American Journal of Biomedical and Life Sciences
2015; 3(2-3): 16-20
Published online August 6, 2015 (http://www.sciencepublishinggroup.com/j/ajbls)
doi: 10.11648/j.ajbls.s.2015030203.13
ISSN: 2330-8818 (Print); ISSN: 2330-880X (Online)

Molecular expression analysis of different inflammatory mediators and their role in breast cancer progression and metastasis

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To cite this article:
Aula Ammar, Amani Halabi, Zuheir Al-Shehabi. Molecular Expression Analysis of Different Inflammatory Mediators and Their Role in Breast Cancer Progression and Metastasis. *American Journal of Biomedical and Life Sciences*. Special Issue: Spectral Imaging for Medical Diagnosis "Modern Tool for Molecular Imaging". Vol. 3, No. 2-3, 2015, pp. 16-20. doi: 10.11648/j.ajbls.s.2015030203.13

Abstract: Introduction: Cytokines include different subfamilies such as interleukins (IL), chemokines, and growth factors. They play an important role in inflammatory conditions such as cancer progression and metastasis. There is an increasing interest in developing strategies to antagonize the function of some cytokine/chemokine to interfere with tumor progression and metastasis, the leading cause of death in most patients. The aim of the research project is to study the molecular characteristics of a sample of Syrian patients with breast cancer and assess the protein and gene expression of different inflammatory mediators and correlate that with the clinicopathological criteria of tumors. Materials and methods: Patient samples will be evaluated histologically (H&E stain) and stained immunohistochemically with antibodies against important molecular markers, cytokines, and different types of activated leukocytes. Immunohistochemistry of CD206, a marker of alternatively activated macrophages in tumors is shown here. PCR and immunohistochemical analysis of cytokines (e.g. IL-2, GM-CSF, IFNγ, M-CSG, IL-4, IL-10, CXCL8, CXCL12, CCL21, CCL19, CCR7) will be further implemented. The staining intensity, localization and distribution within the tumor will be examined and correlated with the gene expression and other clinnicopathological information. Expected results: We expect to get new information about the role of different cytokines in breast cancer progression in addition to get an insight into the possible inter-relationship between these cytokines.

Keywords: Breast Cancer, Tumor Environment, Lymph Node Metastasis, Cytokines, Chemokines, Insert

1. Cancer and Inflammation

The theory of the correlation between cancer and inflammation has evolved from the clinical findings that many chronic inflammatory diseases can increase the probability of cancer development (e.g., inflammation with Helicobacter pylori is associated with gastric cancer) [1]. Moreover, it has been suggested that tumors are "wounds that do not heal"; that means the series of events such as cellular activation (e.g., leukocyte), cytokine secretion and angiogenesis are present in the tumor environment without the occurrence of inflammation resolution [2]. Most current data support the involvement of the inflammatory cytokines in tumor progression rather than playing a role in anti-tumor immunity. For example, induction of the NF-κβ by IL-1 or TNF-α enhances the transcription of many genes involved in tumor development [3]. On the other hand, there are some anti-inflammatory cytokines (e.g., IL-10 and TGF-β) playing complex roles in tumor suppression or progression and may be specific to some tumor types [4].

Cytokines include different subfamilies such as interleukins (IL), chemokines, and growth factors. They play a role in inflammatory conditions, leukocytes recruitment and differentiation, wound healing, cancer progression and metastasis [5,6]. Chemokines are a subset of cytokines consisting of about 45 members divided into 4 subfamilies according to their structure [7]. Many cancers have a complex chemokine networks that influence tumor growth, survival, metastasis and interestingly to this project, leukocyte chemoattraction to the tumor environment. Moreover, studies of human cancer biopsy and mouse cancer models showed that cancer cell chemokine-receptor expression is associated with increased metastatic capacity [8-9]. On the other hand, atypical chemokine receptors, such as CCX-CRK and D6,

may play an opposing role in which they disrupt the chemokine-associated cancer metastasis [10]. Exploring the link between cytokines and metastasis can increase our understanding of the disease and may introduce new approaches for cancer treatment.

