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LOCAL AND SYSTEMIC ANTI-TUMOR IMMUNE RESPONSE FOR BREAST CANCER IS THERE. WHY IT FAILS IN ERADICATION OF THE DISEASE?

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A new model for breast cancer is needed to define the fine dynamic balance between the tumor and the host including various autocrine and endocrine factors which influence proliferation, apoptosis and angiogenesis.
Aim of the study was to measure the systemic and local anti-tumor immune response for breast cancer, to study cytokine network modification (IL-10) (anti-inflammatory) and IL-12 (pro-inflammatory) and to evaluate its correlations with other histopathological parameters.
The study was done on 17 female patients with breast cancer and another control group of 10 patients.
The patients were followed-up for two years.
Significant correlations of studied peripheral blood immune parameters were: Natural Killer (NK) numbers, NK activity (NKA) and peripheral T-lymphocytes in count per minute (cpm) with tissue NK (TNK) and tissue natural Killer Activity (NKA). Significant correlation between NKA and IL-12, and peripheral blood T-lymphocytes with NK and tissue NK activity (T-NKA) was detected. Significant correlations between IL-10, IL-12 and the other parameters were detected.
Significant correlation was noticed between (Local immune response): tumor infiltrating lymphocytes (TILs) and tumor stage, also between other studied parameters of local immune response.
The degree of fibrosis (mechanical tumor control) was correlated negatively with the tumor grade and lymph node number.
The disease free survival (D.F.S) was significantly related to tissue local immune response and systemic immune response through IL-12 modifications. Significant correlation between high levels of IL-10 with NKA and TILs was detected
Recurrence was closely related to the number of positive axillary lymph nodes.
On conclusion: proper function of peripheral T-lymphocytes was crucial for effective destruction of breast cancer cells. NKA was involved in breast cancer disease progression and it depended on levels of IL-12 and the antigen presenting cell (APC).
IL-10 had a predictive role in breast cancer response and exogenous anti-IL-10 may be useful. The amount of IL-12 available was critical for tumor progression. TILs correlates with the tumor stage but failure of tumor eradication may be due to higher levels of IL-10. The mechanical arm was involved in the immune response. Disease. Free. Survival (D.F.S)was related to TNKA and SNKA through the IL-12. Lastly the discriminate factor in DFT was the TNKA.

Key words: local and systemic anti-tumor immune response. Breast cancer - why it fails in eradication of the disease ?

INTRODUCTION

Breast cancer is one of the most commonly occurring cancers in females in the Eastern Mediterranean Region (EMR). Data from Egypt indicated that breast cancer ranked as number 27.3% among females[1].

But, while the incidence of breast cancer has been increasing dramatically, increase in our knowledge about it, improved its treatment, the death rate has not decreased [2,3].

Breast cancer is formed of 3 cell subpopulations, growth fraction, clonogenic fraction[3], non-proliferating fraction (dead, inactive) and rest cells[4] and the growth rate of breast cancer may be exponential model[5], Gompertzian model[6], and stochastic model[7]. All are tumour related but not reflect the host tumor interaction.

All models go hand in hand with the three theories for evolution; Halstaedt's one, breast cancer is a loco-regional disease, systemic theory[8] breast cancer is a systemic disease and the spectrum one[5] which stresses on

importance of local and systemic therapy. Neither of these models explain tumour dormancy, nor they explain timing of the first relapse after primary therapy [3]

Hazards for metastases and death after treatment rise sharply at 2-3 years and then fall to rise to a second peak at about 7-9 years which is true for any stage of the disease. [3] So a new model for breast cancer is needed which takes into account the fine dynamic balance between the tumour and the host including various autocrine and endocrine factors which influence proliferation, apoptosis and angiogenesis[9]

Not all metastases are due to cellular dissemination but it may results from at a transfection phenomenon where nuclear material from the primary malignant clone infecting the wandering cells of the macrophage monocyte system. This mutation is then transported to distant sites where the local mesenchymal cells are transfected with the genetic (mis) information that activates components of the genome to instruct these plastic cells to express the phenotypic picture of a dedifferentiated breast epithelial cell. [10]

Local recurrence either occurs in the breast in the same site of the previous primary or in 90% within the index quadrant [11,12] and it is not due to over looked multi centric foci at the tumour margin[13] and it could be attributed to circulating metastatic cancer cells lodging in the highly vascular surgical bed or from local transfection of surrounding breast epithelium by nuclear material released from the original malignant clone and resulting in insertional mutagenesis[10],[14]

