The Effect of Intraoperative Radiotherapy on Breast Cancer: Focus on the Levels of Angiogenic Factors

Nahid Nafisi
Iran University of Medical Sciences: Tehran University of Medical Sciences
Maryam Mohammadlou (maryam.bio.71@gmail.com)
Semnan University of Medical Sciences and Health Services https://orcid.org/0000-0001-5895-5035
Mohammad Esmaeil Akbari
shahid Beheshti university of Medical Science
Seyed Rabie Mahdavi
Iran University of Medical Sciences: Tehran University of Medical Sciences
Maryam Sheikh
Semnan University of Medical Sciences and Health Services
Mohammad Borji
Shiraz Medical School: Shiraz University of Medical Sciences
Rasoul Baharlou
Semnan University of Medical Sciences and Health Services

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Abstract

Objective: Angiogenesis is one of the hallmarks of cancers that is involved in tumor progression. Angiogenic factors induce the formation of new blood vessels and tumor extension, and finally reduce the survival of patients. Intraoperative radiotherapy (IORT), in which radiation is delivered to the tumor bed can kill cells and change tumor microenvironment. Here, we compared the impact of IORT on the levels of angiogenic factors in the blood and surgical wound fluids (SWF) of the breast cancer patients.

Patients and Methods: Blood and drained wound fluid (WF) samples were collected from the breast cancer patients before and after surgery, followed by quantification of the amounts of TGF-β, EGF, FGF, VEGF and DLL4 in the patients using ELISA.

Results: Our results were indicative of significant differences between the pre-surgery and post-surgery serum levels of EGF, DLL4 and VEGF. In addition, linear regression analysis showed the significant impact of IORT, vascular invasion and lymph node (LN) involvement on the difference between TGF-β levels in the blood before and after surgery-IORT. According to the outcomes of multivariate analysis, IORT changed the levels of EGF and FGF in the blood and WF. Furthermore, logistic regression analyses showed that TGF-β and EGF can be used as predictor markers of the late-stage and LN involvement of the disease. Interestingly, IORT was able to reduce the risk of death and the recurrence rate of disease.

Conclusions: In summary, IORT is a safe and effective treatment that can affect angiogenesis and improve the survival of breast cancer patients.

1 Introduction

Breast cancer is the most common type of cancer in women and the second most prevalent malignancy in the world [1]. Successful early diagnoses and advanced medical treatments have reduced the mortality rate of the disease by almost 40 % over the past 25 years [2]. Angiogenesis is an important phenomenon in a wide variety of normal and cancerous situations. Tumor angiogenesis initiation and development is mainly governed by angiogenic growth factors, such as vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and fibroblast growth factor (FGF) [3]. Several studied have shown that the levels of angiogenic factors and subsequent formation of new blood vessels are among the factors that play roles in breast cancer metastasis and relapse-free or overall survival [4]. Currently, the standard treatment for breast cancer includes conservative surgery followed by radiation therapy [5 6]. It has been demonstrated that surgery leads to the elevated survival, proliferation, and migration of the remaining tumor cells in the breast cancer patients [7,8]. Moreover, during the wound healing process, the immune response or immune cells can promote tumor progression. For example, VEGF and EGF that are secreted by M2 macrophages (tumor-resident macrophages) induce angiogenesis and recruitment of neutrophils which upgrade tumor progression and metastasis [7,9,10]. According to a recent study, surgery induces the regrowth of tumor cells thorough the release of cytokines and activation of myeloid cells [11]. It seems that the post-surgical wound fluid collected from the surgical sites stimulates tumor progression through promoting the proliferation and migration of breast cancer cells [12].

Irradiation of the tumor site is the basis of intraoperative radiation therapy (IORT), which is a technique that directly delivers a single high-dose fraction to the tumor bed during surgery [13]. Tumor microenvironment plays a critical role in the risk of breast cancer recurrence. In addition to killing cancer cells, IORT may also act to alter the environment of an irradiated tumor bed [14]. In a study by Belletti et al., IORT (at a dose of 20 Gy) changed the composition of the wound fluid and annihilated its stimulatory effect on the migration, invasion, and proliferation capabilities of the breast cancer cells [15]. It seems that assessing the impact of IORT on the levels of angiogenic factors has an important role in understanding the effectiveness of treatment on the survival of breast cancer patients. In this study, we have investigated the effect of IORT on the levels of angiogenic factors in the blood and surgical wound fluids (SWFs) of patients who underwent breast-conserving surgery (BCS) and subsequent IORT treatment.

2 Methods And Materials

2.1 Patients and sample collection

The study group consisted of 360 patients, who were diagnosed with breast cancer and recruited in Rasoul-Akram and Khatam-al-Anbya hospitals from 2013 to 2018. They were divided into two groups based on the treatment plan. The Non-IORT group consisted of 229 patients that underwent BCS and the IORT group consisted of 131 patients that were eligible for BCS and subsequently received intraoperative radiotherapy to the tumor bed [16,17]. IORT was performed with the following methodology: following wide tumor excision, a single IORT dose was prescribed to the applicator surface (range 20–50mm) and skin-sparingly applied using 50-kV X-rays with the INTRABEAM miniature X-ray generator (Carl Zeiss Surgical, Oberkochen, Germany). IORT boost with a single dose of 20Gy at the surface of the IORT applicator using 50-kV photons is attenuated down to 5Gy at 1cm distance from the edge of the excision cavity. Patients with previous history of radiotherapy, chemotherapy, and surgery associated with the present disease were excluded from the study. 5 mL peripheral venous blood samples were collected before surgery. One day after surgery, 5 mL peripheral blood and drainage WF samples were collected from both groups and after centrifugation, they were stored at -80°C. All patients are being followed-up every 3–6 months for the first 5 years in the radiation oncology department. The protocol for the present study was approved by the Ethics Committee of Iran University of Medical Sciences. Informed consent was obtained from all subjects who participated in this study. The characteristics of patients have been summarized in Table 1.

