Cellular and Molecular Biology

Tewari *et al. Cell. Mol. Biol.*2016, 62 (2): 116-125
ISSN: 1165-158X
doi: 10.14715/cmb/2016.62.2.20

Original Research

A study on local expression of NF-κB, CCL2 and their involvement in intratumoral macrophage infiltration in breast cancer

B. N. Tewari[1], K. Singh Baghel[5], C. Tripathi[5], P. Dubey[3], M. L. B. Bhatt[3], V. Kumar[1], M. Mati Goel[4], M. P. Singh Negi[6], S. Misra[1,2*]

[1] Department of Surgical Oncology, King George Medical University, Lucknow 226003, India
[2] Department of Surgical Oncology, All India Institute of Medical Sciences, Jodhpur-342005, Rajasthan, India
[3] Department of Radiotherapy, King George Medical University, Lucknow 226003, India
[4] Department of Pathology, King George Medical University, Lucknow 226003, India
[5] Division of Toxicology, Central Drug Research Institute (CSIR), Lucknow 226031, India
[6] Biometry and Statistics Division, Central Drug Research Institute (CSIR), Lucknow 226031, India

Abstract: NF-κB has been implicated in mechanisms promoting inflammation in tumor microenvironment leading to breast cancer metastasis. Owing to critical role of CCL2 during metastasis, particularly in its capacity to act as a chemoattractant for macrophages and their precursors i.e monocytes, we decided to explore if pro-metastatic function of NF-κB could be attributable to CCL2 and/or macrophage infiltration. Through our study we provide experimental and clinical evidence in support of co-ordinated expression of chemokines CCL2, NF-κB and intratumoral macrophage content particularly with respect to breast cancer, with an additional evidence of these three variables being key determinant for poor prognosis and diminished survival amongst breast cancer patients both independently as well in a coordinated manner. The mean fold increase in mRNA expression level of NF-κB and CCL2 indicated that it was over expressed 13.57 and 13.18 fold respectively in tumor tissue as compared to adjacent normal tissue. Among these Immunohistochemistry expression of CD68 marker showed that 62 patients (66.7%) had low/moderate CD68 expression while 31 patients (33.3%) had strong expression. All three variables viz.NF-κB, CCL2 and CD68 showed significant ($p<0.05$ or $p<0.01$ or $p<0.001$) respectively associations with both clinicopathological (except CD68 with stage) and hormone receptors (ER, PR and Her2/neu) and their co-expressions indicating these as predictors of breast cancer. In this study we decipher the possible molecular mechanism by way of which NF-κB may promote breast cancer metastasis. Our study has clinical relevance as it establishes significance of these three variables as potential predictive markers to be employed in breast cancer.

Key words: NF-κB, CCL2, Intratumoral Macrophage (CD68 positive cells).

Introduction

Breast cancer is one of the most common cause of cancer and second leading cause of cancer related death among women worldwide (1). In 2012, an estimated 1.41 million new cancer cases were reported and 8.2 million cancer related death occurred worldwide (2). One of the major underlying cause of breast cancer related deaths is distant metastasis (3). Current medical treatments have provided appropriate palliation but are unable to eradicate metastatic breast cancer (MBC) (4) thereby making an incurable condition. Being largely incurable, the onset of metastasis is one of the biggest obstacles to the successful treatment of cancer (5). Once underway it can neither be reversed nor stopped, therefore, avoiding the onset is of paramount significance. This essentially requires a better understanding with regards to determinants and markers of metastatic transformation. Such knowledge may lead to identification of not only new prognostic markers but also may lead to development of targeted therapeutic regimen against metastatic cancer.

Nuclear factor-κB (NF-κB) is one of the most important transcription factors which play a crucial role in the transcriptional activation of pro-inflammatory cytokines, cell proliferation and survival, inflammatory diseases and various types of cancer (6). NF-κB proteins consist of five members family: which are NF-κB1 (p50 and its precursor p105), NF-κB2 (p52and its precursor p100), RelA (p65), RelB and c-Rel (REL). Nuclear factor-κB (NF-κB) regulates the transcription of many genes for immune response, cell adhesion, differentiation, proliferation, angiogenesis and apoptosis (7). It plays a central role in the regulation of diverse biological processes, including immune responses, development and cell proliferation (8). NF-κB is known to promote multiple cancer related phenomenon such as, cellular transformation, proliferation, tumor neo-vasculogenesis etc. Interestingly, recent studies implicate NF-κB in cancer cell invasion and metastasis as well (9, 10). It is reported that NF-κB activation consequential to underlying inflammation in tumor microenvironment promotes cancer cell invasion and metastasis in many tumors, including breast cancer (11).

CC chemokine ligand 2 (CCL2), also known as monocyte chemotactic protein-1 belongs to CC motif subfamily of chemotactic protein. It is produced by

Received February 15, 2016; **Accepted** February 26, 2016; **Published** February 29, 2016

*** Corresponding author:** Dr. Sanjeev Misra, Department of Surgical Oncology, King George Medical University, Lucknow 226003, India, All India Institute of Medical Sciences, Jodhpur-342005, Rajasthan, India. Email: misralko@gmail.com

macrophages, fibroblasts, and endothelial cells to stimulate chemotaxis of monocyte/macrophages and other inflammatory cells through its cognate receptor, CCR2 (12, 13). CCL2, which is the endogenous ligand of endothelial cell receptor CCR2, bears an angiogenic potential comparable to vascular endothelial growth factor (VEGF-A) (14). In several cancers, CCL2 has been previously shown to be an important determinant of tumor growth (15). CCL2 has been proposed as a possible gene involved in metastatic breast cancer because high expression of CCL2 correlates with a decrease in survival of breast cancer patients. Prognostic investigation revealed that high expression of CCL2 correlated with advanced tumor stage, lymph node metastasis (16) and early relapse (17). The pro-metastasis function of CCL2 has been attributed to its ability to recruit inflammatory monocytes and tumor associated macrophages (TAMs) (18). In agreement with this Bonapace et al (2014) reported that macrophage infiltration in breast cancer correlated with high expression level of CCL2 (19). A recent meta-analysis showed that an increased macrophage density was associated with poor prognosis in more than 80% of breast cancer (20). Despite emerging evidence in favour of role of NF-κB in cancer cell invasion and metastasis, the studies delineating underlying molecular mechanisms are scant and so are specific clinical investigation that would establish the relevance of pathway with respect to cancer cell invasion and metastasis.

In view of the above, current study was planned to substantiate the role of NF-κB in breast cancer metastasis and to decipher the possible molecular mechanism by way of which NF-κB may promote breast cancer metastasis. Owing to critical role of CCL2 during metastasis, particularly in its capacity to act as a chemoattractant for macrophages and their precursors i.e monocytes, we decided to explore if pro-metastatic function of NF-κB could be attributable to CCL2 and/or macrophage infiltration.