Although important in tumor biology, previous studies on chemokines and their receptors in breast cancer had some limitations. In vivo work using patient samples investigated individual or few chemokines that are known to be highly expressed in breast cancer [11]. In addition, few papers compared the expression of chemokines and their receptor between tumors and their matching lymph nodes [12]. More studies are required to analyze the expression of more chemokines and their receptors that may play a role in cancer metastasis, the relationship between different chemokines and other cytokines and, the regulation of expression of inflammatory mediators. This can be performed via studying the protein expression using tissue arrays and by analysing a wide range of gene expression using multiplex PCR and gene microarrays. Functional studies are also required to assess the role of a chemokine axis in a specific process (or processes) during cancer progression and/or metastasis.

2. The Tumor-Stroma Interaction

Most solid tumors are occupied by nonmalignant cells, called stromal cells. The tumor stroma consists of different types of leukocytes (such as tumor-associated macrophages (TAM), dendritic cells (DC) and T cells), endothelial cells and fibroblasts [13-15]. The role of infiltrating leukocytes is important because they can promote tumor progression; they function abnormally in the tumor environment as they become immunosuppressed and modulated to produce growth factors required by the tumor [13]. Previous work supports the correlation between higher numbers of infiltrating TAM and poor patient overall survival in most solid tumors [16].

Macrophages (Mφ) represent a significant population of the inflammatory infiltrate. Mφ become immunosuppressed (known as M2 macrophages) and secrete various cytokines and matrix proteases that can influence tumor progression and metastasis [15]. Published data show that patients with high Mφ densities have a worse prognosis [16]. Technology now allows identification, and isolation, of the different types, and subtypes, of macrophages. Consequently, a new area of research has evolved to fully elucidate and understand tumor-macrophage interactions and identify macrophage-associated molecules specific to different populations, along with their role in regulating tumor invasion and metastasis.

Leukocytes are attracted to tumors by different cytokines and growth factors (GF) present in the tumor environment. Tumor-associated leukocytes differentiate or are 'reprogrammed' by the tumor environment and further play a role in tumor development. TAL produce cytokines, GF and proteases; all enhance the progression of tumors [14]. Tumor-associated macrophages (TAM) are a subset of TAL which function abnormally in the tumor environment as they become immunosuppressed and modulated to produce GF required by the tumor (usually referred to as M2 macrophages) [13].

The regulation of the process of lymph node (LN) metastasis is not fully understood and both cytokines and TAL are involved. Some information is available explaining lymphangiogenesis-associated growth factors (e.g., the VEGF family) and their correlation with LN metastasis [17], yet little information is available about other molecular mechanisms regulating this process. Lymphatic-associated chemokines/chemokines receptors axis, such as CCL-21 and CCL19 and their receptor CCR7, are also important for tumor metastasis [18,19]. Increasing evidence suggests that TAM and their associated cytokines can regulate lymphatic metastasis. Activation of the NF-κβ by IL-1 or TNF-α (cytokines secreted by TAM) upregulates the expression of GF and proteolytic enzymes involved in tumor development [20].

We need to understand how macrophages, and their associated cytokines, contribute to cancer progression and lymph node metastasis. Our project will assess the role of different inflammatory mediators in breast cancer, mainly studying the differentiation of macrophage within the tumor environment, "creating" a cytokine/chemokine profile via the investigation of the gene and protein expression of certain cytokines/chemokines and their receptors, and correlate results with clinical information of the tumors.

3. Methodology

Sample patients: the study started with twenty three patient samples with breast cancer. All patients signed the agreement approved by the institutional ethical board. The mean age of patients was 53.5 years (32-75 years and the mean sie of tumors was 4.8 cm (1.5-8 cm). The histological grade of tumors was as follow: grade I (n = 2, 8.7%), grade II (n = 9, 39.1%), grade III (n = 8, 34.8%). For technical reasons, the tumor grade of four patients was missing. Approximately, 44% (n = 10) of the tumors were invasive ductal carcinoma and 39% of the samples were of the invasive lobular type of tumor. More breast cancer specimens will be included as large sample is required for the validation of results.