Table 1
Demographic, clinical, and laboratory data of breast cancer patients with IORT and Non-IORT.

Variables	Non-IORT (n=229)	IORT (n=131)	*p*
Age	50±63	51±31	NS
Tumor side (%)			NS
Right	116 (78.5)	62 (47.3)	
Left	113 (21.5)	69 (52.7)	
Family history (%)			0.01
Negative	164 (68.8)	78 (65.5)	
Positive	45 (18.8)	41 (34.5)	
Histology (%)			NS
IDC	171 (77.4)	98 (75.4)	
ILC	33 (14.9)	20 (15.4)	
DCIS	13 (5.9)	11 (8.5)	
Other types	4 (1.8)	1 (0.8)	
ER (%)			NS
Negative	81 (37.9)	33 (25.4)	
≤30%	39 (18.2)	25 (19.2)	
30-70%	36 (16.8)	27 (20.8)	
>70%	58 (27.1)	45 (34.6)	
PR (%)			NS
Negative	84 (36.6)	43 (33.1)	
≤30%	53 (24.8)	36 (27.7)	
30-70%	40 (18.7)	16 (12.3)	
>70%	36 (15.7)	35 (26.9)	
HER-2 (%)			NS
Negative	141 (68.1)	91 (71.7)	
Positive	66 (31.9)	36 (28.3)	
Tumor grade (%)			NS
I (well differentiation)	21 (9.1)	16 (13.3)	
II (mod differentiation)	103 (51.8)	74 (61.7)	
III (poor differentiation)	75 (32.7)	30 (25.0)	
Vascular invasion (%)			NS
Negative	142 (64.3)	95 (72.5)	

Values are presented as mean ± standard deviation or number (%). IORT: Intraoperative radiation therapy; DCIS: ductal carcinoma in situ; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

Variables	Non-IORT (n=229)	IORT (n=131)	*p*
Positive	79 (35.7)	36 (27.5)	
Perineural invasion (%)			NS
Negative	192 (86.9)	112 (85.5)	
Positive	29 (13.1)	19 (14.5)	
Calcification (%)			NS
Negative	185 (83.7)	114 (87.0)	
Positive	36 (16.3)	17 (13.0)	
Necrosis (%)			NS
Negative	170 (76.9)	95 (72.5)	
Positive	51 (23.1)	36 (27.5)	

Values are presented as mean ± standard deviation or number (%). IORT: Intraoperative radiation therapy; DCIS: ductal carcinoma in situ; IDC: Invasive ductal carcinoma; ILC: Invasive lobular carcinoma; ER: estrogen receptor; PR: progesterone receptor; HER2: human epidermal growth factor receptor 2.

2.2 Enzyme-linked immunosorbent assay (ELISA)

The amounts of TGF-β, EGF, FGF, VEGF and DLL4 in the patients' sera were measured using ELISA-kits which were purchased from eBiosciences (USA), Thermo Fisher (USA) and Fine Biotech (China), respectively. Briefly, standards were reconstituted to generate stock concentrations of 500, 5000, 10000, 10000 and 5000 pg/mL for TGF-β EGF FGF VEGF and DLL4, respectively. The detection sensitivity for TGF-β, EGF, FGF, VEGF and DLL4 was 8, 1, 15.6, 5 and 46.9 pg/mL, respectively. Briefly, the diluted Capture Antibodies were added to 96-well microtiter plates for overnight. Then Standards or samples were added, and incubated for overnight at 4 ºC. After washing, the detection antibody was added and incubated for 2 hours at room temperature. After a series of washes, Streptavidin-HRP and then, 50 µL of Stop Solution were added. Finally, the plate was read using a micro-plate reader set to 450 nm. Data were expressed in pg/mL.

2.3 Statistical analysis

All statistical calculations were conducted using the Prism 8.0.2 (GraphPad v7, USA) and SPSS (SPSS, v17, USA) software. After assessment of data normality, the serum levels of TGF-β, EGF, FGF, DLL4 and VEGF in the peripheral blood and drainage WF were evaluated compared to the corresponding values from control samples using independent t-test or Mann-Whitney U test, paired t-test or Wilcoxon matched-pairs rank test and One-way ANOVA (or Kruskal–Wallis test). Linear regression and Spearman's correlation coefficient tests were performed to assess the relationships between clinical findings and serum variables. The diagnostic accuracies of disease stage, LN involvement and tumor size were evaluated using the receiver operating characteristic (ROC) analysis and the areas under the ROC curves (AUCs) were compared for each serum variable. Univariate and multivariate logistic regression were analyzed to assess the serum predictor variable associated with the stage and LN involvement diagnoses. Each parameter showed a statistically significant difference from that obtained from the univariate analysis. These factors were further analyzed based on the multivariate logistic regression (backward model) to determine the independent significant risk factors for the stage and LN involvement. To analyze the effects of clinical features on angiogenic factors, univariate and multivariate analyses were performed based on the linear regression modeling. We presented the categorical variables as frequencies and proportions and the results were compared using the chi-squared test. The univariate prognostic analysis was conducted using the Kaplan-Meier method and log-rank test. The significant prognostic factors identified from the univariate analysis were further analyzed in a multivariate Cox proportional hazards model along with the corresponding 95% confidence interval (CI) for each potential risk factor. Results were expressed as mean ± SD. $p<0.05$ was regarded as significant in all statistical analyses.

