1.057	0.523	2.140	Consumers/nonconsumers	0.044*	2.456	1.026	5.880
Alcohol	Consumers/nonconsumers	0.043*	1.853	1.020	3.364	Consumers/nonconsumers	0.242	0.292	0.037	2.290
Smoking	Consumers/nonconsumers	0.811	1.075	0.595	1.942	Consumers/nonconsumers	0.552	1.862	0.240	14.445

(*continued on next page*)

Table 6 (*continued*)

Parameters	Group 1/group 2	Male				Group 1/group 2	Female			
		P value	Hazard ratio	95% confidence interval			*P* value	Hazard ratio	95% confidence interval	
				Lower	Upper				Lower	Upper
Meat	Consumers/nonconsumers	0.780	1.329	0.181	9.742	Consumers/nonconsumers	0.456	2.161	0.286	16.351
Fish	Consumers/nonconsumers	0.235	2.038	0.629	6.599	Consumers/nonconsumers	0.552	1.555	0.362	6.673
Egg	Consumers/nonconsumers	0.488	0.658	0.202	2.146	Consumers/nonconsumers	0.450	1.600	0.473	5.413
Hot food	Consumers/nonconsumers	0.034*	2.080	1.058	4.088	Consumers/nonconsumers	0.005*	3.713	1.492	9.239
Smoked food	Consumers/nonconsumers	0.886	1.065	0.448	2.534	Consumers/nonconsumers	0.058	2.377	0.970	5.824
Fast food	Consumers/nonconsumers	0.578	1.185	0.652	2.153	Consumers/nonconsumers	0.532	1.283	0.587	2.804
Spices	Consumers/nonconsumers	0.125	1.609	0.877	2.951	Consumers/nonconsumers	0.099	2.266	0.856	5.997
Pickle	Consumers/nonconsumers	0.641	1.248	0.492	3.166	Consumers/nonconsumers	0.686	0.799	.270	2.367
Type of tea	Milk tea/red tea	0.693	0.858	0.401	1.837	Milk tea/red tea	0.193	2.189	0.674	7.112
	Both tea/red tea	0.610	0.830	0.405	1.700	Both tea/red tea	0.061	3.136	0.947	10.387
Amount of tea	Medium/low	0.553	0.819	0.423	1.585	Medium/low	0.910	1.051	0.448	2.467
	High/low	0.824	0.911	0.399	2.079	High/low	0.546	1.585	0.355	7.073
Khar	Consumers/nonconsumers	0.289	1.488	0.714	3.101	Consumers/nonconsumers	0.403	1.491	0.585	3.801
Amount of khar	Low/no	0.219	1.608	0.754	3.431	Low/no	0.452	1.512	0.514	4.446
	Medium/no	0.367	1.598	0.577	4.421	Medium/no	0.183	2.274	0.679	7.615
	High/no	0.776	1.145	0.450	2.915	High/no	0.802	1.159	0.367	3.660
TGFβ1 expression in tissue	High/low	0.352	1.633	0.581	4.589	High/low	0.396	1.693	0.502	5.712
TGFβ1 expression in blood	High/low	0.956	0.984	0.544	1.778	High/low	0.108	1.960	0.863	4.453
TGFβR2 expression in tissue	High/low	0.256	0.549	0.195	1.547	High/low	0.020*	0.087	0.011	0.684
TGFβR2 expression in blood	High/low	0.379	0.748	0.391	1.429	High/low	0.401	1.538	0.563	4.204

Note: The hazard ratio represents the ratio of (hazard outcome in group1)/(hazard outcome in group2). For example, while considering gender in this table the hazard ratio 1.174 represents that hazard outcome is 1.174 times higher for female (group 1) compared to male (group 2).
P value <0.05 were considered to be statistically significant and are indicated as * in the table.

signaling pathway depending on the genetic and epigenetic status of target cells and therefore can exert distinct influences on target cells. Collectively, TGF-β1 can involve in different intracellular signaling pathways which are diverse in their consequences and depend on the type of target cells, grade of differentiation, stage of neoplasm transformation, etc.[18]

Considering the divergence in gene expression between tissue and blood level, only tissue level TGF-β1 expression may be considered for ESCC screening because a huge number of patients (91% patients) were observed to have high levels of TGF-β1 expression in their tissue samples. Again, while analyzing TGF-β1 expression in tissue and blood samples with survival outcomes, no significant survival difference was noticed in our study and therefore we cannot link TGF-β1 expression to predict survival of ESCC patients in our studied population.

Again, downregulation of TGF-βR2 was observed in 89% tissue samples and 75% blood samples supporting its high potential as other molecular biomarkers for screening ESCC. Although screening of blood samples is more economic, minimally invasive and easier to conduct compared to screening of tissue samples; however, esophageal biopsy is needed to evaluate and confirm ESCC through histopathology. In this study, we evaluate a section of the tissue that were been screen by histopathology for studying the expression level. As ESCC is localized to certain portion of the esophagus, evaluation of biomarkers in tissue is significant compared to blood has been shown in this study. Moreover, significant survival association was also observed in the tissue level TGF-βR2 expression in female patients. This strongly supports TGF-βR2 expression in tumor tissue as potential biomarkers for ESCC screening as well as survival screening.