Materials and Methods

Patients and clinical specimens

Breast tumor sample were collected after informed consent from 93 patients who underwent surgery for breast cancer at Department of Surgical Oncology, King George's Medical University, Lucknow (India) The ethics committee at King George's Medical University, Lucknow (India) approved the study protocol (#ECM IIB/P17), which followed the Declaration of Helsinki. Immediately after surgical removal, fresh tumor tissue and adjacent normal tissue were separately collected and transferred into formalin for IHC and RNAlater™ (Invitrogen, Carlsbad, CA, USA) for gene expression analysis.

Extraction of RNA and Quality Control

About 30-50 mg of frozen tissues were crushed manually in liquid nitrogen and immediately homogenized (Heidolph Homogenizer, Aldrich) in 1 ml TRI reagent (Molecular Research Center). The homogenized tissue was incubated for 10 min at room temperature followed by the addition of 200 µl chloroform with vigorously mixing for 10-15 sec. The sample was allowed to stand undisturbed for 15 min at room temperature and then centrifuged at 12500 rpm for 20 min at 4°C. The aqueous phase was carefully transferred to a fresh tube and RNA was precipitated with 500 µl isopropyl alcohol. Further, centrifugation at 12000 rpm for 15 min at 4°C followed by one wash at 5000 rpm for 5 min at 4°C with 1 ml 75% ethanol, to recover pure RNA pellet. Finally, the RNA pellet was air dried and re-suspended in RT PCR grade water. RNA concentration and purity was determined at an optical density ratio of 260/280 using the Nanodrop® ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE).Total mRNA with OD260/OD280> 1.8 and OD260/OD230 ≥ 1.8 was used for RT-PCR experiments.

Synthesis of cDNA

High capacity cDNA Reverse Transcription kit (Applied Biosystem, Foster city, USA) was used to synthesize cDNA in a 20µl RT (2XRT Master Mix) reaction mixture including 2 µl of 10X RT buffer, 0.8 µl of 25X dNTP Mix (100 mM), 2.0 µl of 10X RT Random Primer, 1µl of MultiScribe™ Reverse Transcriptase, 1 µl of RNase inhibitor and up to ≥1µg RNA, finally makeup volume 20µl by adding RT-PCR grade water. The mixture was vortex, short spin and run in thermal cycler (Bio-Rad) according to manufacture protocol.

Quantitative real time PCR analysis

Quantitative Real–time PCR analysis was performed on a Light Cycler 480 System (Roche) 96-well plates using SYBR Green qPCR Master Mix (KAPA SYBER FAST q PCR KIT, KAPA BIOSYSTEMS) accordance with manufacturer's protocol. Data were analyzed using the Roche Light Cycler 480 software (Version 1.5). Cp and Ct were calculated by the Second Derivate Maximum Method. The amount of the target mRNA was examined and normalized with respect to β-actin gene mRNA. Relative change in mRNA expression ratio between tumor and matched normal sample were calculated by using $2^{-\Delta\Delta CT}$ method (21).Results represent data from three separate experiments. Forward and Reverse Primer sequence is given in Table 1.

Table 1. Forward and Reverse PCR Primer sequences.

S.NO.	Primer		Sequence (5'-3')
1	NF-κB (Human)	Forward	5'-CTGGCAGCTCTTCTCAAAGC-3'
		Reverse	5'-TCCAGGTCATAGAGAGGCTCA-3'
2	CCL-2 (Human)	Forward	5'-CAGCCAGATGCAATCAATGCC-3'
		Reverse	5'-TGGAATCCTGAACCCACTTCT-3'
3	β-actin (Human)	Forward	5'-TAT TGG CAA CGA GCG GTT C-3'
		Reverse	5'-ATG CCA CAG GAT TCC ATA CCC-3'

Immunohistochemistry

To assess the intratumoral macrophage content CD68 positive status was employed as marker for designating macrophages. Breast tumor tissue slide were deparaffinised, rehydrated and washed. Endogenous peroxidases were blocked using 2% hydrogen peroxide, followed by antigen retrieval in 10 mM citrate buffer pH 6.0 for 30 min. Tissue sections were incubated overnight with the primary antibody (CD68 (KP1): sc-20060 Santa Cruz Biotechnology, California, USA), at 1:50 dilution, Thereafter samples were incubated for 1hr with HRP-conjugated secondary antibody (Jackson) at 1:200 dilution. Antigen was visualized with DAB Peroxidase Substrate Kit (Thermo Fischer Scientific, USA).Finally, tissue specimens were stained with hematoxylin (Thermo Fischer Scientific, USA) to discriminate nucleus from cytoplasm. Thereafter, sections were mounted in DPX (Sigma) and analysed at 400X magnification using Lieca microscope. At least two different sections per sample were analyzed, and staining was annotated as follows: 1^+, low positive (Low), when less than 10% of the cells were positive; 2^+, moderate positive, 11–50% positive cells and 3^+ strongly positive, more than 50% positive cells.

Cell culture and *in vitro* differentiation

Human leukemia monocyte THP-1 cells, human mammary cancer-derived cells (MCF-7 cells) were procured from ATCC. Cells were maintained in RPMI 1640 or DMEM respectively using standard mammalian cell culture methods. THP-1 cells were differentiated to macrophages according to Daigneault et al 2010 (22).

Chemotaxis Assay

For evaluation of migratory potential, the cancer cells were seeded onto matrigel coated 8 µm standing PCF transwell cell culture inserts. These inserts were introduced into standard 12 well cell culture plates. Thereafter THP-1 derived macrophages housed in 0.4µm PET hanging cell culture insert were introduced into 8µm PCF insert harboring cancer cells. The co-cultures were incubated for 24 hrs under standard cell culture environment (5% CO_2, 37°C). After 24hrs the 8µm PCF inserts harboring cancer cells were collected, the non migrated cells at the underside were wiped off using sterile cotton swabs. Migrated cells adhered at the topside of membrane were fixed and mounted in DAPI mounting media (nuclear stain) for qualitative visualization of invasion using Leica DCF450C fluorescence microscope. The quantification was carried out in five different fields of three replica sets.

Statistical analysis

Continuous data were summarized as Mean and SD while discrete (categorical) in number and percentage. The chi-square (χ^2) test was used to assess associations between the variables. Univariate (unadjusted) and multivariate logistic regression analysis was done to assess independent predictor of breast cancer. Cox regression analysis was done to assess independent predictor of overall survival. Difference in overall survival was compared by Log-rank test. A two-tailed p $p<0.05$ was considered statistically significant.

