RESEARCH ARTICLE

Serum Levels of G-CSF and IL-7 in Iranian Breast Cancer Patients

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Abstract

Introduction: Breast cancer cells and tumor stroma produce different cytokines and soluble factors. Cytokines, while playing crucial roles in immune responses to tumors, also favour tumor growth and progression. IL-7 and G-CSF are two cytokines that may exert influences on the pathophysiology of breast cancer. Materials and Methods: Sera were collected from 136 females with breast cancer before receiving chemotherapy or radiotherapy. The control group comprised of 60 healthy age-matched females without any acute or chronic diseases with no family history of breast cancer. Serum levels of IL-7 and G-CSF were measured by commercial enzyme linked immunosorbent assay. Results: While there was no significant difference in the level of G-CSF between patients (92.81±594.54 pg/ml) and controls (0.00 pg/ml), G-CSF level in sera of patients with advanced stages of breast cancer was elevated compared to early stages (p=0.0001). Moreover, the highest level of G-CSF was seen in patients with N3 phase tumors (p=0.0001). IL-7 was slightly but not significantly higher in the control group (0.04±0.11 pg/ml) in comparison with patients (0.02±0.10 pg/ml). Interestingly, a significant increase in the level of IL-7 in patients with skin involvement was observed (p=0.001). Conclusion: Our results showed an elevation of G-CSF in sera of patients with advanced stages of tumor, while IL-7 elevation correlated with skin involvement of breast cancer. IL-7 can be produced by keratinocytes in skin tissue and may be involved in the pathologic establishment of metastatic tumor cells in skin.

Keywords: G-CSF - IL-7 - breast cancer - stage - skin - Iranian

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Introduction

Breast cancer (BC) is a disease that affects over one million women all over the world (Montazeri et al., 2008). Despite the considerable decrease in the mortality rate of breast cancer in some countries, its rate is on the rise in Iran (Taghavi et al., 2012). The mean age of Iranian breast cancer patients is 10 years less than the rest of the world pointing to different carcinogenic exposure or genetic susceptibility in these patients (Montazeri et al., 2008). In spite of extensive clinical use of serum tumor markers like CEA and CA-125, the search for ideal tumor markers for diagnosis, prognosis or treatment of breast cancer is still ongoing. An ideal tumor marker should be tumor specific, measurable with non-invasive methods, and highly sensitive and specific; however, such a tumor marker is still missing (Dehqanzada et al., 2007).

Changes in the levels of cytokines are behavioural criteria of the immune system and tumor cells which can affect or result from the interaction between these entities. It is well known that cancer cells produce different cytokines and growth factors. In a study of 17 cytokines in a subgroup of breast cancer with no estrogen receptor expression, it was shown that the expressions of IL-6, IL-8, G-CSF, IFN-γ, MCP-1 and MIP-1β were elevated in the diseased tissue compared with normal tissue (Chavey et al., 2007). However, in the same study no overexpression of Interleukin-7 (IL-7) was observed. IL-7 is an immune regulatory cytokine that is significantly produced by tumor stromal cells and cells that exist in the inflammatory sites (Roato et al., 2006). IL-7 is essential for early development of lymphocytes and regulates peripheral T cell hemostasis (Roato et al., 2006). As a pleiotropic cytokine, IL-7 induces tropic and anti-apoptotic responses on hematopoietic tissues, particularly lymphocytes. IL-7 is known to induce the development, growth and differentiation of some hematological malignancies, including certain types of leukemias and lymphomas (Roato et al., 2006). However, little is known about its possible effects on solid tumors.

Although IL-7/IL-7R mRNA has been detected in colorectal, esophageal, renal, and head and neck (squamous cell type) carcinoma, there is not much information relating the level of serum IL-7 to the histopathological characteristics of tumors or the disease prognosis (Roato et al., 2006). Recently, it has been suggested that IL-7 exerts lymphangiogenic effects on the vascular endothelium and thus might play an important role in the lymphangiogenesis during the progression and spread of solid tumors. Production of IL-7 by human

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solid tumors can have an effect on tumorigenesis process as shown in specific types of lymphomas and leukemias (Roato et al., 2006). It has been shown that IL-7 is a potent growth factor for both endothelial and breast cancer cells (Al-Rawi et al., 2004). This has led to the proposal that IL-7 may influence breast cancer biology, in terms of development and progression. A recent study has found correlation between IL-7 overexpression and poor prognosis in breast cancer patients (Roato et al., 2006).