The effector mechanisms in cancer immunity are humoral and cell-mediated (CMI)[15]. Of the cell mediated are the innate Natural Killer (NK) and cell-mediated arms (tumour infiltrating lymphocytes (TILs) [16] and the cytokines secreted resulting from the inflammatory reaction to the tumour as cancer is a non healing wound[17]

The key cells involved in tumour immunity are NK, T-cells and macrophages[18]. The T-cell response is Major histocompatibility complex (MHC) dependant either T-helper if MHC-II expressed and T-cytotoxic if MHC-I is expressed but most of tumour cells express MHC-I.[19,20] The deficient expression is related to immature or deficient Antigen presenting cells (APC), dendritic cells. [21,22] The humoral arm kill tumour cells through ADCC or CMC. [23] The NK cells kill tumour cells by direct apoptosis or lymphotoxin release (TNFα)[24] that enhanced by interleukin-2 (IL2) and interferon.[15] The TILs are lymphocytes that infiltrate the tumour and are mainly CD8, CD3-positive and CD4 or mixed[25,26]

The TILs in breast cancer are mainly CD4-positive on stimulation secrete IL-2, interferon and tumour necrosis factor[26] Another study suggested the majority are CD8 (suppressor + cytotoxic)[27,30,31]

The great difference in survival within each stage of breast cancer makes staging less discriminate and this could be explained by biological heterogeneity or insufficient staging. [34,35,36,37]

Finally the surgery, chemotherapy don't spare the normal cells from damage but the immune attack is directed only to these cells possessing tumour antigens.[38]

Aim of this work was to: study the immune response in breast cancer as follows: i) systemic immune response by measuring peripheral blood lymphocytes, natural killer cells (NK) and natural killer activity (NKA)

ii) to study cytokine network modification by measuring IL-10 and IL-12

iii) to study local anti-tumour immune response by measuring tumour infiltrating T-lymphocytes (TILs), NK cells and NKA.

v) to evaluate correlations together and with other histopathological criteria plus their survival functions

PATIENTS AND METHODS

They study was done on 17 female patients with breast cancer their age ranged from (40-65)y. mean age of 52.5 years at Mansoura University hospitals from January 1999 to December 2001. for whom tru-cut needle biopsy of the mass was done for histopathological diagnosis, followed by abdominal uls and skeletal survey. Routine laboratory investigations were done.

Clinical stage was III in 11 patients (64%) and stage II in 4 patients (22%) and stage I in one patient (6%) and stage IV in one patient. This is the common presentation of breast cancer in our hospital. Modified radical mastectomy was done.

Smooth post-operative course apart from seroma in 3 patients (17%) managed by repeated aseptic aspirations. Serum and tumour tissue assays were done as in methods. The tumor tissue was harvested immediately after operation in a special preservative (Methods) and sent for measurement. Histopathological examination of the breast and axilla were done. (Figs. 1, 2, 3, 4, 5)

The patients were followed - up for two years. Another age matched control group consisted of 10 female patients who had benign breast lesions were included where peripheral blood T-lymphocytes, tissue and serum NK cells were measured.

Method: Lymphocyte separation: Lymphocyte separation of mononuclear cells for whole peripheral blood according to[39] then using mononuclear cells for lymphocyte culture with mitogen phytohaemagglutinin which is index to T-cell function. The results were expressed as counts per minute (CPM) [40] and CD16 as surface marker for NK was measured flow cytometry. (Coulter Epics using specific monoclonal Diaclone France).

The test depends on the ability of monoclonal antibody to bind to the surface of cells expressing discrete antigenic determinant, Fluorescein Isothiocyanate (FITC) conjugated mouse monoclonal antihuman CD16 antigens were used from Diaclone Research.

NK cytotoxcity was measured by 4-hour chromium 51 release method on separated peripheral blood mononuclear cells[41]. Percent specific lysis =

$$\frac{\text{CPM experimental} - \text{CPM spontaneous}}{\text{CPM maximum} - \text{CPM spontaneous}} \times 100$$

Spontaneous release was determined by incubation of labelled target cells with medium. Maximum release cells was determined by incubation of target with 0.1 M HCL

The cell line K562 was provided kindly by CAROSELLA Saint Louis, France obtained from American tissue culture collection.