3 Results

3.1 Evaluation of clinical parameters

Among 360 patients under investigation, 229 patients were in the Non-IORT group with the mean age of 50±13 years and 131 patients were in the IORT group with the mean age of 51±10 years. Furthermore, 45 (18.8 %) patients in the Non-IORT group and 41 (34.5%) in the IORT group had family history of BC, while 103 (51.8%) patients in the Non-IORT and 74 (61.7%) ones in the IORT group had grade II tumors (Moderate- differentiation). The most predominant tumor type in the studied patients was ductal, which was observed in 171 patients (77.4%) in the Non-IORT group and 98 patients (75.4%) in the IORT group. Demographic, clinical, and laboratory data of the breast cancer patients in the two studied groups have been shown in Table 1.

3.2 IORT changes the levels of angiogenic factors in the sera of breast cancer patients

We evaluated the levels of TGF-β, EGF, FGF, DLL4 and VEGF in the peripheral blood and WF of breast cancer patients in the IORT and Non-IORT groups. According to the results, significant differences were found in the serum levels of EGF, DLL4 and VEGF measured before and after IORT (Fig. 1). We found that in both IORT and Non-IORT groups, EGF concentration was increased after surgery (Fig. 1B). However, DLL4 was decreased after intervention in the IORT group (Fig. 1D). Similar to the case of EGF, VEGF level was increased in the IORT group after surgery (Fig. 1E).

3.3 Clinicopathological variables affect the angiogenic factors

We used the linear regression models obtained before and after surgery to evaluate the correlations of TGF-β, EGF, FGF, DLL4 and VEGF levels with IORT, vascular invasion, LN involvement as well as tumor size and type and disease stage in the serum and WF of patients. The outcomes of univariate and multivariate linear regression analyses revealed the significant impacts of IORT, vascular invasion and LN involvement on the level of TGF-β in blood (Table 2). In addition, IORT and LN involvement were influential on the serum level of EGF. The FGF level in WF was influenced by IORT, LN involvement and stage of disease. The size of tumor was influential on the difference between the pre- and post-surgery levels of DLL4. On the other hand, the stage of disease affected the difference among the pre- and post-surgery values of VEGF.

Table 2
Linear regression analysis of TGF-β, EGF, FGF, DLL4 and VEGF with IORT, invasion, LN involvement, size of tumor, type of tumor and stage in serum and drain of breast cancer patient before and after surgery.

	Univariate analysis						Multivariate analysis					
TGF-β	**Serum-after**		**Difference**		**Drain**		**Serum-after**		**Difference**		**Drain**	
	B	p	**B**	p	**B**	p	**B**	p	**B**	p	**B**	p
IORT	1293.5	0.13	4177.5	**0.01**	-637.8	0.70	1778.0	0.07	4048.6	**0.02**	-	-
Invasion	2996.9	**0.002**	5035.1	**0.01**	558.5	0.76	2572.0	**0.01**	7204.6	**0.001**	-	-
LN involvement	426.4	0.63	-1938.2	0.29	-1977.0	0.25	-	-	-4242.0	**0.02**	-	-
Size of tumor	1334.1	0.15	-1190.6	0.54	1058.1	0.45	1907.6	**0.05**			-	-
Type of tumor	232.6	0.85	-2773.6	0.31	1633.1	0.38	-	-	-4759.1	0.07	-	-
Stage	1178.6	0.21	-590.4	0.85	-1798.0	0.46	-	-	-	-	-	-
	Univariate analysis						**Multivariate analysis**					
EGF	**Serum-after**		**Difference**		**Drain**		**Serum-after**		**Difference**		**Drain**	
	B	p	**B**	p	**B**	p	**B**	p	**B**	p	**B**	p
IORT	-222.4	**0.03**	182.7	0.13	-159.6	0.31	-294.1	**0.01**	-	**-**	-	-
Invasion	27.1	0.82	154.4	0.26	-133.6	0.44	-	**-**	257.3	0.08	-	-
LN involvement	62.1	0.57	-231.9	0.06	-160.2	0.32	-	-	-270.3	**0.05**	-339.2	0.08
Size of tumor	98.6	0.38	-50.6	0.69	233.1	0.17	-	**-**	-	-	-	-
Type of tumor	-215.9	0.16	-29.5	0.87	-127.2	0.57	-	-	-	-	-	-
Stage	80.1	0.48	-61.0	0.63	184.7	0.27	-	-	-	-	356.1	0.09
	Univariate analysis						**Multivariate analysis**					
FGF	**Serum-after**		**Difference**		**Drain**		**Serum-after**		**Difference**		**Drain**	
	B	p	**B**	p	**B**	p	**B**	p	**B**	p	**B**	p
IORT	69.2	0.14	17.1	0.69	95.1	0.21	109.3	0.06	-	-	186.4	**0.01**
Invasion	75.9	0.16	-5.6	0.90	4.2	0.96	-	-	-	-		
LN involvement	-5.5	0.91	9.1	0.84	-58.4	0.40	-	-	-	-	-153.9	**0.05**
Size of tumor	57.7	0.27	-11.8	0.80	90.1	0.28	99.3	0.08	-	-	-	-
Type of tumor	78.9	0.26	-124.3	0.06	200.5	0.06	-	-	-	-	-	-
Stage	48.1	0.35	-17.1	0.71	48.5	0.55	-	-	-	-	260.2	**0.005**

