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ARTICLE

Tumor-Infiltrating B Cell Immunoglobulin Variable Region Gene Usage in Invasive Ductal Breast Carcinoma

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A major focus of tumor immunology is to reveal the potential role and capacity of immunocompetent cells found in different solid tumor tissues. The most abundant infiltrating cells (TIL), the T lymphocytes have been investigated in details concerning T-cell receptor usage and specificity. However, B cells have hardly been investigated in this respect, although high cellular B-cell infiltration has been correlated with improved patients' survival in some breast carcinomas. This led to our objectives to study variable region gene usage of the tumor-infiltrating B cells in different breast carcinoma types. By defining the immunoglobulin repertoire of the tumor-infiltrating B lymphocytes in the most common invasive ductal carcinoma (IDC) of the breast we compared it to the rare medullary breast carcinoma (MBC). After phenotyping infiltrating ductal carcinomas, B cells were obtained from tumor tissue by microdissection technique. Numerous rearranged TIL-B immunoglobulin heavy chain V genes (VH) were amplified, cloned, sequenced, and comparatively analyzed. Some characteristics were found for both breast carcinoma types. The immunoglobulins produced by TIL-B in ductal carcinoma are highly matured and oligoclonal. We conclude that Ig variable region gene usage reveals similar and distinguishable characteristics of TIL-B immunoglobulin repertoires, which are representative of the nature of the immune responses in invasive ductal and medullary breast carcinomas.(Pathology Oncology Research Vol 11, No 2, 92–97)

Key words: immunoglobulin variable region, breast ductal carcinoma, tumor-infiltrating lymphocytes

Introduction

Beside other solid tumors, breast carcinomas also have various amounts of tumor- infiltrating T and B (TIL-T, TIL-B) lymphocytes. The most common form of breast cancer (80%) is ductal carcinoma.[1] It can be either 'in situ' (DCIS, ductal carcinoma in situ), when it is restricted to the ducts of the mammary gland. A more malignant form is the invasive ductal carcinoma (IDC), in which the tumor cells invade the surrounding tissue. DCIS and IDC has a better prognosis when tumor-infiltrating lymphocytes appear in the tumor tissue.[2] Breast carcinoma cells are often closely associated with TILs in the primary tumor and metastatic tumors in the axillary lymph nodes. Although the role of lymphocytic infiltration in primary carcinomas has not been revealed, it may reflect a host response to the tumor.[3,4] In tumor tissues T lymphocytes are the most abundant cells in general, so studies were concentrated on T cells.[5-8] Lymphocytic infiltrates in ductal breast carcinomas consist largely of T cells with higher cytotoxic/helper T cell ratio as it was reported by some authors.[9-11] However, predominant helper T subpopulation was stated by others.[5,12] Variable numbers of macrophages, natural killer cells and B cells were also found in

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Abbreviations: Aa: amino acid, CDR: complementary determining region, DCIS: ductal carcinoma in situ, FDC: follicular dendritic cell, FR: framework region, IDC: invasive ductal carcinoma, Ig: immunoglobulin, MBC: medullary breast carcinoma, PBMC: peripheral blood mononuclear cell, TIL-T: tumor-infiltrating T lymphocytes, TIL-B: tumor-infiltrating B lymphocytes, VH: Ig heavy chain variable region