Interleukin-10 and Interleukin-12: Quantitative assay by ELISA technique supplied Diaclone France.

Statistical methods

Mean, standard deviation were used to describe data. Mann-Whitney u test was used to test for difference in quantitative variables between the two groups. Kendall's non-parametric correlation was used to test for linear relationship between variables. P was considered significant if less than 0.05. These tests were run on an IBM compatible personal computer using the Statistical Package for Social scientists (SPSS) for windows 7.5 (SPSS Inc., Chicago, IL, USA).

RESULTS

From January 1999 to December 2001 at Mansoura University hospitals, 17 female patients with breast cancer were studied. Their age ranged from 40-65 years with a mean age of 52.5 years. Their clinico-pathological criteria are shown in (Table 1).

Another control group consisted of 10 female patients with matched age for the study group was included.

The following measurements were done for both of the groups:

1) In the serum: (systemic immune response)

T-lymphocytes count per minute CPM - Natural killer (NK) - Natural Killer Activity (NKA)

2) In the breast tissue: (Local immune response)

Tumour infiltrating lymphocytes (TILs) – (TNK) – (TNKA)

3) In the serum: (Cytokines) modification network:

Interleukin-10 (IL -10) - Interleukin-12 (IL-12)

The correlation of studied parameters in relation to the control group is shown in (Table 2), (Figs.6 & 7).. Significant difference is noted between the study and the control group. While (Tables 3,4). and (Fig, 8) show significant correlations of studied peripheral blood parameters which are NK numbers with NKA, peripheral blood lymphocytes (CPM). NKA with IL-12, peripheral blood T-lymphocytes with tissue NK and tissue NK (T-NK) activity.

Tables.(5,6). and (Fig. 9). define the significant correlation of studied tissue cellular immune responses with different parameters. (Table 7) shows correlation of grade and stage with immuno-pathological parameters. (Table 8) signifies the correlation of serum cytokines IL-10 and IL-12 with different parameters

Table (9) shows the significant histopathological correlations. (Table 10) displays the survival function of our patients.

Table (1): *Clinico - pathological parameters of the study group*

Case number	*Stage*	*Grade*	*Bl.v inv.*	*Lymph. Inv.*	*Fibrosis*	*L.N n=*	*Rec.*	*D.F.T*
1	III	3	I	I	I	8	1	12
2	II	1	0	0	3	0	0	24
3	III	3	0	1	3	4	1	22
4	III	2	0	0	3	2	0	24
5	III	2	0	0	3	3	0	24
6	II	2	0	0	2	5	1	20
7	III	3	I	1	1	9	1	12
8	II	2	0	0	2	4	1	20
9	III	3	I	1	1	13	1	14
10	II	2	0	0	2	4	1	20
11	I	2	0	0	3	0	0	24
12	IV	3	I	1	1	10	1	14
13	III	2	0	0	2	3	0	24
14	III	2	0	1	2	5	0	24
15	III	2	0	0	2	4	1	22
16	III	2	0	1	2	6	0	18
17	III	2	I	0	1	8	0	18

- Grade 1= I - Fibrosis 1 = Mild
2 = I 2 = moderate
3 = III 3 = Excess
- Bl. v. inv 0 No - Recurrence: 0 no recurrence
=
lymph. inv 1 invasion 1 recurrence

Table (2): *Peripheral blood and tissue parameters in the study versus the control group.*

	SNK%		*SNKF*		*TNK%*		*TNKF*		*T.L.C cpm*		*TIL cpm*	
	P	C	P	C	P	C	P	C	P	C	P	C
Mean	30.14	7.78	29.76	8.69	6.68	0	20.71	0	28273.64	10548.12	18323.73	0
P value	0.001		0.001		0.001		0.001		0.001		0.001	

SNK% = serum NK %
- SNKF = function
- TNK = tissue NK
- TNKF = function
- T.L.C = total lymphocytic count (count per minute)
- P = Patient n = 17
- C = Control n = 10

Table (3): ***Correlation of studied peripheral blood cellular immune response with other parameters.***

	SNK	*SNKA*	*Lymph. Count*
Serum NK		0.02	0.04
Serum NKA	0.002		
Lymph. Count	0.04		
TNK			
TNKA			0.03
TILs			
IL-10			
IL-12		0.001	
Grade			
Fibrosis			
Vascul. Invasion			
Lymph. Invasion			
Lymph node			
Stage			