IORT: Intraoperative radiation therapy; LN: lymph node

The difference was evaluated using subtraction each angiogenic factor in before and after surgery

	Univariate analysis						Multivariate analysis					
	Univariate analysis						Multivariate analysis					
DLL4	**Serum-after**		**Difference**		**Drain**		**Serum-after**		**Difference**		**Drain**	
	B	p	**B**	p	**B**	p	**B**	p	**B**	p	**B**	p
IORT	-216.7	0.49	-895.7	0.13	-955.7	0.34	-	-	-	-	-	-
Invasion	-386.6	0.27	-31.1	0.96	1501.4	0.17	-	-	-	-	-	-
LN involvement	-108.1	0.74	154.5	0.80	1069.6	0.30	-	-	-	-	-	-
Size of tumor	207.9	0.53	1026.8	**0.05**	685.2	0.53	-	-	1098.1	**0.04**	-	-
Type of tumor	235.1	0.61	86.1	0.92	-472.2	0.74	-	-	-	-	-	-
Stage	-67.8	0.83	901.9	0.09	984.7	0.36	-	-	-	-	-	-
	Univariate analysis						**Multivariate analysis**					
VEGF	**Serum-after**		**Difference**		**Drain**		**Serum-after**		**Difference**		**Drain**	
	B	p	**B**	p	**B**	p	**B**	p	**B**	p	**B**	p
IORT	-6.5	0.65	13.6	0.37	4.8	0.91	-	-	-	-	-	-
Invasion	-11.1	0.49	-6.61	0.70	-25.2	0.60	-	-	-	-	-	-
LN involvement	6.2	0.67	6.31	0.69	-58.1	0.19	-	-	-	-	-	-
Size of tumor	-1.3	0.89	-3.8	0.68	-40.5	0.31	-	-	-25.4	0.08	-	-
Type of tumor	-17.7	0.31	1.9	0.91	-17.7	0.31	-	-	-	-	-	-
Stage	-2.7	0.83	-1.4	0.91	-42.8	0.28	-	-	32.1	**0.04**	-	-
IORT: Intraoperative radiation therapy; LN: lymph node												
The difference was evaluated using subtraction each angiogenic factor in before and after surgery												

3.4 Angiogenic factors as valuable biomarkers predict end stages of disease

Univariate and multivariate logistic regression analyses were used to assess the serum levels of angiogenic factors as predictors of the pathological N stage before surgery. Both of univariate and multivariate analyses demonstrated that TGF-β and EGF can be used as end stage predictors. However, only EGF could predict LN involvement in the breast cancer patients, based on the univariate and multivariate analyses (Table 3).

Table 3
Logistic regression analysis of variables before surgery to predict stage and pathological N stage

	Univariate analysis			Multivariate analysis		
Stage (late vs. early)	**B**	p	**OR (95% CI)**	**B**	p	**OR (95% CI)**
TGF-β (high vs. low)[a]	1.627	**0.05**	5.09 (0.99- 26.18)	1.86	**0.04**	6.48 (1.06- 39.34)
EGF (high vs. low)[b]	1.738	**0.01**	5.68 (1.50- 21.49)	1.96	**0.007**	7.12 (1.69- 29.95)
FGF (high vs. low)[c]	0.76	0.42	2.15 (0.32- 14.31)	-	-	-
DLL4 (high vs. low)[d]	-0.76	0.49	0.46 (0.05- 4.20)	-	-	-
VEGF (high vs. low)[e]	0.39	0.61	1.48 (.32- 6.70)	-	-	-
LN involvement (pos vs. neg)						
TGF-β (high vs. low)[a]	0.21	0.79	1.23 (0.25- 6.10)	-	-	-
EGF (high vs. low)[b]	1.66	**0.01**	5.30 (1.39- 20.12)	1.74	**0.01**	5.71 (1.49- 21.84)
FGF (high vs. low)[c]	0.07	0.93	1.079 (0.16- 7.0)	-	-	-
DLL4 (high vs. low)[d]	0.21	0.79	1.23 (0.25- 6.10)	-	-	-
VEGF (high vs. low)[e]	0.63	0.36	1.88 (0.47- 7.42)	-	-	-

a >3000 vs. ≤3000 pg/mL; b >900 vs. ≤900 pg/mL; c >600 vs. ≤600 pg/mL; d >5000 vs. ≤5000 pg/mL; e >100 vs. ≤100 pg/mL; OR: odds ratio; LN: lymph node; Neg; negative; Pos: positive; Early: stage 1 and 2; Late: stage 3 and 4.