Granulocyte-colony stimulating factor (G-CSF) is a member of glycoprotein molecules which stimulates the production and differentiation of white blood cells. G-CSF mobilizes hematopoietic stem cells, progenitors and mature cells into peripheral blood and stimulates granulocyte differentiation (Natori et al., 2002). Although some data suggest that G-CSF administration enhances tumor angiogenesis and growth, the evidence implicating this factor in tumorigenesis is far from conclusive (Shojaei et al., 2009). Since the original finding of G-CSF producing tumors, this cytokine has been found in different cancers (Ikeda et al., 2005). G-CSF, M-CSF or CA-125 plasma levels are shown to be elevated in ovarian, bladder, lung (non small cell type) and colorectal cancers (Nishimura et al., 1996; Mroczko et al., 2001; 2002; Asano et al., 2002; Ławicki et al., 2006). G-CSF producing malignant tumors have been associated with poor clinical outcome (Matsumoto et al., 2010). Moreover, G-CSF maybe responsible for prominent leukocytosis occasionally observed in patients with malignant non-hematopoietic tumors (Nasu et al., 2004).

Regulation of neutrophil function and activity against tumor cells are the two facets of G-CSF during tumor development and/or metastasis (Zygier et al., 2010) making it a candidate marker in diagnosis, prognosis and/or treatment of tumor (Dehqanzada et al., 2007). Therefore, we aimed to investigate the levels of IL-7 and G-CSF in Iranian breast cancer patients for the further possibility of their use in the screening, diagnosis or treatment of this cancer

Materials and Methods

The research was approved by ethics committee of Shiraz University of medical science (SUMS). The patients were informed about the aim of this study as well as safety and security measures before their consents were obtained. Cases were selected among women with breast cancer who referred for operation to hospitals affiliated to Shiraz Medical School since April 2009 till May 2010. 136 cases with age between 25-83 years were entered to the study. None of the patients had been treated with chemotherapy or radiotherapy before sample collection.

The group of 136 breast cancer patients (all females) and 60 healthy controls (all females) were matched based on their age (Table 2). The mean age of the breast cancer patients was 49.7±12.4 years (Range=25-83 years) and the mean age of healthy individuals was 49.3±11.1 yrs (Range=21-82 years, P=0.507).

Samples

Four ml blood was collected from peripheral veins of patients on the day before surgery. The samples were brought to Shiraz Institute for Cancer Research immediately. Samples were centrifuged and sera were preserved in -20˚C till analysis was done. On the day of operation tissue biopsies were assessed by collaborative pathologist.

Cancer was staged according to tumor-node-metastasis (TNM) by American Joint Committee on Cancer Classification and stage grouping. The patients were classified according to their pathologic characteristics including histological tumor type, tumor size, in situ component, histological grade, tumor necrosis, peritumoral invasion, axillary lymph node involvement, perinodal fat infiltration and TNM staging for further analysis. Clinical characteristics of the tumors are shown in Table 1.

ELISA assays

Plasma level of G-CSF was measured by a commercial enzyme linked immunosorbent assay (ELISA) (ebiosciences, Austria) according to the manufacturer's instructions. The sensitivity of this assay was 11 pg/ml and range of detection was between 39.1-2500 pg/ml.

Serum level of IL-7 was measured using a commercial ELISA assay (Abcam, UK) according to the manufacturer's instructions. The sensitivity of this assay was less than 3 pg/ml and the range of detection was between 6.25-200 pg/ml.

Statistical analysis

Student's t-test was used for the analysis of age distribution between case and control groups. One-way ANOVA or t-test was used in the comparisons between the two groups using SPSS software (11.5, Chicago, Illinois). When the data points were not enough in categories, normality of data was checked and parametric or non parametric (Kruskal-Wallis and Mann-Whitney) analyses were done. Statistically significant differences were defined as comparisons resulting in $p<0.05$.

Results

A higher level of G-CSF (92.8±594.5 pg/ml) was found in the sera of patients with breast cancer compared with healthy age/sex matched controls (0.00±0.00 pg/ml), (p=0.071). There was no significant difference between the level of IL-7 in sera of patients (0.02±0.10 pg/ml) and healthy controls (0.04±0.11 pg/ml). A higher percentage of BC patients (10 out of 136, 7.35%) had some levels of G-CSF in their sera while 0 out of 60 (0%) healthy controls were found positive for G-CSF (ROC curve cut off point=95.7 pg/ml). Conversely, a higher number of healthy controls (14 out of 60, 23.3%) had some levels of IL-7 in their sera compared to the BC patients (15 out of 136, 11.0%).