Results

Basic characteristics of breast cancer patients

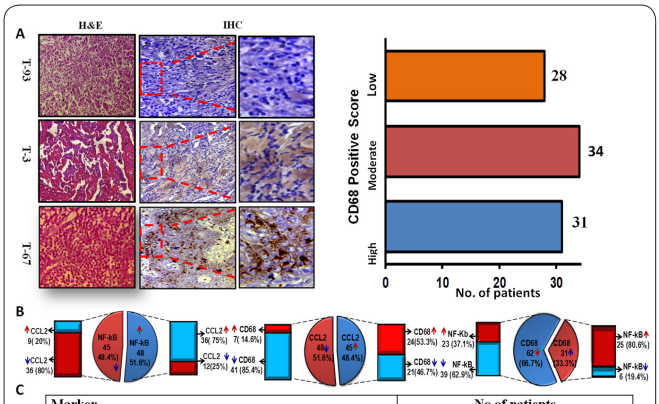
The demographic, clinicopathological and hormone receptor expressions of 93 breast cancer patients are summarized in Table 2. The age of patients ranged from 25-70 yrs with mean (± SD) 46.04 ± 11.54 yrs and median 45 yrs. Among patients, ≤45 yrs, 42 (45.2%) were premenopausal and >45 yrs, 51 (54.8%) with post menopausal, fourteen patients (15.1%) had tobacco addiction and 35 (37.6%) were non vegetarian. Furthermore, 37 (39.8%) patients had tumor size T3-T4 and 72 (77.4%) patients had lymph node metastasis and 51 (54.8%) were in stage III/IV. Of total 48 (51.6%) patients were ER positive, 43 (46.2%) were PR positive and 57 (61.3%) were Her2/neu positive. Moreover, 45 (48.4%) patients had no co-expression (ER, PR and Her2/neu), 19 (20.4%) patients had triple negative (–ve) and 29 (31.2%) had triple positive (+ve) disease.

Association between mRNA fold expression level (ΔΔCt) of NF- κB, CCL2 and intratumoral macrophage content in breast cancer specimens

Immunohistochemical analysis of macrophage marker (CD68), frequency distribution (%) of mRNA fold expressions (ΔΔCt) of CCL2 and NF-κB were studied and results are summarized in Figure 1A&B. The mRNA fold expression of CCL2 and NF-κB in patients ranged from 0.00-56.30 and 0.01-53.08 respectively with mean

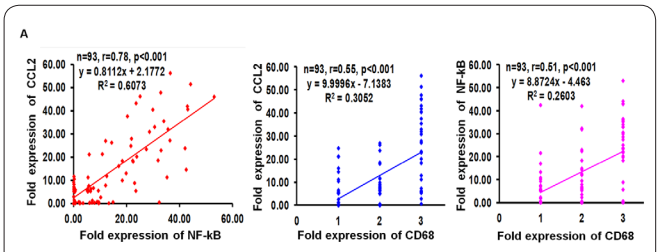
Table 2. Demographic, clinico-pathological and hormone receptor expression of breast cancer patients (n=93).

Variables	No of patients (n=93) (%)
Age (yrs) (Mean ± SD, range, median):	46.04 ± 11.54, 25-70, 45
≤45	51 (54.8)
>45	42 (45.2)
Menopause:	
Pre-menopausal	42 (45.2)
Post-menopausal	51 (54.8)
Tobacco:	
No	79 (84.9)
Yes	14 (15.1)
Diet:	
Veg	58 (62.4)
Nonveg	35 (37.6)
Tumor Size:	
T1-T2	56 (60.2)
T3-T4	37 (39.8)
Lymph node metastasis:	
Absent	21 (22.6)
Present	72 (77.4)
Stage:	
I-II	42 (45.2)
III-IV	51 (54.8)
ER:	
-ve	45 (48.4)
+ve	48 (51.6)
PR:	
-ve	50 (53.8)
+ve	43 (46.2)
Her2/neu:	
-ve	36 (38.7)
+ve	57 (61.3)
Coexpression§ (ER,PR and Her2/neu):	
No	45 (48.4)
Triple -ve	19 (20.4)
Triple +ve	29 (31.2)



Marker	No of patients (n=93) (%)
NF-kB fold expression (Mean ± SD, range, median):	13.57 ± 13.91, 0.01-53.08, 7
Low (ΔΔCt ≤7)	45 (48.4)
High (ΔΔCt >7)	48 (51.6)
CCL2 fold expression (Mean ± SD, range, median):	13.18 ± 14.48, 0.00-56.30, 7
Low (ΔΔCt ≤7)	48 (51.6)
High (ΔΔCt>7)	45 (48.4)
CD68 expression:	
Low+Moderate	62 (28+34) (66.7)
Strong	31 (33.3)

Figure 1. Immunohistochemistry detection of tumor-associated macrophages (CD68 marker) in breast cancer specimens. A: Representative images of H&E. staining and Immunohistochemistry.(T-93) Micrograph shows low infiltration of macrophage, (T-3) Micrograph shows Moderate infiltration of macrophage and (T-67) Micrograph shows High infiltration of macrophage in Breast Cancer Tissue, Magnification ×400. **B**: Schematic distribution of breast cancer patients (n=93) with respect to fold change expression of selected markers. **C:** Distribution of macrophage marker expression (CD68) and mRNA fold change expressions of chemokine (CCL2) and inflammatory variables (NF-κB) of breast cancer patients (n=93).



Marker gene	n	NF-kB expression			CCL2 expression			CD68 expression		
		Low	High	p value	Low	High	p value	Low/Moderate	Strong	p value
NF-kB expression										
Low (≤7)	45	-	-	-						
High (>7)	48									
CCL2 expression:										
Low (≤7)	48	36 (80.0)	9 (20.0)	<0.001	-	-	-	-	-	-
High (>7)	45	12(25.0)	36 (75.0)							
CD68 expression:										
Low/Moderate	62	39 (86.7)	6 (13.3)	<0.001	41 (85.4)	7 (14.6)	<0.001	-	-	-
Strong	31	23 (47.9)	25 (52.1)		21 (46.7)	24 (53.3)				

Figure 2. Association between fold change (tumor tissue vs matched normal) in mRNA expression level of NF-kB, CCL2, and IHC expression of macrophage (CD68) in human breast cancer specimen. A: Correlation between fold change expressions of CCL2 vs NF-κB, CCL2 vs CD68 and NF-κB vs CD68 marker genes of breast cancer patients (n=93). **B:** Inter correlation of frequency distribution of macrophage marker CD68 by Immunohistochemistry and mRNA fold change expressions of chemokine (CCL2), inflammatory variables (NF-κB) of breast cancer patients (n=93).

(± SD) 13.18 ± 14.48 and 13.57 ± 13.91 respectively and median 7 (Figure 1C). The mean fold expressions of NF-κB and CCL2 indicate that it over expressed 13.57 and 13.18 fold respectively in cases (tumor tissue) as compared to controls (matched or adjacent normal tissue). Further, median expression showed that 45 (48.4%) patients had ≤7 (low) NF-κB fold expression and 48 (51.6%) patients had >7 (high) fold expression. Forty eight (51.6%) patients had ≤7 (low) CCL2 fold expression and 45 (48.4%) patients had >7 (high) fold expression. The frequency distribution of macrophage content (CD68 positive cells) showed that 62 (66.7%) patients had low/moderate expression and 31 (33.3%) had strong expression (Figure 1B & 1C).The correlation analysis also revealed a significant and direct association between the variables (NF-κB vs. CCL2, r=0.78, p<0.001; CD68 vs. CCL2, r=0.55, p<0.001; CD68 and NF-κB, r=0.51, p<0.001) with highest being between NF-κB and CCL2 (Figure 2A)**.** The inter correlation of frequency distribution of CD68, CCL2 and NF-κB showed a significant and positive (direct) associations with each other. Further, chi-square (χ^2) analysis of frequency distribution showed significant associations between the variables (NF-κB vs. CCL2, p<0.001; CD68 vs. CCL2, p<0.001; CD68 vs. NF-κB, p<0.001) (Figure 2B). Further, the quantitative assessment also revealed significant and positive association with each other indicating interrelatedness between these variables (Table 3).