As can be seen from the Fig. 2, using ROC analysis, DLL4 and EGF can be used to differentiate patients with late stages, LN involvement and larger tumor size from early stage, LN free and smaller tumor size, respectively. DLL4 with 70% specificity and sensitivity (AUC=0.7, p=0.0002) (Fig. 2A) could be employed to differentiate early from late stages to predict the stage of disease. On the other hand, EGF with 64% specificity and sensitivity (AUC=0.64, p=0.008) (Fig. 2B) could be utilized to differentiate LN free from >1 LN involvement for predicting LN involvement. In addition, tumor size could be predicted with 62% specificity and sensitivity using DLL4 (AUC=0.62, p=0.02) (Fig. 2C).

3.5 Clinicopathological variables predict poor prognosis

To assess the impact of clinical risk factors on the overall survival and recurrence-free survival, univariate and multivariate cox regression were used in breast cancer patients. Cox risk regression models revealed that age, tumor grade, estrogen receptor (ER) and progesterone receptor (PR) were independent risk factors for a poor prognosis of the breast cancer. As presented in Table 4, Ages over 40 years and high-grade tumors led to 2.51 and 1.60 time increases in the risk of death, respectively. In addition to the negative ER state that led to reduced overall- and recurrence-free survivals, the negative PR state increased the risk of recurrence.

Table 4
Univariate and multivariate Cox regression analyses of clinical risk factors in breast cancer patients

	Overall survival				Recurrence-free survival			
	Univariate analysis		Multivariate analysis		Univariate analysis		Multivariate analysis	
	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p	HR (95% CI)	p
Age (>40 vs. ≤40.)	1.94 (1.18-3.18)	**0.008**	2.51 (1.43-4.38)	**0.001**	0.79 (0.47-1.34)	0.39	-	-
Size of tumor (>2 cm vs. ≤2)	1.22 (0.78-1.9)	0.37	-	-	1.47 (0.83-2.60)	0.17	-	-
LN involvement (pos vs. neg)	0.95 (0.63-1.43)	0.83	-	-	0.93 (0.57-1.53)	0.79	-	-
Vascular invasion (pos vs. neg)	0.74 (0.49-1.11)	0.14	-	-	1.06 (0.65-1.72)	0.80	-	-
Stage (late vs. early)	1.16 (0.67-2.03)	0.58	-	-	1.26 (0.61-2.61)	0.52	-	-
Grade (high vs. low)	1.36 (0.89-2.07)	0.147	1.60 (1.00-2.55)	**0.05**	1.51 (0.91-2.51)	0.10	-	-
ER (pos vs. neg)	0.68 (0.45-1.03)	0.06	0.63 (0.39-1.00)	**0.05**	0.84 (0.72-0.99)	**0.04**	-	-
PR (pos vs. neg)	0.68 (0.45-1.02)	0.06	-	-	0.51 (0.32-0.83)	**0.006**	0.63 (0.60-0.99)	**0.04**

HR: hazard ratio; CI: confidence interval; LN: lymph node; ER: estrogen receptor; PR: progesterone receptor; Neg; negative; Pos: positive

3.6 IORT increases the survival and decreases the recurrence rate in breast cancer patients

To determine whether IORT could contribute to the improved overall and recurrence-free survival, we plotted Kaplan Meier survival curves for the patients. As observed in Fig. 3, IORT reduced the risk of death (HR= 0.33, p=0.023) (Fig. 3A) and the recurrence rate (HR= 0.29, p=0.021) (Fig. 3B) in comparison to the Non-IORT group.

4 Discussion

Tumor angiogenesis is a highly complex process that involves accurate communication of tumor cells with their host organs or tissues, which is controlled by the interplay of a wide range of factors [18]. Angiogenic factors encourage the formation and development of blood vessels by cancer cells to expand tumors [19]. Several studies have shown that angiogenic factors have a significant role in tumor growth and expansion. According to the outcomes of immunohistochemical analyses, the members of VEGF family and their receptors are expressed in almost half of human cancers. Moreover, a significant association between the expression of VEGF and prognosis has been described in colorectal, breast, lung, head and neck squamous cell carcinomas, Kaposi sarcoma and malignant mesothelioma. These researches have also demonstrated that the levels of angiogenic factors in a tissue indicate the aggressiveness of tumor cells, and thus have predictive value in recognizing patients with poor prognosis who are at high risk [20].

Several studies have shown that overexpression of angiogenic factors such as PD-ECGF, bFGF, TGF-β, angiogenin, and COX-2 in different cancers are correlated with the advanced tumor stage and decrease patient survival [21]. Aside from the VEGF family, FGFs are also known as a family of potent angiogenic motivators associated with the risk of breast cancer [22]. While FGF-1 is known as an acidic polypeptide, FGF-2 is a bFGF polypeptide and plays a pivotal role in the stimulated proliferation and

differentiation of endothelial cells. In addition, a significant association between high serum or urine levels of bFGF and progressive disease in patients with different types of cancers has been reported [22,23].

TGF-β is secreted by both of normal and cancerous cells. Depending on the stage of breast cancer development and progression, it can act as either a pro- or anti-oncogenic protein. TGF-β is a highly oncogenic factor in the late stage, aggressive and metastatic breast cancers [24]. According to recent studies, the high expression of angiogenesis-related proteins is associated with adverse clinicopathological parameters in the early-stage breast cancer patients [25].