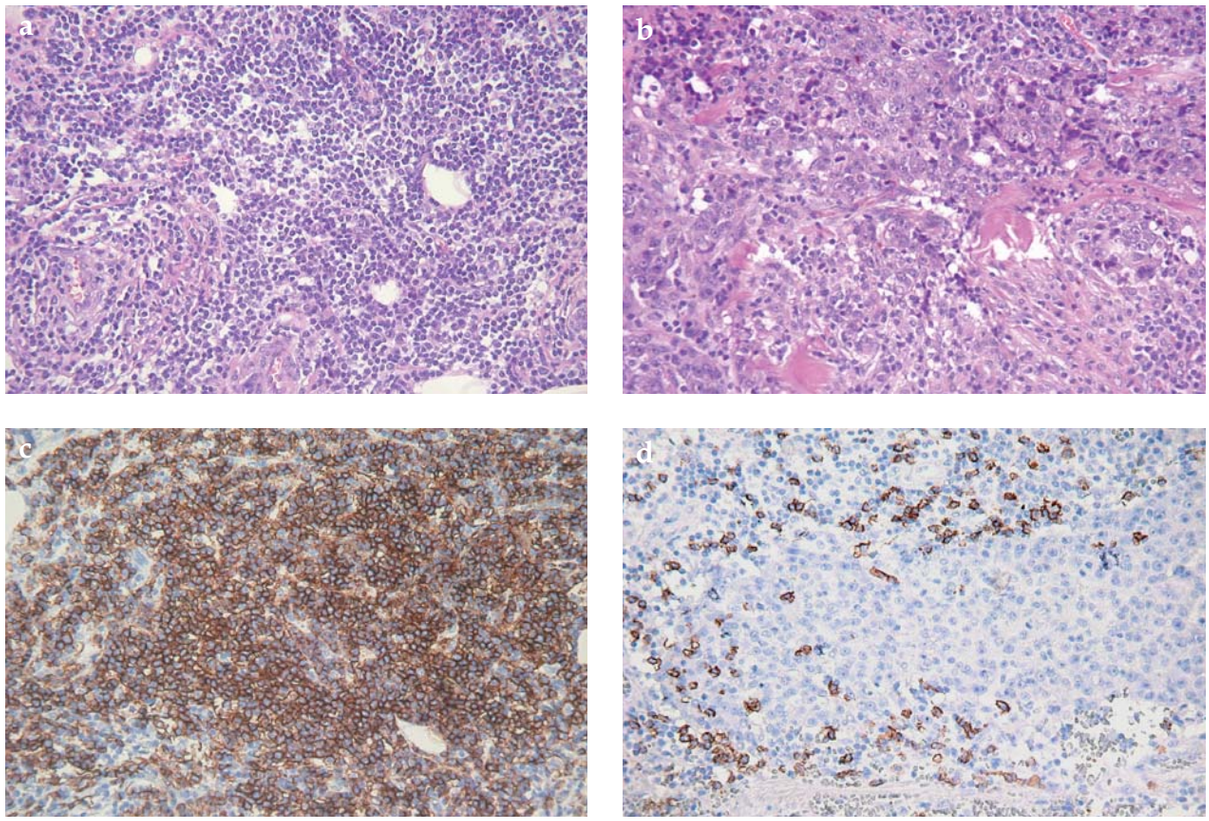

Figure 1. *Identification of B cells in medullary and invasive ductal carcinoma of the breast. B cells were labeled with anti-CD20 and visualized by HRP-DAB reaction (x100).* ***a****: histology of medullary carcinoma,* ***b****: histology of invasive ductal carcinoma,* ***c****: CD20-positive B cells in medullary breast carcinoma,* ***d****: CD20-positive B cells in ductal breast carcinoma*

breast cancer,[10,12] where limited number of B cells formed small aggregates at the periphery of the tumor.[13] In contrast to tumor-infiltrating T cells, B cells are poorly characterized in their specificity and immunoglobulin (Ig) repertoire. All works that increase the knowledge about tumor-infiltrating B lymphocytes help to explore the nature of the B-cell response to breast cancer.

Contrary to IDC, medullary breast carcinomas (MBC) are relatively rare breast tumors, diagnosed in up to 5% of cases,[14] and originate from the medullar tissue of the breast. The tumor is infiltrated and surrounded with lymphocytes and plasma cells; in its most exuberant expression, it is classically designated "medullary carcinoma with lymphoid stroma". An inverse correlation between the extent of cellular infiltration and tumor size and grade, and a correlation of increased B- and plasma cell infiltration with better survival was found in medullary breast carcinomas.[15,16] This may be indicative of a favorable and partially successful host response against the tumor. The infiltrates have been noted to be confined to the tumor bed itself without extending into the adjacent normal breast tissue, suggesting some maladjustment between tumor and host.[17] MBC has a better prognosis then any of the IDCs.