For immunohistochemistry staining of CD206 antibody, 5-µm thick sections were deparaffinized in xylene, and dehydrated in ethanol baths (100%-30%) then in distilled water. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide in methanol for 20 min. Antigen retrieval was performed by boiling sections in Tris-EDTA (pH=9) for 20 min. Background sniper (Biocare Medical, California, USA) was used to block nonspecific binding, followed by adding the anti-CD206 primary antibodies (1:20, R&D systems, USA) at 4°C overnight. Primary antibodies were washed using TBS, then incubated with HRP-conjugated anti-goat antibody (1:500, R&D systems, USA) for 1h at room temperature. After washing in TBS, sections were incubated for 1 min with diaminobenzidine (DAB, Biocare Medical, California, USA), counterstained in hematoxylin, dehydrated

and mounted using canada balsam. Tonsil tissues were used as positive controls for CD206 expression. Negative controls were stained using the same procedure but excluding the primary antibody.

The density of CD206-positive macrophages was categorized into three groups: no staining (0), low (1) and high (2). The density of inflammatory infiltrate in tumor sections was also examined and tissues were categorized into two categories: low (1) and high (2) according to the stromal infiltration with immune cells. All samples were examined by two independent assessors, blinded to tumor data, with reanalysis of any discrepancies.

Using cross tables and Chi-squared test, the relationships between categorized data of CD206 and the inflammatory infiltrate and the tumor grade, A p value of ≤0.05 defined a significant relationship. Statistical analysis was performed using PASW Statistical package, version 18.0 (SPSS Inc. USA).

Studying the expression of other proteins of interest (IL-2, GM-CSF, IFNγ, M-CSG, IL-4, IL-10, CXCL8, CXCL12, CCL21, CCL19, CCR7) is subject to available funds and is planned to be conducted using immunohistochemistry whereas the gene expression of specific cytokines will be investigated using reverse transcription polymerase chain reaction (RT-PCR). A designed bioinformatics algorithms will be used for the expression analysis of the cytokine networks.

4. Current and Future Work Plan

Breast cancer sections were stained with antibodies targeting cathepsins B and S, both have been previously shown to be expressed in tumor associated macrophages and correlated with the tumor stage mainly in pancreatic cancer [21]. We tested the expression of these proteases in human breast cancer and our results mainly showed that both tumor cells and macrophages express Cts B and S however tumor cells preferentially express Cts B rather than Cts S [22].

We further looked at type II macrophages by staining tonsil sections (positive controls) and tumor sections with anti-CD206. In tonsils, CD206 positive macrophages were mainly present in the marginal (T-cells) zones of germinal centers which are areas of humoral responses (IL-4 and IL-10 expressing zones) as shown in Fig 1 (a,b). In breast cancer, CD206 positive macrophages were found lining the necrotic areas of tumors (Fig 1 c,d) as well as in tumor stromal and humoral adipose tissues (Fig1, e and f, respectively).

Table 1. *correlation between the densities of CD206 macrophages and the inflammatory infiltrate.*

Inflammatory Infiltrate	CD206 staining			
	absent	Low	High	p value
Low	6	1	1	0.05
High	3	8	2	

When assessing the densities of CD206 macrophages, approximately 42% of samples did not show any CD206 positive cells and the same percentage showed low densities of CD206 positive macrophages whereas about 14% of samples were highly infiltrated with CD206 macrophages. As would be expected, CD206 macrophages represent a significant population of the inflammatory infiltrate and this was shown by the correlation studies between categories of CD206 expressing tumors and the inflammatory infiltrate (chi squared test, p=0.05, table 1).

Correlation studies between macrophage densities (3 categories) and tumor grade (3 categories) did not show any significant correlation which is mainly due to the small sample size (p>0.05, data not shown). The sample size, which was a limitation in our work, will be expanded in the ongoing work for future statistical correlation studies.

Sections used in the study were also stained with antibodies targeting CD31 (an endothelial cell marker) and the D2-40 (a marker for lymph endothelium) to assess the density of the tumor vasculature. The density of blood vessels and lymphatic vessels will be correlated with chemokines with pro-angiogenic activity (e.g.IL-8 (CXCL-8)) and will be correlated with lymphovascular invasion and lymph node involvement.