Radiation can cause damage to the microenvironment of both cancerous and normal cells (like endothelial cells). There are conflicting reports on the consequences of radiation. Some studies indicate that radiation may enhance tumor invasiveness and metastasis. These observations may be explained by the fact that cancer cells are destroyed or damaged by radiation, thereby secreting a variety of soluble factors that promote angiogenesis and improve migration and invasion of cancer cells [26,27]. Destruction of epithelial cells by radiation depends on the beam dose. It has been generally reported that higher doses in the range of 2–15 Gy have an anti-angiogenic effect, while lower doses about 0.5–0.8 Gy appear to be pro-angiogenic [28,29]. However, it can be more complicated than it seems. For instance, it has been reported that a single high dose (20 Gy) of radiation to the mammary gland decreases the local vessel density in a mouse model of breast cancer relapse, after injection of tumor cells [30]. Radiation has been reported to alter the expression of cytokines in the wound fluid [15]. Furthermore, IORT changes the expression of miRNA223, thereby reducing EGF expression and EGF receptor activation, a cascade that normally inhibits the growth of breast cancer cells and decreases the risk of local tumor recurrence in mice models [31].

In the present research, the concentration of EGF in both IORT and Non-IORT groups was increased after surgery. Our results also indicated that IORT decreases the DLL4 level. In addition to VEGF, EGF level was also increased after IORT intervention. Interestingly, IORT and LN involvement were influential on the EGF level. EGF was indeed a significant LN involvement predictor. Moreover, IORT, vascular invasion and LN involvement have significant impacts on the serum levels of TGF-β and EGF. The FGF level in WF was influenced by IORT, LN involvement and stage, while the size of tumor and the subsequent TNM stage affected the difference between the pre- and post-surgery levels of DLL4 and VEGF expression. DLL4 is a critical factor in vascular maturation and tumor angiogenesis and plays a key role in VEGF signaling [32]. A recent study has shown that VEGF secretion by tumor cells is essential for tumor development in the early-stage of breast tumors [33].

An attractive finding obtained from the ROC analysis was that DLL4 and EGF levels can be used to differentiate the late stages of disease from early stages, LN involvement from free LN, and high tumor size from low tumor size. On the other hand, these two biomarkers could predict the end-TNM-stage. According to the results of previous studies, the serum level of TGF-β is an early marker for predicting fibrosis after surgery and before radiotherapy. The serum levels of TGF-β in patients who had undergone IORT after surgery were significantly higher than those of the patients that had only undergone breast-conserving cancer surgery, suggesting that this alteration in the TGF-β level was the outcome of IORT [34].

Keegan et al. have demonstrated that young patients with breast cancer are associated with more advanced stages, such as higher T and N stages [35]. On the contrary, our Cox risk regression analyses showed that age more than 40 years is a risk factor for the overall survival. In addition, tumor grade, estrogen receptor (ER) and progesterone receptor (PR) were other independent risk factors for a poor prognosis of breast cancer and had negative impacts on the overall and recurrence-free survival of patients. Another interesting finding was the effect of IORT on the overall and recurrence-free survival.

We demonstrated that treatment with IORT reduces the risk of death and the recurrence rate in comparison to the Non-IORT group. Furthermore, several randomized trials have demonstrated excellent early tumor control, survival, and cosmetic outcomes following IORT in the breast cancer patients [36]. Vaidya et al. have performed a prospective randomized study on the IORT treatment versus the whole-breast radiotherapy based on a four-year dataset. They demonstrated a local recurrence rate of 1.2% in the IORT group versus 0.95% in the external beam radiotherapy group [16]. In another research, they have reported a 5-year risk for local recurrence in the conserved breast equal to 3.3% for targeted intraoperative radiotherapy (TARGIT) versus a value of 1.3% for the adjuvant whole-breast external beam radiotherapy (EBRT). However, the mortality due to breast cancer in the TARGIT vs EBRT groups were the same [37].

In our study, the mean follow-up duration was 24 hours after treatment, while a follow-up of 48 or 72 hours may lead to more accurate results on the effect of IORT on the angiogenic factors. However, patients have been following up for more than 5 years now. Overally, IORT was found to offer a potential survival advantage and it can help to reduce recurrence.

5 Conclusion

Overally, this study, in addition to the well-known tumoricidal effects of IORT, provides a biological basis for intervention that demonstrates the effects of this treatment on reducing tumor recurrence through alterations in the tumor microenvironment and angiogenic factors. IORT can be regarded as an innovative approach for the delivery of efficient radiation to the tumor bed and increase the survival of breast cancer patients with less toxic effects. These findings may also help us in early detection of end-stage of disease for faster interventions, based on the levels of angiogenic factors as predictor markers for prognosis of breast cancer.

Declarations

Statement of ethics

The investigation was carried out following the rules of the Declaration of Helsinki. Institutional Review Board (human subject committee), Semnan University of Medical Sciences approved the protocol (IR.SEMUMS.REC.1398.58). Each and all subjects gave written consent.

Conflict of interest

The authors have no conflicts of interest to declare.

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Author contribution

Nahid Nafisi and Rasoul Baharlou conceived and planned the experiments. Maryam Sheikh and Rasoul Baharlou carried out the experiments. Maryam Mohammadlou wrote the manuscript. Nahid Nafisi and Mohammad Borji contributed to sample preparation. Mohammad Esmaeil Akbari and Seyed Rabie Mahdavi contributed to the interpretation of the results. All authors provided critical feedback and helped shape the research, analysis and manuscript.