The two forms of breast cancer are different in incidence, characteristics and prognosis. Additional useful information might be gathered by comparing our findings from analysis of TIL B-cell antibody repertoire and antigen recognition with that of other works, and different breast cancer types.

Materials and methods

Selection of tumor samples and immunohistochemistry

Tumor samples of patients with invasive ductal breast carcinoma and medullary breast carcinoma undergoing therapeutic excision were tested by immunohistochemistry to detect the most common infiltrating cells in the tumor tissue. Patients have not received previous chemotherapy, radiotherapy or endocrine manipulation. Sections were stained with mouse monoclonal antibodies specific for B cells (anti-CD20, 1:50, DAKO, CA), T cells (anti-CD3, 1:100, DAKO), follicular dendritic cells (FDC) (anti-FDC, 1:100, DAKO), and plasma cells (Wue-1, Greiner,

Wurzburg, Germany). The detection system was HRP-conjugated anti-mouse Ig (DAKO) and DAB substrate, counterstained with Mayer's hematoxylin (Sigma Chemical Co, St Louis, MO). Selected tumor samples from the patient with IDC were embedded in Tissue-Tek OCT Compound (Sakura Zoeterwoude, the Netherlands) and were snap frozen in liquid nitrogen immediately after surgery. Serial frozen sections (8 μm) were cut with a cryostat and mounted on coated slides (Menzel Glaser, Germany). Sections were air-dried, fixed in acetone, and stored at –80ºC with a desiccant.

Microdissection of tumor-infiltrating B cells, and DNA extraction

B-cell clusters were excised from frozen sections (8 μm) under sterile distilled water using sterile skin-prick lancets controlled by micro-manipulators (Narishige, Tokyo, Japan) under a Nikon Diaphot (Melville, NY) inverted microscope. Stained clusters of B cells were microdissected, each cluster separated in a distinct tube using a fresh lancet, in order to avoid DNA contamination from the nearby B-cell clusters. The excised tissue was digested in 10 μg/ml proteinase-K (Sigma) at 50ºC for 1 h, and the enzyme was inactivated thereafter.

Amplification and cloning of rearranged heavy chain V genes

Rearranged VH genes were amplified by nested PCR with high fidelity system (Boehringer Mannheim, Germany). The primers were designed to amplify all known functional, rearranged human Ig VH genes according to Marks.[18] Amplification and cloning of rearranged heavy chain V genes were carried out by a slightly modified method that was used earlier.[19] Discrete PCR bands with the expected size (between 300-400 base pairs) were cut and extracted (Qiagen, Chatsworth, CA) from gel before ligated to TA cloning system (Invitrogen, Carlsbad, CA).

Sequencing, data processing – bioinformatic analysis

Insert-bearing clones prepared using QIAprep spin mini-prep kits (Qiagen) were sequenced in both directions with forward and reverse primers using BigDie kit in ABI automated cycle sequencing (Applied Biosystem, Foster City, CA), or sent to DNASHEF technologies (Edinburgh, UK). Sequences were compared with the human VBASE directory of Ig genes[20] using DNAPLOT to identify the best matching germline gene segments. Retrieval and junction analysis of further germline sequences was made with IMGT (http://imgt.cines.fr: 8104). Verified sequences were compared with ClustarX software and displayed with free licensed Bioedit editor program (/BIOEDIT/bioedit.html).