TILs = tumour infiltrating lymphocytes

Table (4): ***Correlation between SNK, serum lymphocytes, and SNA activity and other immuno-pathological parameters.***

		SNK	*Peripheral lymphocytes*	*SNKA*
SNK	τb	1.000	0.052	-0.406
	P		0.773	0.023
TNK	τb	0.052	1.000	-0.030
	P	0.773		0.869
SNKA	τb	0.406	-0.030	1.000
	P	0.023	0.869	
TNKA	τb	-0.089	-0.022	0.170
	P	0.620	0.901	0.343
S. lymphocytes	τb	-0.096	0.119	0.147
	P	0.592	0.509	0.410
TILs	τb	0.362	0.119	0.147
	P	0.592	0.509	0.410
Grade	τb	-0.133	0.113	0.112
	P	0.519	0.585	0.586
Stage	τb	-0.010	0.170	-0.248
	P	0.961	0.404	0.220
Fibrosis	τb	0.124	-0.213	-0.114
	P	0.536	0.288	0.565
L.N	τb	0.023	0.108	0.061
	P	0.901	0.559	0.739
DFT	τb	0.089	-0.307	-0.161
	P	0.640	0.106	0.395

τb = Kendall's correlation coefficient

Table (5): ***Correlation of studied tissue cellular immune response with other parameters***

	TNK	*TNKA*	*TILs*
SNK			
SNKA			
S. lymphocytes			
IL-10			
IL-12			
Grade			0.04
Fibrosis		0.01	
Vascular invasion			
Lymph. Invasion			
L. N		0.001	
Stage			0.01

TILs = tumour infiltrating lymphocytes - TNK = tissue NK - TNKA = tissue NKA - L.N = lymph node

Table (6): ***Correlation between TNK, TNKA and TILs and other immuno pathological parameters.***

		TNKA	*TNKA*	*TILs*
SNK	τb	-0.089	-0.096	0.362
	P	0.620	0.592	0.043
TNK	τb	-0.022	0.119	-0.030
	P	0.901	0.509	0.869
SNKA	τb	0.170	0.147	-0.206
	P	0.343	0.410	0.249
TNKA	τb	1.000	0.125	0.376
	P		0.483	0.035
S. lymphocytes	τb	0.125	1.000	0.29
	P	0.483		0.869
TILs	τb	-0.376	0.029	1.000
	P	0.035	0.869	
Grade	τb	-0.337	-0.519	0.132
	P	0.102	0.012	0.520
Stage	τb	-0.080	-0.406	-0.030
	P	0.695	0.044	0.883
Fibrosis	τb	0.592	0.28	-0.079
	P	0.003	0.233	0.691
L.N	τb	0.061	-0.652	-266
	P	0.739	0.000	0.157
DFT	τb	-0.161	0.572	0.257
	P	0.395	0.003	0.174

τb = Kendall's correlation coefficient

Table (7): ***Correlation between grade and stage and other immuno-pathological parameters*.**

		Grade	*Stage*
SNK	Ta	-0.133	0.010
	P	0.519	0.961
TNK	Ta	0.113	0.170
	P	0.585	0.404
SNKA	Ta	0.112	-0.248
	P	0.586	0.220
TNKA	Ta	-0.337	0.080
	P	0.102	0.695
S. lymphocytes	Ta	-0.519	-0.406
	P	0.012	0.044
TILs	Ta	-0.132	-030
	P	0.520	0.883
Grade	Ta	1.000	0.521
	P		0.026
Stage	Ta	0.521	1.000
	P	0.026	
Fibrosis	Ta	-0.536	-0.403
	P	0.020	0.075
L.N	Ta	0.613	0.463
	P	0.004	0.026
DFT	Ta	-0.611	-0.270
	P	0.005	0.207

τb = Kendall's correlation coefficient

Table (8): ***Correlation of cytokines IL-10 and IL-12 with other parameters*.**

	IL-10	*IL-12*
SNK		
SNKA	0.03	0.02
S. lymphocytes	0.01	0.03
TNK		
TNKA		
TILs		
Grade		
Fibrosis		
Vas. Invasion		
Lymph. Invasion		
L. N		
Stage	0.05	0.09
IL-10		0.05
IL-12	0.05	