NF-κB inhibition in MCF-7 cells diminished CCL2 mRNA expression level compromise the macrophage chemoattracting ability

Result showed that when quiescent breast cancer cells were treated with EGF (5nM) to activate NF-κB pathway, there was 6 fold increase in the mRNA expression levels of CCL2. Addition of QNZ (NF-κB inhibitor) resulted in a concentration dependent decrease in the mRNA expression levels of CCL2 gene. Even in the quiescent cell, where NF-κB activation would ideally be at basal levels, the exposition to QNZ (NF-κB inhibitor) resulted in significant down-regulation of CCL2 expression (Figure 3A). These findings clearly indicate that NF-κB could be an inducer of CCL2 gene with in breast cancer cells. CCL2 is a potent chemoattractant for macrophages. Thus down-regulation of CCL2 in breast cancer cells particularly following NF-κB inhibition should eventually result in impeded macrophage chemotaxis towards breast cancer cells. In order to ascertain this, chemotaxis of THP-1 derived macrophages towards quiescent or NF-κB activated breast cancer cells was evaluated in absence or presence of NF-κB inhibitor. Human breast cancer cells MCF-7 were grown in lower well of the modified boyden chamber. EGF treatment was employed for NF-κB activation.

Results revealed that THP-1 derived macrophages exhibited an enhanced chemotactic response towards EGF treated breast cancer cells (MCF-7) as compared

Table 3. Inter correlation of macrophage expression and fold expression (ΔΔCt) of chemokine and inflammatory genes of breast cancer patients (n=93).

Marker genes	NF-κB	CCL2	CD68
NF-kB	1.00		
CCL2	0.78***	1.00	
CD68	0.51***	0.55***	1.00

***- p<0.001 Values represent in table are pearson correlation coefficient.

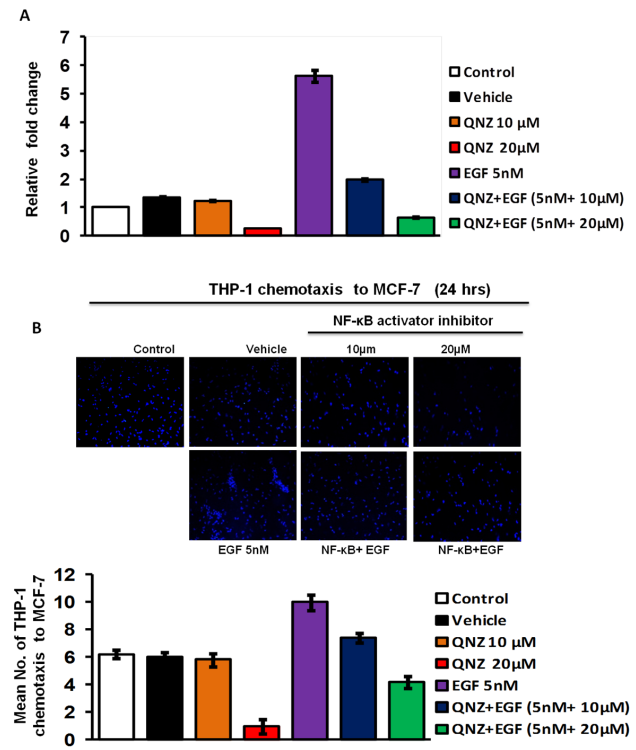


Figure 3. Effect of NF-κB inhibition in MCF-7 cells on the chemotaxis of THP-1 differentiated macrophages A: Quantitative RT-PCR revealed mRNA fold change expression levels of chemokine CCL2 at different concentration of NF-κB inhibitor. **B:** Representative images from the *in vitro* cell migration assay revealed that EGF enhanced THP-1 differentiated macrophages chemotaxis to NF-κB inhibited cancer cells (MCF-7).Bars represent mean no. of THP-1 chemotaxis towards MCF-7 ±SE (*P<0.05.).

to that towards mock control or vehicle control cells, which in turn were markedly impeded in presence of NF-κB inhibitor in a characteristic concentration dependent manner. Furthermore even in the quiescent cells where the NF-κB signalling will functional at basal levels, the higher concentration of NF-κB inhibitor significantly impeded the macrophage chemotaxis towards breast cancer cells. The results clearly indicated that NF-κB signalling potentiates chemoattractant attributes of breast cancer cells leading to enhanced chemotaxis of macrophages towards breast cancer cells. Furthermore, our findings indicate that hindering NF-κB signalling with in breast cancer cells compromises their chemoattractant attributes resulting minimized chemotaxis of macrophages towards these cells (Figure 3B). Collectively the in vitro findings corroborated the observation from human breast cancer specimen and substantiated the association between NF-κB, CCL2 and macrophage infiltration.

Both of the results revealed elevated NF-κB and CCL2 levels significantly correlated with increased intra-tumoral infiltration by macrophages (CD68 positive cells). Collectively these results pointed towards the possibility of existence of NF-κB -CCL2-macrophage axis, wherein NF-κB induced CCL2 expression with in tumor cells may result in macrophage chemotaxis towards cancer cells.

Association of mRNA expression level NF-κB, CCL2 and intratumoral macrophage content with breast cancer risk factors

The associations of variables (NF-κB, CCL2 and

Table 4. Association of variables with demographic, clinicopathological and expressions of hormone receptors of breast cancer patients (n=93).