In this study, tumor tissue was collected with an endoscope and the expression level of TGF-β1 and TGF-βR2 were observed. Endoscope visualizes lumps or tumors that may be cancerous, but all lumps or tumors are not cancerous. Therefore, further histopathologic evaluation of biopsy samples taken from the suspicious lesions is required to confirm the diagnosis of cancer and this is a time-consuming and labor intensive process. Moreover, multiple biopsies were taken from patients to improve the accuracy of cancer diagnosis, which may be critical for patients. On the other hand, expression analysis is fast, easy to conduct, and less laborious which facilitates one time confirmation of cancer eradicating necessity of multiple biopsies.

The results of our findings have some consistencies and inconsistencies with the findings from previously conducted studies. This may due to variation in sample size, geographical regions, environmental factors, associated pathologic conditions, genetic, and/or epigenetic factors and ethnicity differences. These all play an essential role in the development of carcinogenesis.[20-22] High level of TGF-β expression was reported in many advanced breast cancer cases.[15] TGF-β expression is deregulated in colorectal cancer and elevated expression was found to associate with malignancy.[23] High level of TGF-β expression was reported in many gastrointestinal cancers. In gastric cancer, high TGF-β levels were observed and associated with lymph node metastasis.[14] Additionally, TGF-β was reported as a potential marker for detection of early stages of hepatocellular carcinoma having higher sensitivity than other traditional biomarkers.[24] Reduced level of TGF-βR2 expression was also observed in tumor progression of prostate cancer and hepatocellular carcinoma.[25-27] Downregulation of TGF-βR2 expression was also observed in gastric and breast cancers.[28] But increased level of TGF-βR2 expression was observed in many pancreatic cancer subtypes.[29]

In India, the role of dietary habits in developing esophageal cancer has been studied recently and much attention has been given to Northeast India, especially Assam has the highest incidence of esophageal cancer among all states of the country.[30] Betel nut chewing is one of the most alarming risk factors of carcinogenesis among Asian population.[31] Consumption of tobacco (chewing or smoking) and alcohol are also reported as 2 important risk factors for esophageal cancer.[32,33] Consumption of spices, hot food, some locally prepared food of Assam, eg, kalakhar, etc. were reported to have positive associations with esophageal cancer development.[30] Smoked food like smoked meat, smoked fish, etc. which contains nitrosamines, a potent carcinogen, also increases the risk of developing cancer.[32,34] Additionally, consumption of very hot beverages like hot tea consumption was also reported to have association with increased risk of esophageal cancer.[35]

Among all parameters, histopathology grading, consumption of betel nut and smoked food showed significant levels of association with TGF-β1 expression and dysphagia grading showed significant association with TGF-βR2 expression in both blood and tissue samples and while analyzing both male and female patients separately. These results strongly support their association with change in expression of the studied genes in ESCC.

Esophageal cancer is reported as the sixth most common cause of cancer-associated death worldwide having a relatively low survival rate. Histopathology grade, tumor stage, dysphasia grade, and all other clinicopathological factors influence the survival of esophageal cancer.[36] In this study, it was found that the survival of male patients is significantly associated ($P < 0.05$) with a habit of consumption of alcohol, hot food, differences in tumor stage, and lymph node staging and present or absence of metastasis while the survival of female patients is significantly associated with the habit of chewing tobacco, consumption of hot food, smoked food, difference in tumor stage, and the presence or absence of metastasis. Moreover, the univariate model of Cox regression analysis also supports this survival data. The hazard (mortality) rate was found significantly higher in male having tumor stage 4, consuming alcohol, hot food, having a tumor in the lower part of the esophagus, metastatic male and female patients, females having histopathology grade 3, females consuming hot food and tobacco and females having node stage 3. This kind of hazard outcomes also indicates individual influence of these parameters on the mortality rate of ESCC patients. While analyzing TGF-β1 and TGF-βR2 expression with survival and hazard outcomes, significant association was only seen in the TGF-βR2 expression in tissue samples of female patients. This strongly supports TGF-βR2 expression in tumor tissue as potential biomarkers for ESCC screening.

The Northeast India is distinct from another part of the country containing a distinct genetic pool.[21,37] Different genetic, environmental, and host factors like individual diet, nutrition, food habits, lifestyle, etc. that influence the high incidence of esophageal cancer in this area are yet to be determined.[21,38,39] This kind of molecular level study will help us to understand the role of TGF-β1 and TGF-βR2 gene on ESCC progression and its interaction with various other risk factors for developing ESCC in Northeast India.

Conclusion

This study shows the association of TGF-β1 and TGF-βR2 expression in ESCC. Survival outcomes and hazard analysis support the importance of some clinicopathologic and lifestyle factors on esophageal cancer development, whereas expression study suggests their association with the change in the expression of the studied genes in ESCC patients. Moreover, expression of both TGF-β1 and TGF-βR2 in tissue samples may be a prospective biomarker for screening of ECSS among Northeast Population. This comparative study of gene expression profile between blood and tissue samples of cancer patients will provide a better knowledge toward a comprehensive understanding of esophageal cancer biology. In this study, a total of 76 ESCC patients and an equal number of age- and sex-matched healthy individuals were evaluated. However, further study targeting larger population and cohort is needed for a strong support to the findings of the current study.

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Supplementary materials

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