There was a significant statistical difference in the level of G-CSF (p=0.0001) between stage 3c and other stages. The mean concentration of this cytokine increased dramatically in this stage and decreased in stage 4 (Table 2). There was, however, no significant difference between IL-7 concentrations in different stages. In general, mean G-CSF concentration increased significantly from low

stages (stages I and II) to high stages (stages III and IV) while mean IL-7 concentration decreased from low to high stages (Table 2).

Although in 108/117 (92.3%) of patients no skin involvement of the tumor was observed, a significant increase in the level of IL-7 in patients with skin involvement was observed (p=0.001). The difference in the level of G-CSF between patients and controls did not reach the significant level. The mean level of IL-7 and G-CSF in patients with skin involvement was 0.13 pg/ml and 143.0 pg/ml while the levels of these cytokines in patients without skin involvement were 0.01 pg/ml and 104.9 pg/ml, respectively. We also observed a huge increase in the level of G-CSF in patients who showed some degree of lymph node involvement (190.3±872.1 pg/ml vs. 14.6±65.7 pg/ml) (Table 2). However, this difference was mostly related to an increase in the N3 phase (p=0.0001, Table 2). Mean IL-7 concentration increased from N0 to N2 but decreased from N2 to N3.

In comparison with control healthy women, concentration of IL-7 was less in breast cancer patients but the difference did not reach the significant level (Table 2). Concentration of G-CSF was elevated in patients' sera but we did not detect any G-CSF in the sera of control group. However, this difference did not reach the significant level due to the high standard deviation of the data in this group.

Although there was no significant difference between the level of IL-7 in sera of patients with right-sided and left-sided breast tumors (0.23±0.10 vs. 0.03±0.11 pg/ml), a considerable increase in the level of G-CSF in sera of patients with right-sided tumor compared to left-sided tumors was observed (218.0±976.6 vs. 27.7±102.5 pg/

Table 1. Clinicopathological Characteristics of Patients

Characteristics	N=136	%	IL-7(pg/ml) Mean ± SD	G-CSF(pg/ml) Mean ± SD	Characteristics	N=136	%	IL-7(pg/ml) Mean ± SD	G-CSF (pg/ml) Mean ± SD
Tumor type					In situ component				
In situ	4	2.94	0.00±0.00	0.00±0.00	Is seen	32	23.5	0.00±0.02	7.05±39.9
Invasive	118	86.8	0.02±0.10	106.9±637.4	Not seen	6	4.41	0.00±0.00	0.00±0.00
Others	14	10.3			Unknown	98	72.1		
Histological grade					Tumor stage				
Well	34	25	0.03±0.12	37.4±133.0	Stage1	22	16.2	0.00±0.00	18.8±61.1
Moderate	48	35.3	0.02±0.10	8.69±42.2	Stage2a	48	35.3	0.02±0.10	8.41±58.3
Poorly	35	25.7	0.02±0.08	312.3±1147.3	Stage2b	22	16.2	0.04±0.11	29.4±137.9
Unknown	19	14			Stage3a	18	13.2	0.06±0.16	0.00±0.00
Stage					Stage3c	10	7.35	0.02±0.04	1094.0±2005.7[c]
Low	110	80.8	0.02±0.10	13.3±77.1	Stage4	3	2.2	0.00±0.00	72.1±125.0
High	13	9.55	0.01±0.03	858.2±1794.6[a]	Unknown	13	9.55		
Unknown	13	9.55							
Tumor size (Diameter)					Nipple involvement				
>2 cm	68	50	0.01±0.07	23.6±99.2	Positive	14	10.3	0.03±0.04	31.5±80.3
<2 cm	54	39.7	0.03±0.12	166.8±832.6	Negative	54	39.7	0.05±0.14	218.0±932.2
Unknown	14	10.3			Unknown	68	50		
Tumor side					Skin involvement				
Right breast	49	36	0.02±0.10	218.0±976.6	Positive	9	6.61	0.13±0.21[d]	143.0±352.5
Left breast	70	51.5	0.03±0.10	27.7±102.57	Negative	108	79.4	0.01±0.08	104.9±659.3
Unknown	17	12.5			Unknown	19	14		
Perilymphatic involvement					Perinodal fat infiltration				
Is seen	52	38.2	0.02±0.10	85.9±505.3	Positive	19	14	0.02±0.04	496.2±1532.3
Not seen	69	50.8	0.03±0.11	118.1±712.4	Negative	116	85.3	0.03±0.11	27.5±127.0
Unknown	15	11			Unknown	1	0.73		
Perineural involvement					Axillary involvement				
Is seen	22	16.2	0.02±0.10	173.7±766.1	Not seen	51	37.5	0.01±0.09	16.0±68.7
Not seen	99	72.8	0.02±0.11	88.8±598.6	Is seen	60	44.1	0.03±0.11	196.7±886.0
Unknown	15	11			Unknown	25	18.4		
Perivascular involvement					Tumor necrosis				
Is seen	22	16.2	0.00±0.03	213.0±770.2	Is seen	64	47.1	0.01±0.06	164.4±852.0
Not seen	99	72.8	0.03±0.12	80.1±595.8	Not seen	54	39.7	0.03±0.13	38.8±159.1
Unknown	15	11			Unknown	18	13.2		
Tumor calcification					Sentinel node involvement				
Is seen	32	23.5	0.04±0.12	7.05±39.9	Negative	10	7.35	0.00±0.00	0.00±0.00
Not seen	87	64	0.02±0.09	142.4±739.8	Positive	11	8.08	0.00±0.00	0.00±0.00
Unknown	17	12.5			Unknown	115	84.5		
N (TNM)					Number of involved LN				
N0	56	41.1	0.01±0.09	14.6±65.7	n=0	56	41.1	0.01±0.09	14.6±65.7
N1	32	23.5	0.02±0.09	20.2±114.4	n>0	62	45.5	0.03±0.11	190.3±872.1
N2	17	12.5	0.07±0.16	0.00±0.00	Unknown	18	13.2		
N3	13	9.55	0.01±0.03	858.2±1794.6[b]	Case	136	100	0.02±0.10	92.8±594.5
Unknown	18	13.2			control	60/60	100	0.04±0.11	0.00±0.00