Variables	N	NF-kB			CCL2			CD68		
		Low	High	p value	Low	High	p value	Low/ Moderate	Strong	p value
Age (yrs):										
≤45	51	26 (51.0)	25 (49.0)	0.581	26 (51.0)	25 (49.0)	0.893	34 (66.7)	17 (33.3)	1.000
>45	42	19 (45.2)	23 (54.8)		22 (52.4)	20 (47.6)		28 (66.7)	14 (33.3)	
Menopause:										
Pre-menopausal	42	19 (45.2)	23 (54.8)	0.581	23 (54.8)	19 (45.2)	0.581	28 (66.7)	14 (33.3)	1.000
Post-menopausal	51	26 (51.0)	25 (49.0)		25 (49.0)	26 (51.0)		34 (66.7)	17 (33.3)	
Tobacco										
No	79	36 (45.6)	43 (54.4)	0.197	42 (53.2)	37 (46.8)	0.477	53 (67.1)	26 (32.9)	0.838
Yes	14	9 (64.3)	5 (35.7)		6 (42.9)	8 (57.1)		9 (64.3)	5 (35.7)	
Diet										
Veg	58	32 (55.2)	26 (44.8)	0.092	33 (56.9)	25 (43.1)	0.189	40 (69.0)	18 (31.0)	0.545
Nonveg	35	13 (37.1)	22 (62.9)		15 (42.9)	20 (57.1)		22 (62.9)	13 (37.1)	
Tumor size:										
T1-T2	56	34 (60.7)	22 (39.3)	0.003	35 (62.5)	21 (37.5)	0.010	45 (80.4)	11 (19.6)	0.001
T3-T4	37	11 (29.7)	26 (70.3)		13 (35.1)	24 (64.9)		17(45.9)	20 (54.1)	
Lymph node metastasis:										
Absent	21	16 (76.2)	5 (23.8)	0.004	18 (85.7)	3 (14.3)	<0.001	20 (95.2)	1 (4.8)	0.002
Present	72	29 (40.3)	43 (59.7)		30 (41.7)	42 (58.3)		42 (58.3)	30 (41.7)	
Stage:										
I-II	42	27 (64.3)	15 (35.7)	0.005	28 (66.7)	14 (33.3)	0.008	32 (76.2)	10 (23.8)	0.077
III-IV	51	18 (35.3)	33 (64.7)		20(39.2)	31 (60.8)		30 (58.8)	21 (41.2)	
ER:										
-ve	45	29 (64.4)	16 (35.6)	0.003	36 (80.0)	9 (20.0)	<0.001	39 (86.7)	6 (13.3)	<0.001
+ve	48	16 (33.3)	32 (66.7)		12 (25.0)	36 (75.0)		23 (47.9)	25 (52.1)	
PR:										
-ve	50	34 (68.0)	16 (32.0)	<0.001	37 (74.0)	13 (26.0)	<0.001	44 (88.0)	6 (12.0)	<0.001
+ve	43	11 (25.6)	32 (74.4)		11 (25.6)	32 (74.4)		18 (41.9)	25 (58.1)	
Her2/neu:										
-ve	36	24 (66.7)	12 (33.3)	0.005	25 (69.4)	11 (30.6)	0.006	31(86.1)	5 (13.9)	0.002
+ve	57	21(36.8)	26 (63.2)		23 (40.4)	34 (59.6)		31 (54.4)	26 (45.6)	
Coexpression§ (ER,PR and Her2/neu):										
No	45	34 (75.6)	11 (24.4)		32 (71.1)	13 (28.9)		37 (82.2)	8 (17.8)	
Triple -ve	19	10 (52.6)	9 (47.4)	<0.001	14 (73.7)	5 (26.3)	<0.001	18 (94.7)	1 (5.3)	<0.001
Triple +ve	29	1 (3.4)	28 (96.6)		2 (6.9)	27 (93.1)		7 (24.1)	22 (75.9)	

§: coexpression of ER, PR and Her2/neu, -ve: negative, +ve: positive

macrophage content) with breast cancer risk factors (demographic, clinicopathological and hormone receptor expressions) are summarized in Table 4. All three variables showed significant ($p<0.05$ or $p<0.01$ or $p<0.001$) associations with clinicopathological and hormone receptors (ER, PR and Her2/neu) except macrophage content (CD68 positive cells) with stage. Further, all three marker also showed high association ($p<0.001$) with co-expression of hormone receptors.

Independent association of NF-κB, CCL2 and intratumoral macrophage markers of breast cancer patients

To find out independent association of variables with risk factors, the demographic, clinicopathological and hormone receptor expressions were further subjected to separately univariate (unadjusted) and multivariate (adjusted) logistic regression analysis and summarized

Table 5. Univariate (unadjusted) and multivariate (adjusted) association of NF-κB, CCL2 and CD68 expressions with clinical characteristics of breast cancer patients using logistic regression analysis (n=93).

	NF-κB				CCL2				CD68			
	Univariate		Multivariate		Univariate		Multivariate		Univariate		Multivariate	
Variables	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value
Age (yrs):												
≤45	Ref		Ref		Ref		Ref		Ref		Ref	
>45	0.79 (0.35-1.80)	0.582	0.24 (0.05-1.25)	0.091	1.06 (0.47-2.40)	0.893	2.72 (0.43-17.31)	0.289	1.00 (0.42-2.38)	1.000	0.96 (0.15-5.99)	0.966
Menopause:												
Pre-menopausal	Ref		Ref		Ref		Ref		Ref		Ref	
Post-menopausal	1.26 (0.56-2.86)	0.582	3.41 (0.72-16.24)	0.124	0.79 (0.35-1.80)	0.582	0.41 (0.08-2.20)	0.289	1.00 (0.42-2.38)	1.000	1.25 (0.22-7.22)	0.804
Tobacco												
No	Ref		Ref		Ref		Ref		Ref		Ref	
Yes	2.15 (0.66-6.99)	0.203	2.95 (0.61-14.38)	0.180	0.66 (0.21-2.08)	0.479	0.44 (0.09-2.26)	0.328	0.88 (0.27-2.90)	0.838	0.47 (0.09-2.31)	0.351
Diet												
Veg	Ref		Ref		Ref		Ref		Ref		Ref	
Nonveg	0.48 (0.20-1.13)	0.094	0.13 (0.03-0.56)	0.006	0.57 (0.24-1.33)	0.191	0.23 (0.05-1.01)	0.051	0.76 (0.32-1.84)	0.545	0.93 (0.24-3.58)	0.917
Tumor Size:												
T1-T2	Ref		Ref		Ref		Ref		Ref		Ref	
T3-T4	0.27 (0.11-0.66)	0.004	1.04 (0.26-4.21)	0.952	0.33 (0.14-0.79)	0.011	0.63 (0.13-2.96)	0.554	0.21(0.08-0.52)	0.001	0.18 (0.03-0.96)	0.045
Lymph node metastasis:												
Absent	Ref		Ref		Ref		Ref		Ref		Ref	
Present	0.21 (0.07-0.64)	0.006	0.26 (0.05-1.30)	0.100	0.12 (0.03-0.44)	0.001	0.10 (0.02-0.71)	0.021	0.07 (0.01-0.55)	0.011	0.05 (0.00-0.59)	0.018
TNM Stage:												
I-II	Ref		Ref		Ref		Ref		Ref		Ref	
III-IV	0.30 (0.13-0.71)	0.006	0.34 (0.07-1.56)	0.165	0.32 (0.14-0.76)	0.009	0.51 (0.10-2.64)	0.420	0.45 (0.18-1.10)	0.080	4.55 (0.66-31.51)	0.125
ER:												
-ve	Ref		Ref		Ref		Ref		Ref		Ref	
+ve	0.28 (0.12-0.65)	0.003	1.23 (0.33-4.59)	0.757	0.08 (0.03-0.22)	<0.001	0.11 (0.03-0.46)	0.003	0.14 (0.05-0.40)	<0.001	0.58 (0.12-2.82)	0.497
PR:												
-ve	Ref		Ref		Ref		Ref		Ref		Ref	
+ve	0.16 (0.07-0.40)	<0.001	0.12 (0.03-0.44)	0.001	0.12 (0.05-0.31)	<0.001	0.30 (0.08-1.11)	0.072	0.10 (0.03-0.28)	<0.001	0.13 (0.03-0.56)	0.006
Her2/neu:												
-ve	Ref		Ref		Ref		Ref		Ref		Ref	
+ve	0.29 (0.12-0.70)	0.006	0.27 (0.07-0.97)	0.045	0.30 (0.12-0.72)	0.007	0.41 (0.11-1.54)	0.186	0.19 (0.07-0.57)	0.003	0.22 (0.04-1.10)	0.065
Coexpression§:												
No	Ref		Ref		Ref		Ref		Ref		Ref	
Triple -ve	0.01 (0.00-0.10)	<0.001	0.01 (0.00-0.09)	<0.001	0.03 (0.01-0.14)	<0.001	0.01 (0.00-0.12)	<0.001	0.07 (0.02-0.22)	<0.001	0.05 (0.01-0.23)	<0.001
Triple +ve	0.03 (0.00-0.29)	0.002	0.02 (0.00-0.24)	0.003	0.02 (0.01-015)	<0.001	0.01 (0.00-0.07)	<0.001	0.02 (0.00-0.16)	<0.001	0.01 (0.00-0.09)	<0.001