Table (9): ***Correlation among studied histopathological parameters***

	Grade	*Fibrosis*	*Lymph. Invasion*	*Vas. Invasion*	*L.N*	*Stage*
Grade		0.01			0.02	0.01
Fibrosis	0.01				0.01	
Lymp. Inv.						
Vas. Inv.						
L.N	0.001	0.001	0.01	0.05		0.02
Stage	0.02				0.03	

Table (10): ***Survival functions of studied patients***

	O. F. S	*D. F. T*	*Recur.*
SNK			
SNKA	NS		
S. lymph. Count	NS		
TNK	NS		
TNKA	0.05	0.03	
TILs	0.04		
Grade		0.05	
Fibrosis	0.02	0.001	
Lymph. Invasion	NS	0.001	
Vas. Invasion	NS	0.05	
L.N	0.04	0.03	0.04
Stage	0.001		
IL-10	NS	0.001	
IL-12	0.03	0.02	

D.F.S = Disease free survival - D.F.T = Disease free survival
Rec. = Recurrence

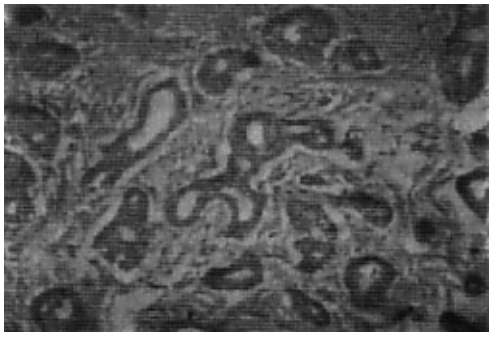

Fig.(1): well differentiated infiltrating duct carcinoma. Evident tubular differentiation. (H&Ex100).

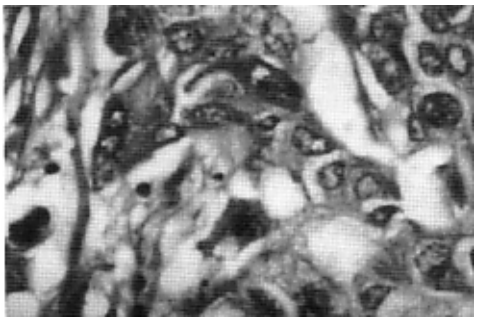

Fig.(2): High grade infiltrating duct carcinoma. Prominent cellular atypia with frequent mitoses. (H&Ex100).

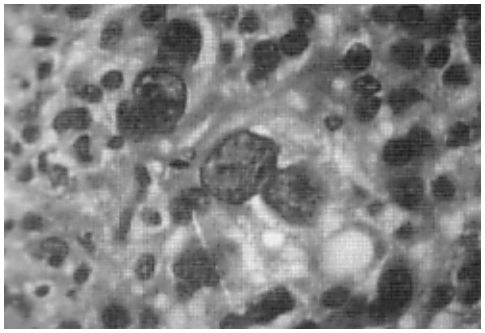

Fig.(3): Medullary carcinoma. Mature lymphocytes at the periphery of neoplastic syncteal sheets. (H&Ex100).

Fig.(4): Lymphatic tumor emboli. Tumor sheets within lymphatic spaces (H&Ex100).

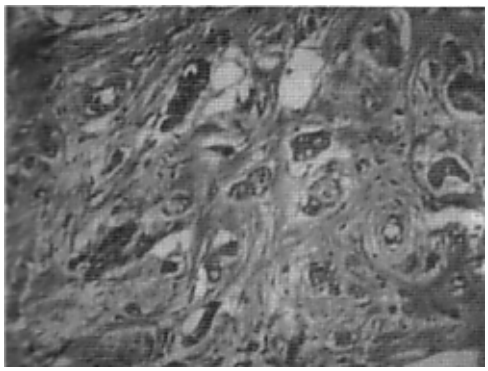

Fig.(5): Sclerosing G II infiltrating duct carcinoma. ((H&Ex100).