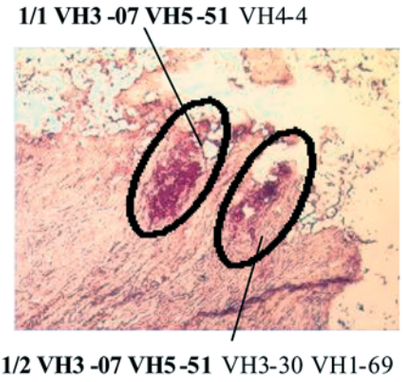

Figure 2. *Microdissection from ductal carcinoma, tumor 1. Immunohistochemical staining and analysis of B cells. Two adjacent dark stained clusters (marked with ellipse) were dissected. The numbers represent the VH germline origin of the Igs found in the particular cluster; VH3-07 was found in both clusters.*

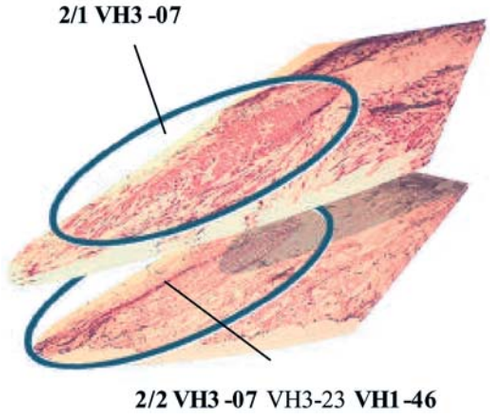

Figure 3. *Microdissection from ductal carcinoma, tumor 2. Immunohistochemical staining and analysis of B cells. Two dissections were made at different depths of the same dark stained cluster (marked with ellipse). The numbers represent the VH germline origin of the Igs found in the cluster; VH3-07 was found at both levels.*

Results

Phenotyping of cells infiltrating ductal and medullary breast carcinoma

The distinct phenotype of the ductal and medullary carcinoma is well known concerning the density of infiltrating leukocytes. Infiltrating B cells were present in the invasive ductal carcinoma as well when compared to a medullary breast carcinoma (*Figure 1*). Although with lower density than in medullary carcinoma, in invasive ductal breast carcinoma areas were observed containing variable numbers of infiltrating cells, scattered or forming clusters sometimes. B cells could be seen after histological staining at the tumor

periphery. These clusters could easily be microdissected and separated one by one. With this approach the clonal spreading of cells can be followed along the tumor tissue through the different clusters. T cells, B cells, plasma cells and follicular dendritic cells were detected, frequently in the same areas and forming clusters.

The B cell clusters were microdissected from two distinct tumors from the same breast of one patient with invasive ductal carcinoma. From one tumor, two B-cell clusters from the same slide were cut to analyze the spread of lymphocytes between clusters (parallel dissection – *Figure 2*). From the second tumor, sections from distinct depth of the same cluster were dissected in order to analyze the migration inside the cluster (sequential or consecutive dissection – *Figure 3*).

***Table 1.* Gene family distribution of sequences from ductal carcinoma**

VH germline Number	*N° of sequences*			
	Tumor 1 (parallel clusters)		*Tumor 2 (consecutive dissection) cluster 3*	
	Cluster 1 (1/1)	*Cluster 2 (1/2)*	*Dissection 1 (2/1)*	*Dissection 2 (2/2)*
VH3-07	10	7	12	7
VH5-51	4	1	–	–
VH3-30	–	1	–	–
VH4-4	1	–	–	–
VH1-69	–	1	–	–
VH3-23	–	–	–	2
VH1-46	–	–	–	11

Ig family distribution named with the VH germline number of origin found in three clusters (four dissections) microdissected from two tumor lumps (tumor 1 and tumor 2) of one patient.

The immunoglobulin repertoire of invasive ductal breast carcinoma

The microdissected B-cell clusters were amplified with genomic nested PCR. Sixty-five bacterial colonies with immunoglobulin inserts were sequenced from two adjacent clusters from the same section (*Figure 2*), and 58 clones from sequential sections of a third cluster from the second tumor (*Figure 3*). Only 25 and 32 sequences turned out to be immunoglobulin sequences, respectively. Immunoglobulin repertoire of TIL-B cells in invasive ductal breast carcinoma tumors were analyzed comparatively at DNA and amino acid level.