§: coexpression of ER, PR and Her2/neu, -ve: negative, +ve: positive

in Table 5. In univariate analysis, all three variables showed significant (p<0.05 or p<0.01 or p<0.001) associations with clinicopathological (except CD68 with stage) and hormone receptors (ER and PR), Her2/neu and their co expressions indicating these as markers of breast cancer. In multivariate analysis, NF-κB also showed significant (p<0.05 or p<0.01 or p<0.001) associations with diet, PR, Her2/neu and co-expression (triple –ve/+ve). Similarly, CCL2 showed significant associations (p<0.05 or p<0.01 or p<0.001) with node status, ER and co expression (triple –ve/+ve). Further, macrophage content (CD68 positive cells) showed significant (p<0.05 or p<0.01 or p<0.001) association with size of tumor, node status, PR and co expression (triple –ve/+ve), indicating NF-κB, CCL2 and macrophage content the independent markers of breast cancer.

Association of NF-κB, CCL2 and intratumoral macrophage content (CD68 positive cells) with survival of breast cancer patients.

After treatment, the patients were followed up to 4 yrs (48 months). The median follow up was 38 months. During the periods, 13 (14.0%) patients left the treatment (LTF), 59 (63.4%) patients were alive and 21 (22.6%) patients died due to disease accounting total 72 (77.4%) live (LTF + Live). To find out prognostic significance of markers, the univariate (unadjusted) and multivariate (adjusted) Cox regression analysis were done between overall survival and predictors (demographic, clinicopathological, hormone receptors and markers) and summarized in Table 6. In univariate analysis, tumor size, lymph node metastasis, stage, ER, PR, Her2/neu coexpression (triple –ve/+ve), NF-κB, CCL2 and macrophage content (CD68 positive cells) showed significant (p<0.05 or p<0.01 or p<0.001) associations with overall survival. However, in multivariate analysis NF-κB showed significant (p<0.05 or p<0.01 or p<0.001) and an independent prognostic predictor in breast cancer patients along with tumor size, lymph node metastasis, stage, ER, Her2/neu and coexpression of hormone receptors. In contrast, CCL2 showed significant (p<0.05 or p<0.01 or p<0.001) and an independent prognostic predictor in breast cancer patients along with tumor size, lymph node metastasis, stage, ER and coexpression of hormone receptors. Conversely, CD68 showed significant (p<0.05 or p<0.01 or p<0.001) and an independent prognostic predictor in breast cancer patients along with tumor size, TNM stage, ER and coexpression of hormone receptors.

The 4 year overall survival in 93 breast cancer patients were further evaluated on NF-κB fold expression (Low vs. High), CCL2 fold expression (Low vs. High) and CD68 expression (Low/Moderate vs. Strong) and summarized in Table 7 and also depicted in (Figure 4 A,

Table 6. Univariate (unadjusted) and multivariate (adjusted) association of demographic, clinicopathological, hormone receptor and variables with overall survival of breast cancer patients using Cox regression analysis (n=93).

Predictors	Univariate		Multivariate					
			NF-κB		CCL2		CD68	
	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value	OR (95%CI)	p value
Age (yrs):								
≤45	Ref		Ref		Ref		Ref	
>45	0.97 (0.64-1.47)	0.898	0.99 (0.48-2.03)	0.977	0.62 (0.31-1.26)	0.184	0.72 (0.35-1.47)	0.360
Menopause:								
Pre-menopausal	Ref		Ref		Ref		Ref	
Post-menopausal	1.13 (0.75-1.70)	0.571	0.96 (0.49-1.87)	0.907	1.38 (0.70-2.71)	0.349	1.22 (0.62-2.40)	0.567
Tobacco:								
No	Ref		Ref		Ref		Ref	
Yes	1.07 (0.60-1.90)	0.817	0.8 (0.44-1.48)	0.491	1.13 (0.62-2.07)	0.690	1.02 (0.56-1.86)	0.940
Diet:								
Veg	Ref		Ref		Ref		Ref	
Nonveg	1.08 (0.70-1.65)	0.740	1.24 (0.74-2.09)	0.408	1.07 (0.63-1.79)	0.813	1.04 (0.62-1.76)	0.880
Tumor size:								
T1-T2	Ref		Ref		Ref		Ref	
T3-T4	0.44 (0.28-0.68)	<0.001	0.76 (0.42-1.40)	0.002	0.71 (0.39-1.30)	0.027	0.67 (0.36-1.24)	0.041
Lymph node metastasis :								
Absent	Ref		Ref		Ref		Ref	
Present	0.57 (0.34-0.93)	0.005	0.84 (0.44-1.63)	0.025	0.77 (0.39-1.51)	0.032	0.69 (0.35-1.35)	0.276
stage:								
I-II	Ref		Ref		Ref		Ref	
III-IV	0.56 (0.37-0.86)	0.007	0.95 (0.52-1.74)	0.019	0.92 (0.49-1.71)	0.030	0.92 (0.48-1.75)	0.037
ER:								
-ve	Ref		Ref		Ref		Ref	
+ve	0.75 (0.50-1.13)	0.009	1.08 (0.60-1.96)	0.011	1.52 (0.83-2.79)	0.021	1.23 (0.69-2.17)	0.041
PR:								
-ve	Ref		Ref		Ref		Ref	
+ve	0.59 (0.39-0.89)	0.029	0.84 (0.47-1.52)	0.564	0.63 (0.37-1.08)	0.093	0.59 (0.34-1.03)	0.065
Her2/neu:								
-ve	Ref		Ref		Ref		Ref	
+ve	0.59 (0.39-0.91)	0.023	0.82 (0.47-1.43)	0.037	0.70 (0.40-1.21)	0.197	-0.67 (0.38-1.19)	0.173
Coexpression§:								
No	Ref		Ref		Ref		Ref	
Triple -ve	0.42 (0.26-0.68)	0.004	1.02 (0.55-1.90)	0.025	0.60 (0.31-1.14)	0.039	0.45 (0.24-0.84)	0.042
Triple +ve	0.42 (0.23-0.77)	<0.001	0.79 (0.39-1.54)	0.003	0.61 (0.29-1.28	0.002	0.46 (0.21-1.03)	0.011
NF-κB:								
Low	Ref		Ref					
High	0.27 (0.17-0.43)	<0.001	0.32 (0.18-0.55)¥ 0.28 (0.15-0.52)¶	<0.001 <0.001	-	-	-	-
CCL2:								
Low	Ref				Ref			
High	0.46 (0.30-0.71)	<0.001	-	-	0.65 (0.33-0.93)¥ 0.58 (0.39-1.21)¶	0.026 0.001	-	-
CD68:								
Low/Moderate	Ref						Ref	
Strong	0.52 (0.33-0.81)	0.004	-	-	-	-	1.08 (0.56-2.09)¥ 0.94 (0.53-1.68)¶	0.810 0.045

§: coexpression of ER, PR and Her2/neu, -ve: negative, +ve: positive, Odds ratio evaluated against ref group

¥-odds ratio after adjusting age, menopause, tobacco habit, diet, tumor size, node status, stage, ER, PR and Her2/neu

¶-odds ratio after adjusting age, menopause, tobacco habit, diet, tumor size, node status, stage, and coexpression (ER, PR and Her2/neu

Table 7. Distribution of overall survivals according to NF-κB, CCL2 and Macrophage content (CD68 Positive cells) expression of breast cancer patients (n=93).