[a]Significantly elevated (p=0.001); [b](p=0.0001); [c](p=0.0001); [d](p=0.001)

Table 2. Levels of G-CSF and IL-7

Stage	N	IL-7(pg/ml) Mean±SD	G-CSF(pg/ml) Mean±SD
Levels of G-CSF and IL-7 in different stages of breast cancer			
Stage 1	22	0.00±0.00	18.80±61.1
stage 2a	48	0.02±0.10	8.41±58.3
stage 2b	22	0.04±0.11	29.40±137.9
stage 3a	18	0.06±0.16	0.00±0.00
stage 3c	10	0.02±0.04	1094.00±2005.7[#]
stage 4	3	0.00±0.00	72.10±125.0
Levels of G-CSF and IL-7 in sera of BC patients with high stage compared to those with low stage			
Low stages	110	0.02±0.10	13.30±77.1
High stages	13	0.01±0.03	858.20±1794.6[#]
Levels of G-CSF and IL-7 in BC patients with lymph node involvement compared to those without lymph node involvement			
n=0	56	0.01±0.09	14.60±65.7
n>0	62	0.03±0.11	190.30±872.1
Higher levels of G-CSF in sera of BC patients with N3 in TNM classification			
N0	56	0.01±0.09	14.60±65.7
N1	32	0.02±0.09	20.20±114.4
N2	17	0.07±0.16	0.00±0.00
N3	13	0.01±0.03	858.20±1794.6[#]
Levels of G-CSF and IL-7 in sera of BC patients compared to control group			
Case	136	0.02±0.10	92.80±594.5
Control	60	0.04±0.11	0.00±0.00

[#]Significantly elevated (p=0.0001)

ml).

The highest level of G-CSF was observed in patients with tumors greater than 2 cm (154.8±790.6 pg/ml) as well as poorly differentiated tumors (312.3±1147.3 pg/ml) but the differences were not significant.

We did not observe any significant differences in the levels of IL-7 and G-CSF based on the tumor necrosis, tumor calcification, peritumoral lymphatic invasion, perineural invasion, vascular invasion and axillary involvement.

Discussion

In our study there was no significant difference in the levels of G-CSF and IL-7 between breast cancer patients and healthy individuals. Interestingly, there was a significantly higher level of IL-7 in tumors with skin involvement (p=0.001). Skin involvement is one of the most distressing presentations of locally recurrent breast cancer and few studies have identified effective mediators in this setting (Franchina et al., 2012). A previous study reported that IL-7 can be produced by skin tissue (Kim et al., 2011). Production of IL-7 by keratinocytes in skin is suggested to play a role in the survival of dendritic epidermal T cells (DETCs) in epidermis (Matsue et al., 1993; Takashima et al., 1995). DETCs are shown to exert both anti-tumor and immunosuppressive activities in vitro and in vivo (Kaminski et al., 1993;Cavanagh and Halliday, 1996). Specifically, a DETC line is shown to induce specific immunologic tolerance in vivo and inhibit the proliferation of naive T cells in response to Ag-bearing dendritic cells in vitro (Love-Schimenti and Kripke, 1994). Moreover, a pathogenic role of DETCs in inflammatory skin disease as well as cutaneous cell lymphomas is suggested (Heufler et al., 1993).