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Figures

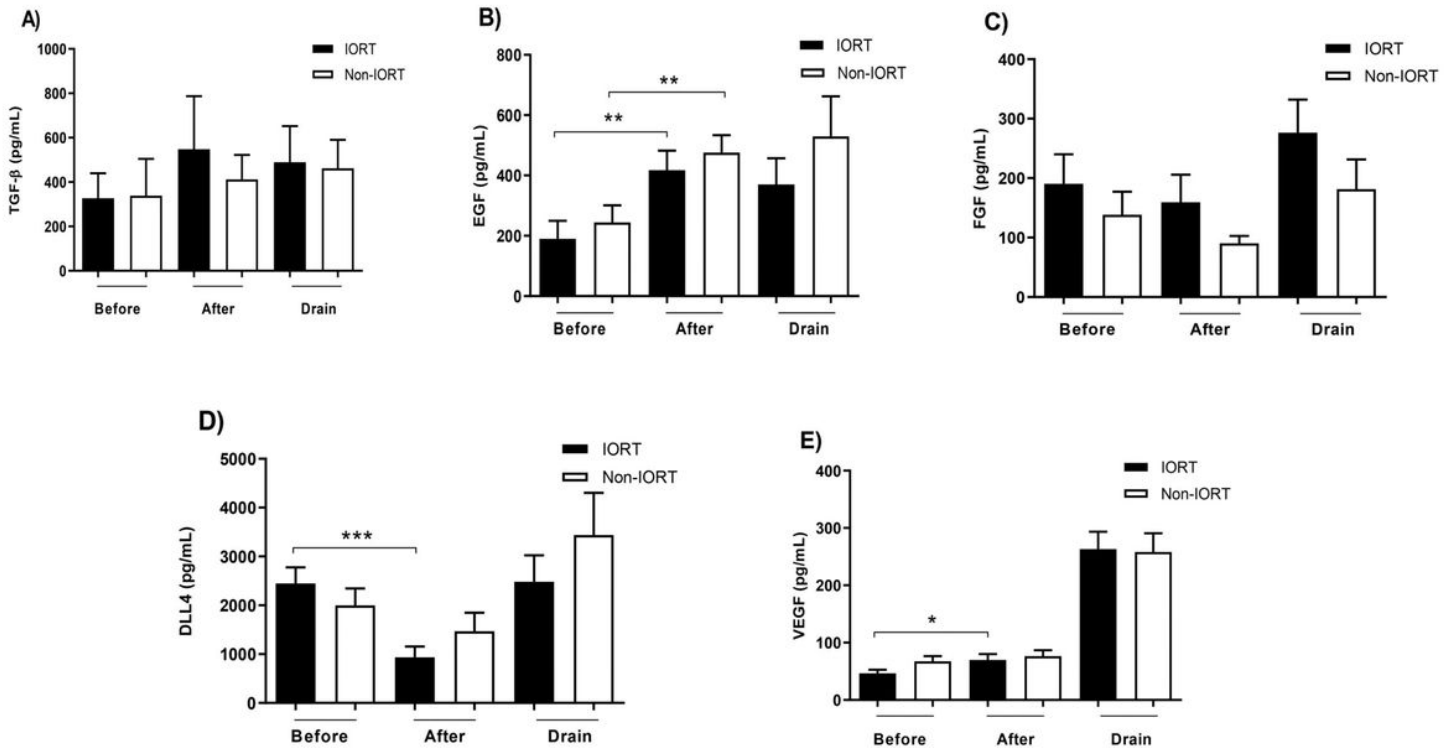

Figure 1

The level of TGF-β, EGF, FGF, DLL4 and VEGF in the peripheral blood and WF of breast cancer patients with and without IORT. Significant difference was found in the serum level of EGF (B), DLL4 (D) and VEGF (E) between before and after IORT. Results were analyzed with nonparametric Wilcoxon matched-pairs rank test and two-tailed Mann-Whitney U test. Values are the mean ± SEM; * = p<0.05; ** = p<0.01.