Gene expression	N	Mean	SE	95% CI Lower	95% CI Upper	Median	SE	95% CI Lower	95% CI Upper
NF-κB:									
Low	45	39.11	1.08	37.00	41.22	40.00	0.41	39.19	40.81
High	48	28.27	1.27	25.78	33.77	28.00	1.15	25.75	30.26
CCL2:									
Low	48	37.33	1.20	34.99	39.68	39.00	0.50	38.03	39.97
High	45	29.44	1.42	26.66	32.23	29.00	1.68	25.71	32.29
CD68:									
Low/Moderate	62	35.27	1.24	32.84	37.71	39.00	0.71	37.61	40.39
Strong	31	30.00	1.57	26.93	33.07	30.00	1.58	26.90	33.10
Overall	93	33.52	1.01	31.54	35.49	38.00	1.74	34.59	41.41

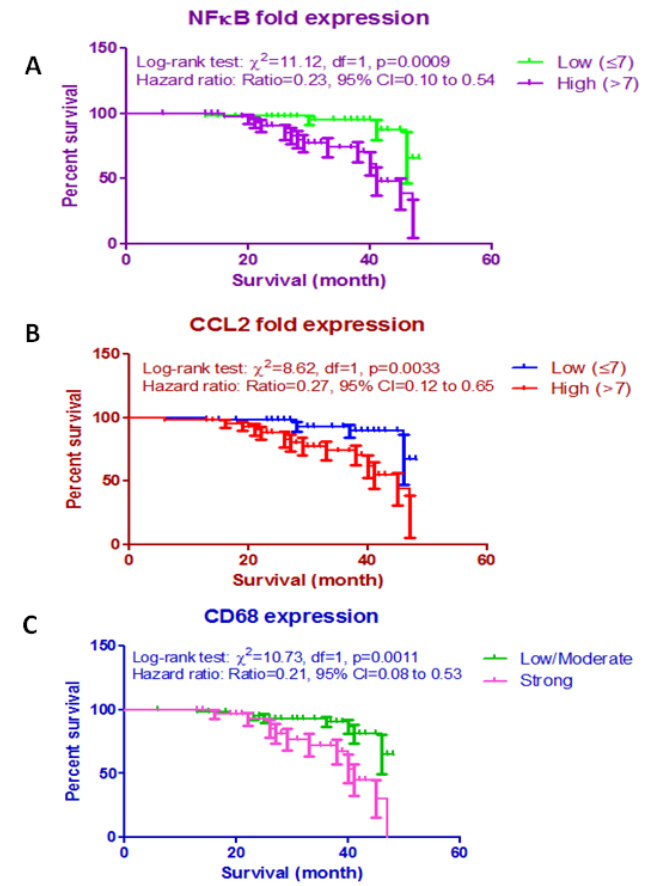


Figure 4. Four year overall survivals according to NF-κB, CLL2 and macrophage expressions of breast cancer patients. **A:** Overall survivals according to NF-κB gene expressions of breast cancer patients. **B:** Overall survivals according to CCL2 gene expressions of breast cancer patients. **C:** Overall survivals according to macrophage (CD68) expressions of breast cancer patients.

B & C). The NF-κB (Log-rank test: χ^2=11.12, p=0.0009; Hazard ratio: ratio=0.23, 95% CI=0.10 to 0.54), CCL2 (Log-rank test: χ^2=8.62, p=0.0033; Hazard ratio: ratio=0.27, 95% CI=0.12 to 0.65) and macrophage content (CD68 positive cells) (Log-rank test: χ^2=10.73, p=0.0011; Hazard ratio: ratio=0.21, 95% CI=0.08 to 0.53), showed significant association with overall survival suggesting that patients with higher expression had significantly lower survival.

Discussion

Current study was planned so as to substantiate the role of NF-κB in breast cancer metastasis, particularly via CCL2 up regulation and consequent influx of TAMs. Owing to critical role of CCL2 during metastasis, particularly in its capacity to act as a chemoattractant for macrophages and their precursors i.e monocytes, attempt was made to evaluate if pro-metastatic function of NF-κB were on account upregulated CCL2 levels and elevated tumoral macrophage content.

We observed that there exist an association between mRNA expression level of NF-κB with CCL2 mRNA expression level and with intratumoral macrophage content (CD68 +ve cells). The frequency distribution of mRNA expression level of CCL2, NF-κB and intratumoral macrophage content exhibited a significant and positive (direct) association with each other indicating crosstalk between these variables. Further, the quantitative assessment also revealed significant and positive correlations amongst each other indicating for interdependence.

To validate the interdependence of these variables as revealed during analysis of human breast cancer specimen, and to further dissect the cause and effect relationship amongst them, detailed in-vitro experiments were carried out in MCF-7 human breast adenocarcinoma cells. Compared to quiescent cells, the cells where NF-κB was active (EGF stimulated), the CCL2 mRNA levels were markedly elevated. These results clearly substantiated the ability of NF-κB to modulate CCL2 expression levels and thus corroborated the association of NF-κB with CCL2 expression levels observed in human breast cancer specimen. CCL2 is known to promote intra tumoral macrophage content (23).In agreement with this Bonapace et al reported that macrophage infiltration in breast cancer correlated with high expression level of CCL2 (19).Since our results indicated that NF-κB upregulated CCL2 expression levels in breast cancer cells, it appeared plausible that this may culminate into enhanced chemotaxis of macrophages towards breast cancer cells. In agreement with this, we observed that compared to quiescent breast cancer cells, the cells having activated NF-κB chemoattracted THP-1 derived macrophages to a greater extent which was markedly diminished in presence of NF-κB inhibitor. The results clearly indicated that NF-*κ*B promoted macrophage chemoattracting properties of breast cancer cells. This was in agreement with our human breast cancer specimen analysis where we observed a strong correlation between NF-κB and macrophage content. It is well documented that NF-κB potentiates metastasis; however the underlying mechanisms and their clinical relevance

are not clear and warrant further investigation. Our study points that heightened macrophage infiltration could be one of the key mechanisms by which NF-κB may promote breast cancer metastasis as macrophages are known to actively promote cancer cell invasion and metastasis.