We observed that patients with well differentiated tumors had the highest level of IL-7. IL-7/IL-7Rα-Fc administration inhibits tumor growth and increases survival in lung cancer by promoting afferent and efferent antitumor responses (Andersson et al., 2011). Moreover, cytotoxic T lymphocytes (CTLs) generated under IL-7 stimulating conditions can diminish the pulmonary metastatic sarcoma in mice (Jicha et al., 1991). It is possible that the attempts by the immune system to counteract the tumor growth is a reason for this increase in IL-7 level, however, as the tumor progresses to moderate and poorly differentiated phase, the immune parameters fade and are replaced by more inflammatory type of cytokines in favor of tumor (Ravishankaran and Karunanithi, 2011). This was portrayed in our observation that the level of G-CSF decreases in the transition from well to moderate differentiation phase but increases when the tumor progresses to the poorly differentiated phase. The change in the tumor environment and the factors produced by the tumors are the consequence of a dynamic process. It is definitely very simplistic to attribute the progression or regression of a tumor to only two cytokines. However, it is worth noting that the IL-7 and G-CSF showed an inverse or different pattern of elevation in relation to correlates of tumor progression and metastasis such as stage, lymph node involvement, and skin involvement. However, at the same time, both cytokines increased at the beginning of tumor expansion (T1 to T2) but this increase did not continue.

In general, serum concentration of G-CSF increased at the higher stages of breast cancer. Moreover, the patients with tumors at N3 phase had the highest level of G-CSF in their sera. These observations are in accordance with previous reports on different tumors (Yamano et al., 2007).

Elevation of G-CSF, GM-CSF and CA 15-3 plasma levels in stage II of breast cancer patients before surgery is already reported (Ławicki et al., 2009). Another study showed that G-CSF expression increases in higher stages of tumor in gastric cancer (Yamano et al., 2007). We also observed that G-CSF level in sera of patients with advanced stages of breast cancer was elevated while it was not detected in the early stages. Previously, Chavey et al. (2007) reported the same observation in estrogen receptor negative breast tumors (Chavey et al., 2007). The reported increased level of G-CSF in breast cancer may have tumor promoting or immunosuppressive effects. A study on breast cancer patients receiving G-CSF adjuvant therapy has shown an increased level of CA 15-3 in patients with resected tumors who received chemotherapy (Pentheroudakis et al., 2004). This increase which was triggered by G-CSF was suggested to be mediated by Neutrophils (Pentheroudakis et al., 2004). CA 15-3 on the other hand is an epitope of Mucin 1 (MUC1) with immunoregulatory and intercellular adhesion modulatory effects (Taylor-Papadimitriou et al., 1999). Therefore, it is possible that increased levels of G-CSF may be exploited by tumor to suppress the immune system. In this regard, it is shown that ectopic expression of G-CSF in a mammary tumor cell line can promote their growth and augment

granulocytic myeloid-derived suppressor cell (MDSC) accumulation (Waight et al., 2011). Indeed, production of G-CSF by mammary tumor cells is already shown (Kowanetz et al., 2010). There is also one possibility that the tumor-derived G-CSF would be structurally different from that of physiologically produced G-CSF (Ghaderi et al., unpublished data).

Mean concentration of G-CSF in patients with positive peritumoral vascular invasion was more than its level in those without involvement but this difference was not statistically significant. It is suggested that peritumoral vascular invasion has a major role in prognosis of breast cancer (Sabatier et al., 2011). To elucidate the significance of G-CSF level in the peritumoral vascular invasion and prognosis of breast cancer in Iranian patients more studies are needed.

Collectively, our results showed an elevation of IL-7 and G-CSF in different stages of tumor growth and progression. The current information on the IL-7 level in breast cancer do not correspond. In one study IL-7 mRNA and protein could not be detected in breast cancer (Maeurer et al., 1997). In another study, however, the levels of IL-7, IL-7R, and its signalling intermediates were shown to be overexpressed in the aggressive breast tumors (Al-Rawi et al., 2004). Moreover, there are multifaceted aspects of G-CSF function to be considered in relation to tumor progression and defence against breast tumors. A molecular and genetic analysis of G-CSF variants in breast tumors would shed more light on the current controversy.

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