The sequences showed homology with 7 different germline VH-genes (*Table 1*), which means an oligoclonal appearance in the tumor, suggesting a non-random infiltration. The majority of sequences belonged to the VH3 family with identical V, D and J segment and junction sequences, and therefore originated from a single clone of B cells using germline gene VH3-07. The cells of this B-cell clone were spread over all 3 different clusters (4 dissections), suggesting that it may recognize an antigen that is widely expressed in the tumor. Other Ig V genes were found only in one or other of the tumors. The somatic mutation analyzes are demonstrated in a mutation distribution diagram (*Figure 4*). In the

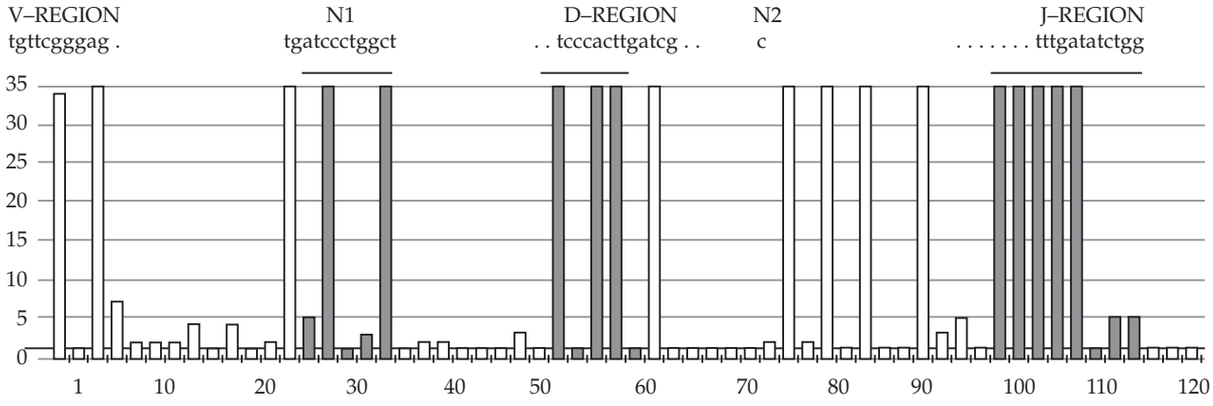

Figure 4. *A. CDR 3 region of VH3-07 clone from ductal carcinoma (IMGT junction analysis). High number of N nucleotide insertions and deletions, the verge of the mini-genes mark somatic maturation. Approximate Aa dissimilarity pattern of ductal carcinoma-derived Ig variable region group compared to germline VH3-07. The abscissa shows the Aa number, while the ordinate the total number of sequences from VH3-07 group (36) named after the germline origin. The high columns show the number of sequences which carry Aa deviation at a certain position. Value 1 means identity with germline, and 0 represents deletion. The horizontal line in the column represents the additional amino acid. The commonly shared mutations at the CDR region show identical origin, while the differences between the group members (small columns) mainly in FR regions suggest an ongoing maturation process.*

CDR regions of the VH3-07 group, all the sequences share the same mutations compared to the germline even in the CDR 3 region according to IMGT junction analysis. Differences among the group members, which mainly occurred in the framework region, suggest that the B cells may be undergoing "*in situ*" maturation. Database search did not reveal full-length homology with germline, and no known specificity could be suggested.

Discussion

Until recently, previous studies about tumor-infiltrating B cells focused on static, *in vitro* phenotypic, or functional evaluation of either isolated mononuclear infiltrating cells, or on histopathological and immunohistochemical investigations of tumor biopsy tissues. The antigenic phenotypes of the inflammatory infiltrates have been found "essentially similar" between MBC and IDCs, emphasizing the importance of the immune reaction rather than the histologic type of the tumor. The prominence of IgG versus the usual IgA plasma cells in MBC and IDC tumors alike, and the confinement of reactions to the tumor beds, suggest a specific response to one or more tumor-derived neoantigens in the tissues.[24-26] The tumor-infiltrating B-cell cluster regions in the IDC tumor tissue had all the components required for an antigen-driven B-cell proliferation, that is, T cells and FDCs were present as well.