Metastasis is the biggest hurdle during clinical management of cancer. It is one of the major cause of poor prognosis and adverse clinical outcome (24).Approximately 90% of breast cancer related deaths are due to distant metastasis and it is the major determinant for diminished survival of patients (25).Since all the three variables studied by us were documented to promote metastasis and they exhibited significant correlation amongst themselves, we further studied their association with patient survival rates. The four year survival data of CCL2, NF-κB and CD68 macrophage marker showed statistically significant independent association with overall survival suggesting that patients with higher expressions had significantly lower survival. Thereby substantiating the clinical significance of these variables with respect to survival rate of breast cancer patients. Clinical evidence indicate that high-infiltration of TAMs correlated with shorter survival in patients with breast cancer (26).

Onset of metastasis is one of the biggest obstacles to the successful treatment of cancer. Thus, in the clinical sense avoiding the metastasis becomes physician's primary concern. This essentially demands identification of key molecular determinants of metastasis which in turn may serve as early biomarkers/prognostic markers to be employed by physicians for clinical management of cancer. Through our study we provide experimental and clinical evidence in support of co-ordinated expression of the inflammatory chemokines CCL2, NF-κB and intratumoral macrophage content particularly with respect to breast cancer, with an additional evidence of these three variables being key determinant for poor prognosis and diminished survival amongst breast cancer patients both independently as well in a coordinated manner. Our study has clinical relevance as it establishes significance of these three variables as potential predictive or prognostic markers to be employed during clinical management of breast cancer.

Acknowledgement

The first author (Brij Nath Tewari) is thankful to the Indian Council of Medical Research (ICMR), New Delhi, India, for awarding Senior Research Fellowship (ICMR-SRF) (SRF IRIS ID No.: 2012-00220) under the guidance of the corresponding author. We also thankful to all patients for their participation in the study.

References

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, Forman D. Global Cancer Statistics 2011. CA. Cancer J. Clin. 2011; 61:69–90.
2. Torre LA, Bray F, Siegel RL, Ferlay J, Lortet-tieulent J, Jemal A. Global Cancer Statistics 2012. CA. Cancer J. Clin. 2015; 65:87–108.
3. Weigelt B, Peterse JL, van 't Veer LJ. Breast cancer metastasis: markers and models. Nat. Rev. Cancer. 2005; 5:591–602.
4. Ellis MHD, Lippman ME. In: Treatment of metastatic disease. Harris JLM, Morrow M, et al. (eds.), Diseases of the Breast, 2nd edition. Philadelphia: Lippincott-Raven, 2000, pp. 749–799.
5. Lu J, Steeg PS, Price JE, Krishnamurthy S, Mani SA, Reuben J, et al. Breast cancer metastasis: Challenges and opportunities. Cancer Res 2009; p. 4951–3.
6. Tas SW, Vervoordeldonk MJBM, Tak PP. Gene therapy targeting nuclear factor-kappaB: towards clinical application in inflammatory diseases and cancer. Curr Gene Ther 2009; 9:160–70.
7. Beinke S, Ley SC. Functions of NF-κB1 and NF-κB2 in immune cell biology. Biochem J 2004; 382:393–409.
8. Xiao G, Fu J. NF-κB and cancer: a paradigm of Yin-Yang. Am J Cancer Res 2011; 1:192–221.
9. Helbig G, Christopherson 2nd KW, Bhat-Nakshatri P, Kumar S, Kishimoto H, Miller KD, et al. NF-kappaB promotes breast cancer cell migration and metastasis by inducing the expression of the chemokine receptor CXCR4. J Biol Chem 2003; 278:21631–8.
10. Shishodia S, Aggarwal BB. Nuclear factor-kappaB: a friend or a foe in cancer? Biochem Pharmacol 2004; 68:1071–80.
11. Chaturvedi MM, Sung B, Yadav VR, Kannappan R, Aggarwal BB. NF-κB addiction and its role in cancer: "one size does not fit all". Oncogene 2011; 30:1615–30.
12. Mizutani K, Sud S, McGregor N a, Martinovski G, Rice BT, Craig MJ, et al. The chemokine CCL2 increases prostate tumor growth and bone metastasis through macrophage and osteoclast recruitment. Neoplasia 2009; 11:1235–42.
13. Rukset A, Bedia A, Sibel BK, Canan C, Seyma S, Leman MY, et al. Association of CCL2 and CCR2 gene variants with endometrial cancer in Turkish women. In vivo (Brooklyn) 2010; 24:243–8.
14. Goede V, Brogelli L, Ziche M, Augustin HG. Induction of inflammatory angiogenesis by monocyte chemoattractant protein-1. Int J Cancer 1999; 82:765–70.
15. Conti I, Dube C, Rollins BJ. Chemokine-based pathogenetic mechanisms in cancer. Novartis Found Symp 2004; 256:29–52,266–9.
16. Lebrecht A, Grimm C, Lantzsch T, Ludwig E, Hefler L, Ulbrich E, et al. Monocyte chemoattractant protein-1 serum levels in patients with breast cancer. Tumour Biol 2004; 25:14–7.
17. Ueno T, Toi M, Saji H, Muta M, Bando H, Kuroi K, et al. Significance of macrophage chemoattractant protein-1 in macrophage recruitment, angiogenesis, and survival in human breast cancer. Clin Cancer Res 2000; 6:3282–9.
18. Qian B-Z, Li J, Zhang H, Kitamura T, Zhang J, Campion LR, et al. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. Nature 2011; 475:222–5.
19. Bonapace L, Coissieux M-M, Wyckoff J, Mertz KD, Varga Z, Junt T, et al. Cessation of CCL2 inhibition accelerates breast cancer metastasis by promoting angiogenesis. Nature 2014; 515:130–3.
20. Bingle L, Brown NJ, Lewis CE. The role of tumour-associated macrophages in tumour progression: Implications for new anticancer therapies. J Pathol 2002; p. 254–65.
21. Pfaffl MW, Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001; 29:e45.
22. Daigneault M, Preston JA, Marriott HM, Whyte MKB, Dockrell DH. The Identification of Markers of Macrophage Differentiation in PMA-Stimulated THP-1 Cells and Monocyte-Derived Macrophages. Doherty TM, editor. PLoS One 2010; 5:e8668.
23. Kitamura T, Qian B-Z, Soong D, Cassetta L, Noy R, Sugano G, et al. CCL2-induced chemokine cascade promotes breast cancer metastasis by enhancing retention of metastasis-associated macrophages. J Exp Med 2015; 212:1043–59.
24. Schroeder A, Heller DA, Winslow MM, Dahlman JE, Pratt GW, Langer R, et al. Treating metastatic cancer with nanotechnology. Nat Rev Cancer 2011; 12:39–50.
25. Chaffer CL, Weinberg RA. A perspective on cancer cell metastasis. Science 2011; 331:1559–64.
26. Zhang Y, Cheng S, Zhang M, Zhen L, Pang D, Zhang Q, et

al. High-infiltration of tumor-associated macrophages predicts unfavorable clinical outcome for node-negative breast cancer. PLoS One 2013; 8:e76147.