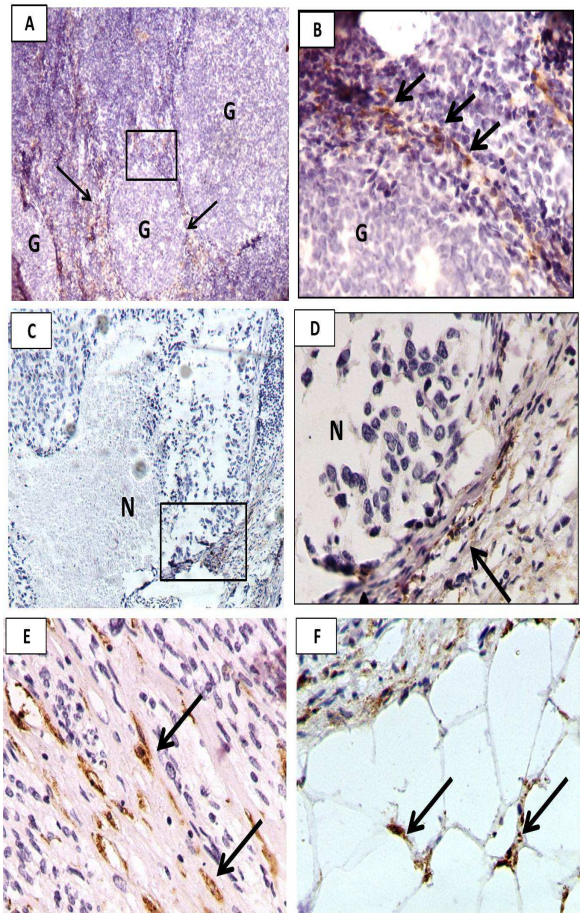

Figure 1. *Immunohistochemical staining of CD206 macrophages in tonsils and breast cancer. A & B. CD206 macrophages in marginal zones of germinal centers (x100 and x400 respectively), C&D show CD206 macrophages lining the necrotic areas (N). Rectangle in A and C are shown at higher magnifications in B and D, respectively (x400).*

Current work is focused on identifying the macrophage differentiation profile in the tumor environment using CD68 as a pan-macrophage marker in addition to the CD206

mentioned above. We also tend to assess the distribution of macrophages in a tumor sections in a larger cohort of patients. Milestone aims are to correlate the differentiation profile of macrophages with the cytokine and chemokine profiles of type 1 and 2 immune status (such as IL-2, GM-CSF, IFNγ, M-CSG, IL-4, IL-10, IL-8 , CXCL12, CCL21, CXCR4 and CCR7). The cytokine expression profile will be studied using RT-PCR. The protein expression will further be assessed for the cytokines of interest using IHC. The staining of chemokines involved in tumor metastasis in both tumor sections and corresponding lymph nodes (with and without metastasis) is considered novel. We would like to show how lymph nodes containing tumor metastasis differentially express specific chemokines and how the nested tumor cells express the corresponding receptor.

Correlation studies using chi-squared test were used for our pilot experiments, however a new bioinformatic algorithm (in collaboration with Dr Issa Ibrahim, Biomedical Sciences, Damascus Iniversity) will be used to produce a cytokine expression profile and find out the possible inter-relationships between the different cytokine and the chemokine axis.

5. Concluding Remarks

It is very important to understand which factors may be involved in tumor metastasis. We consider the study of the expression of inflammatory cytokines/chemokines associated with active (Th1) and suppressed (Th2) immune response will lead to identify novel marker for cancer treatment. We designed our experiments so that changes in the gene expression of cytokine/chemokines will be compared among normal breast tissues (controls), primary breast tumors (non-metastatic, metastatic to lymph nodes) and islets of metastasizing tumors in lymph nodes. This experimentation will be followed by functional studies to assess the role of significant inflammatory players.

6. Acknowledgements

We would like to thank Dr Tamim Hammad, School of Pharmacy, Tishreen University for his unlimited support and administrative guidance.

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