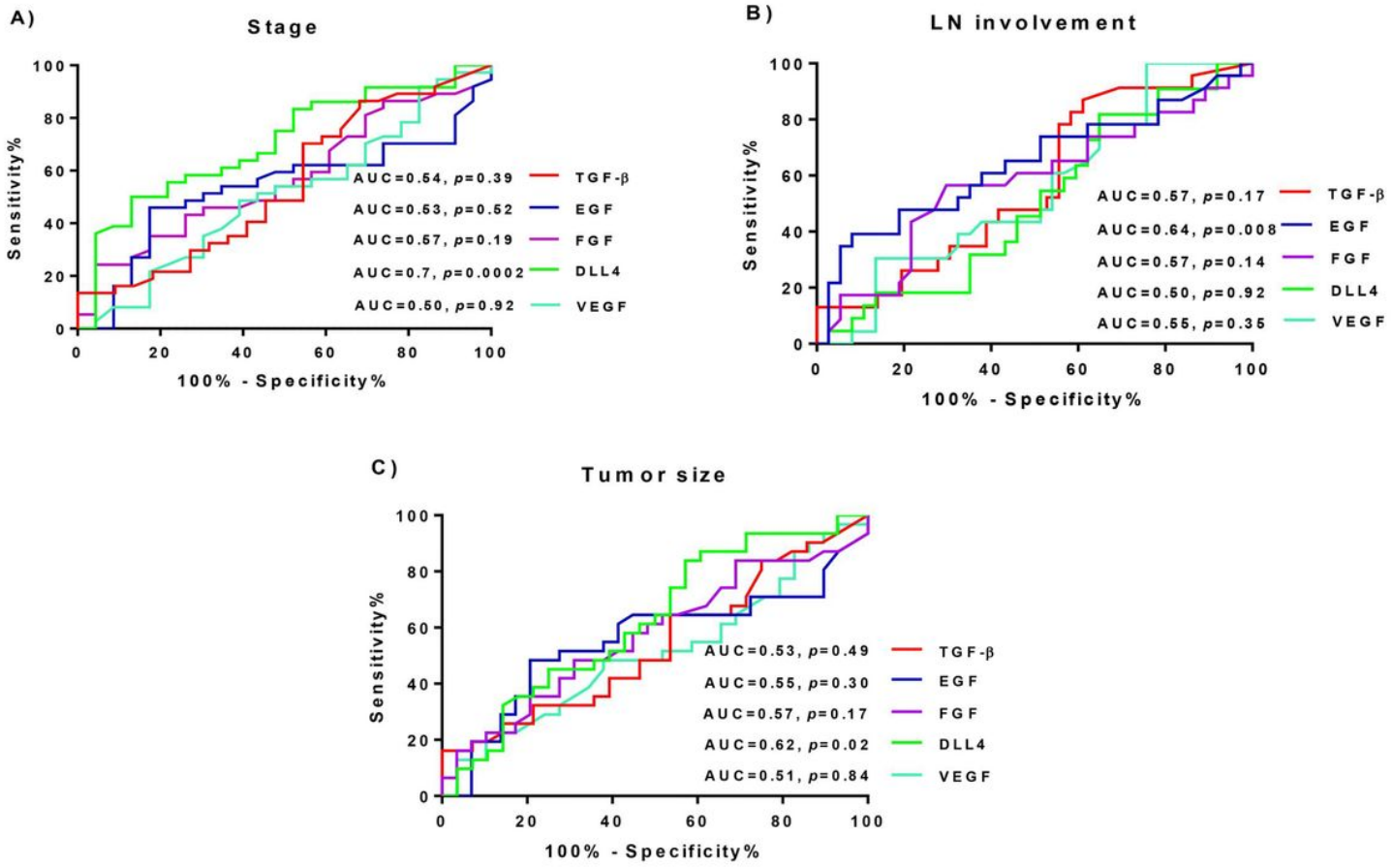

Figure 2

Receiver operating characteristics (ROC) curve analyses. A) As diagnostic biomarkers differentiating early from late stage. B) Diagnostic biomarkers differentiating LN free from >1 LN involvement. C) Diagnostic biomarkers differentiating tumor size ≤2 from >2 cm. AUC: Area under the curve; LN: Lymph node.

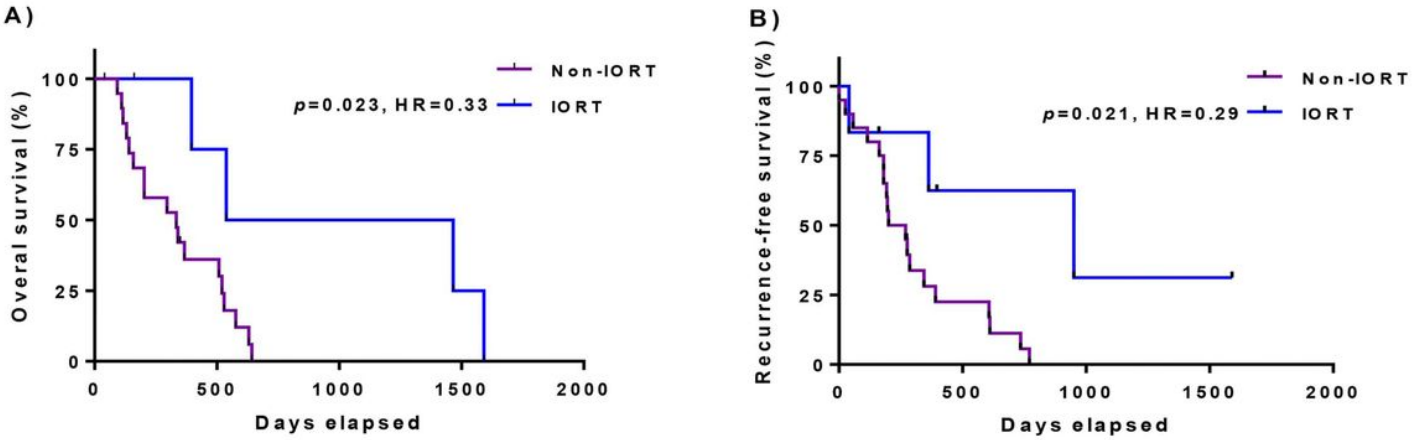

Figure 3

Kaplan-Meier analysis of survival rate (A) and recurrence rate (B) in IORT (N=5) and Non-IORT (N=20) groups. Significant difference was determined by log-rank (Mantel Cox) analysis. IORT: intraoperative radiotherapy