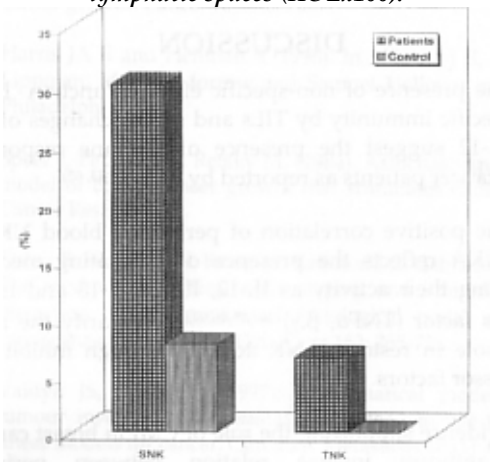

Fig.(6): SNK and TNK percent in patients and control groups.

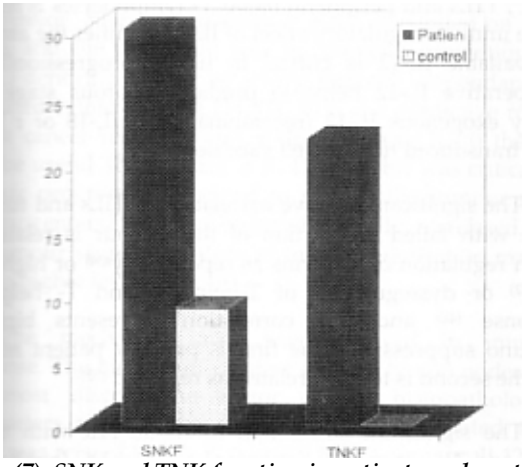

Fig.(7): SNK and TNK function in patients and control groups.

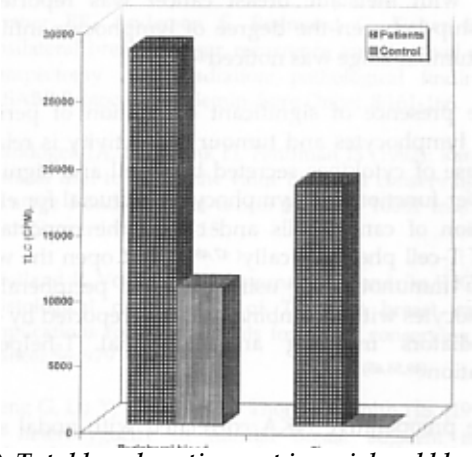

Fig.(8): Total lymphocytic count in peripheral blood and tissue of patients and control groups.

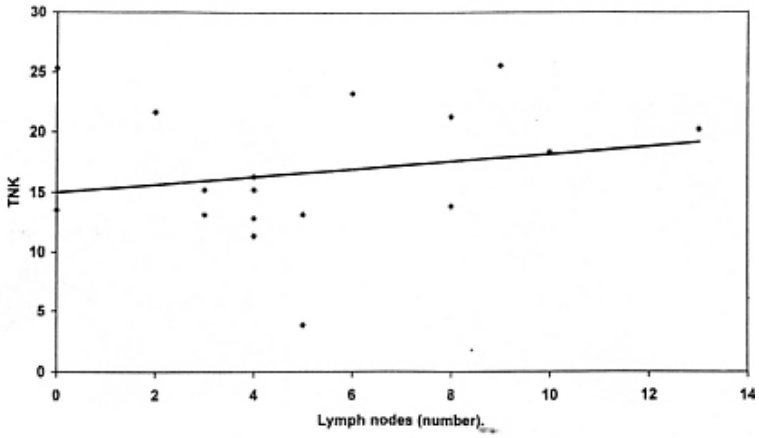

Fig.(9): Correlation between positive lymph nodes number and TNK in patient group.

DISCUSSION

The presence of non-specific effector function by NK and specific immunity by TILs and serum changes of IL-10 and IL-12 suggest the presence of immune response in breast cancer patients as reported by [27, 30, 42, 43, 65]

The positive correlation of peripheral blood NK with their NKA reflects the presence of circulating mediators enhancing their activity as IL-12, IL-15, IL-18 and tumour necrosis factor (TNFα, β,γ) [44,45,46,59,61] so clarify the role of levamisole in restoring NK activity through inhibition of suppressor factors.