The purpose of this study was to elucidate the potential immune recognition and immune response capacity of the TIL-B cells in a novel respect. Our objective was to determine further characteristics of tumor-infiltrating B cells concerning their immunoglobulin repertoire in invasive ductal breast carcinoma, and evaluate it in comparison to medullary breast carcinoma.

Of the cloned sequences obtained from the microdissected B-cell clusters originating from the IDC tumors, sufficient amount of data could be gathered to draw consequences. Ig V genes isolated from members of the same germline VH, DH and JH exons and identical sequences at the VH-D and D-JH junctions indicated that they derived from the same progenitor B cells. The presence of shared and unshared somatic mutations within the V genes allows the construction of genealogical trees of clonally related B cells.

The sequences from the IDC showed homology with 7 different germline VH genes. The majority of sequences turned out to be originating from a single clone of B cells using germline gene VH3-07. Our findings are in accordance with those of others, where the most common VH germline gene used by TIL-B belonged to the VH3 and VH4 families. However, VH1 gene segments were used in TIL-B IgG (based on our studies, in accordance with those of others) and tumor-draining lymph node IgG H chains, but no VH1 occurred in the PBMC repertoire.[15] Analysis of the sequences from both IDC and MBC clearly demonstrates oligoclonal expansion of B cells, while in the case of IDC, Ig H chains are undergoing somatic hypermutation within the clusters of lymphocytes in the tumors but not in MBC. It is known that B cells undergoing a germinal center response, i.e. clonal proliferation accompanied by somatic hypermutation and affinity selection, are driven by antigen, which is required at both the initial trigger and at the later stage of affinity selection.[27] The observation of clonally related sets of mutated V genes expressed by B cells within the cell clusters shows that TIL-B cells undergo a similar process of antigen-driven clonal proliferation and affinity maturation *in situ*. Our data represent an oligoclonal expansion of TIL-B IgG VH, suggesting an antigen-driven selection. These data are in accordance with those found by other methodological approaches in breast and cervical cancers.[15,16,28] The mutation pattern of CDR and FR regions were compared by IMGT junction analysis. Differences, which mainly occurred in the framework region, suggest that the B cells may be undergoing "*in situ*" maturation. A characteristic distribution of heavy chain families was found, that is VH3, VH1, and VH5 (IDC) vs. VH3, VH4, VH5, VH1 (MBC) in the order corresponding their prevalence. Although the VH gene segments VH3-23 and VH4-4 are frequently overrepresented in general, comprising up to 20% of the adult immune repertoire, only one out of more than 40 VH clones could be found.[21,22,29,30] The appearance of rare immunoglobulin families (VH5, VH1) in both types of tumors also indicate the presence of antigen and ongoing immune response, while the characteristics of these antigens seem to be distinct.

The immunoglobulin repertoire of tumor-infiltrating B cells in IDC was compared to that of the medullary breast carcinoma TIL-B cells for similar germline gene usage, clonality and for evidence of antigen dependence, that is somatic mutation within the CDR and FR regions. Recent findings concerning tumor antigen specificity of TIL-B in MBC give further evidence supporting earlier results[23] based on sequence data analysis, and give an important new aspect to the potential role and capacity of tumor-infiltrating B cells.[31]

Our results provide additional information to results of the available few other investigations concerning the characteristics and potential use of TIL-B immunoglobulin repertoire analysis. We could identify some main similarities and differences in the TIL B cell repertoire of invasive ductal breast carcinoma compared to that of medullary breast carcinoma. This might lead to a better understanding of the nature of B-cell immune response in different breast carcinomas.

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