Evidence supporting the role of CMI in breast cancer is the significant inverse relation between peripheral lymphocytes count and chance of recurrence. An altered proportion of T-cell subsets in the peripheral blood of patients with metstatic breast cancer was reported. No relationship between the degree of lymphocyte infiltration and the tumour stage was noticed.[27]

The presence of significant correlation of peripheral blood T lymphocytes and tumour NK activity is related to the release of cytokines secreted by T-cell and signify that the proper function of T-lymphocytes is crucial for effective destruction of cancer cells and clarify the importance of study of T-cell phenotypically[47,48,52] and open the way for adoptive immunotherapy using activated peripheral blood T-lymphocytes with recombinant IL-2 as reported by 49 also for mediators inducing an oligoclonal T-helper cell proliferation[49,58,65]

The preoperative NKA correlated with nodal status , vascular invasion, tumor stage so NKA are involved in disease progression and host immunity contributes to metastasis development as reported by 45. Moreover the significant correlation of NKA with interleukin – 12 but not NK count denotes the upregulation of NK is dependant on the TL-12 levels as reported by[49, 59].

Moreover, the correlation of NK with NKA and with peripheral blood lymphocytes suggests the important role of antigen presenting cell (APC) dendritic cells as reported by [22, 50, 58] and the DC capture by the apoptic tumors and present their antigens MHCI and II pathways for recognition by CD4 and CD 8 cells [51].

The significant high level of IL 10 with their significant correlation with NKA and TILs but low IL12 is related to the inhibition of macrophage co-stimulatory effect inhibiting NKA and TILs function so the IL 10 level has a predictive role in breast cancer response to immunotherapy and justify exogenous anti- TL 10 therapy [52,58,60].

The significant correlation of IL-12, serum and tissue NAK , T1Ls and peripheral blood T- lymphocytes is related to the immunoregulatory effect of IL-12 signifies the amount of available IL-12 is critical in tumor progression[53] so preoperative IL-12 helps to predict tumorous stage and justify exogenous IL-12 (recombinant) r, IL-18 or r IL-12 gene transduced tumour cell vaccine [59].

The significant positive correlation of TILs and tumour stage with failed eradication of the tumour is related to down regulation of integrins as reported by[54] or higher IL 10 [52] or dysregulation of T-helper I and T- helper 2 response [46] and that correlation represents biphasic immuno suppression , the first is primary patient related and the second is tumour related as reported [55].

The significant correlation of tissue NK with tissue NKA and TILs is mostly antigenic based on DC (antibody – dependant cell mediated cytotoxicity) as reported by [44] so DC is helpful in screening and therapy of breast cancer patients.

The degree of fibrosis correlated negatively with the tumour grading and lymph node number so the mechanical arm is involved in the immune response.

Analysis of two-years outcome revealed 47% recurrent disease. The recurrence is closely related to the number of the positive axillary lymph nodes.

The significant correlation of tumour size with D-F-S seems to be indirect through the lymph node metastases and not through tumor grade due to lack of correlation of tumour grade and tumour size as reported by[35,56] and maxillary lymph node status was the discriminant factor among the histopathological parameters as reported by [37,56].

The D-F-S is significantly correlated with tissue NKA and serum NKA through the IL-12 level so IL-12 helps to predict patients prognosis and the type of therapy as reported by [44, 53, 57, 58,60 , 61].

Also correlation with TILs as reported by [52,5,62] is related to their cytotoxic activity and the cox proportional hazard was the TILs as reported by [66,67,68].

Also , the survival function of D-F-T revealed medium D-F-T [17-76] months, median 20 months (12-14 month). And the DFT was significantly correlated with tissue NKA, lymph node number through lympho-vascular invasion and the fibrosis through IL-10 but the discriminant factor was TNKA.

CONCLUSIONS

Proper function of peripheral blood T-lymphocytes was crucial for effective destruction of breast cancer cells rising the importance of using it activated in adoptive immunotherapy NKA was involved in breast cancer progression. The upregulation of NKA depended on IL-12 levels and the important role of antigen –presenting cell (APC) dendritic cell for achieving antibody dependant cell mediated cytotoxicity (A.D.C) IL 10 had a predictive role in breast cancer response and exogenous anti IL-10 therapy may be useful The amount of IL-12 available was critical for tumour progression , helped to predict the tumour stage and exogenous IL-12 may be beneficial. TILs correlated with the tumour stage but failure of tumour eradication may be due to higher levels of IL-10

The mechanical arm was involved in immune response. The number of positive axillary lymph nodes was the most discriminant factor among histopathological parameters and the recurrence was closely related to it. D.F.S was related to T N K A and S N K A through IL12 The discriminant factor in DFT was the TNKA

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