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BCA-1, A B-cell chemoattractant signal, is constantly expressed in cutaneous lymphoproliferative B-cell disorders

M. Mori^a, C. Manuelli^a, N. Pimpinelli^{a,*}, B. Bianchi^a, C. Orlando^b, C. Mavilia^c, P. Cappugi^a, E. Maggi^c, B. Giannotti^a, M. Santucci^d

^aDepartments of Dermatological Sciences, University of Florence Medical School, Via degli Alfani, 37, 50121 Firenze, Italy

^bClinical Pathophysiology—Clinical Biochemistry Unit, University of Florence Medical School, Via degli Alfani, 37, 50121 Firenze, Italy

^cInternal Medicine—Section Immunoallergology, University of Florence Medical School, Via degli Alfani, 37, 50121 Firenze, Italy

^dHuman Pathology and Oncology, University of Florence Medical School, Via degli Alfani, 37, 50121 Firenze, Italy

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Abstract

We analysed the immunophenotypic and molecular expression of BCA-1 (B-cell-specific chemokine) and CXCR5 (BCA-1 receptor) in normal skin and different cutaneous lymphoproliferative disorders (cutaneous T-cell lymphoma (CTCL); cutaneous B-cell lymphoma (CBCL); cutaneous B-cell pseudolymphoma (PCBCL)), with the aim of investigating their possible involvement in the pathogenesis of cutaneous B-cell disorders. BCA-1 and CXCR5 were constantly expressed in CBCL and PCBCL, but not in normal skin and CTCL. BCA-1 and CXCR5 were constantly coexpressed by CD22+ B-cells, while CD35+ follicular dendritic cells coexpressed BCA-1 in PCBCL cells only. In low grade CBCL, as compared with high grade CBCL, the intensity of CXCR5 expression on neoplastic CD22+ cells was lower than that of BCA-1. The image analysis of reverse transcriptase-polymerase chain reaction (RT-PCR) products showed a significant quantitative difference between PCBCL/low grade CBCL and high grade CBCL. The above findings, although only observed in a small series of patients, are in keeping with findings in MALT gastric and gastric MALT lymphomas, adding further evidence of the close similarities between CBCL and MALT lymphomas.

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Keywords: Skin, Lymphoma, Pseudolymphoma, B-cell, Chemokine

1. Introduction

Recent progress in chemokine (chemoattractant cytokine) research has greatly advanced the understanding of the migration properties of lymphoid cells. Two functionally distinct subfamilies of chemokines, referred as “inflammatory” and “housekeeping” chemokines, have recently been recognised [1–4]. While the former are produced by activated tissue cells and leucocytes at sites of inflammation, the latter regulate the localisation of lymphoid cells during lymphopoiesis, antigen-priming and immune surveillance [1–4]. B-cells show a high capacity to recirculate between blood, lymphoid and extralymphoid compartments [5–7]. By contrast to T-cells, migration responses of B-cells to chemokines are not well characterised, although data from the current

literature indicate that both housekeeping and inflammatory chemokines act on B-cells [8–10]. The recently discovered chemokine, BCA-1 (also regarded as CXCL13), is the most effective B-cell chemoattractant [11–12]. It selectively binds to CXCR5, a chemokine receptor with predominant expression on blood and tonsillar B-cells, as well as on a subset of memory CD4+ T-cells [13].

It has recently been demonstrated that both BCA-1 and CXCR5 are highly expressed in *Helicobacter pylori* (Hp)-induced Mucosa-Associated Lymphoid Tissue (MALT) lymphoma, showing a distinctive pattern of expression [14]. This findings suggest a possible pathogenic role for BCA-1 in the natural history of MALT lymphoma. No information is currently available concerning the expression of BCA-1 and CXCR5 in cutaneous lymphoproliferative disorders. The clinico-pathological features of primary cutaneous B-cell lymphomas (CBCL) are extensively dealt with elsewhere in Refs. [15–18]. In short, CBCL are mostly characterised by a

* Corresponding author. Fax: +39-55-2758303.

E-mail address: pimpi@unifi.it (N. Pimpinelli).

homogeneous clinical presentation and behaviour, with a good response to local radiotherapy, low tendency to extracutaneous spread and excellent prognosis. According to the European Organization for Research and Treatment of Cancer (EORTC) classification of primary cutaneous lymphomas [19], CBCL are divided into three subgroups. So-called follicular centre cell (FCC) lymphoma and immunocytoma (IC)/marginal zone lymphoma (MZL), due to putative histological similarities with their purported nodal counterparts, and they have an indolent clinical behaviour. These entities, FCC and IC/MZL, are regarded as follicular lymphoma and extranodal MZL (MALT-type lymphoma), respectively, in the World Health Organization (WHO) classification of lymphoid neoplasms [20]. The different morphologies are often found in different lesions of the same patient or even in different areas of the same lesion and this hampers their strict categorisation into specific sub-groups, such as FCC lymphoma and IC/MZL. An alternative possibility of a unitary interpretation of low grade CBCL has been suggested. In fact, we have documented that the histological pattern in CBCL is largely related to the age and growth rates of the skin lesions [15–18], with a characteristic multiphasic progression of the histological/immunohistological pattern from “early” (patchy/nodular infiltrate, with distinct compartmentalisation of neoplastic, small-medium centrocyte-like B-cells and reactive T-cells, that are often prominent) to “late” phases (diffuse infiltrate of neoplastic, large transformed centrocyte-like B-cells, with few admixed reactive T-cells). These multiphasic histological features have no correlation with either the clinical course or the prognosis of the disease. Moreover, most CBCL—despite variable morphological features—show a rather uniform immunophenotype (CD5-, CD10-) and genotype (lack of *bcl-1/bcl-2* and *c-myc* gene rearrangements) of the neoplastic cells. The close similarities between primary CBCL and MALT lymphomas and the evidence for an acquired B-cell arm of Skin-Associated Lymphoid Tissue (SALT) [21] led us to tentatively designate these CBCL as SALT-related B-cell lymphomas [16,18]. Recently, however, more refined definitions of FCC lymphoma [22] and MZL [23] have been suggested, as the prerequisite for a better clinicopathological differential diagnosis. Subtle classification problems apart, in this paper we will refer to this prominent group of CBCL as “low grade CBCL”. In addition, a third, much smaller subgroup with intermediate prognosis (the so-called large B-cell lymphoma of the leg) is identified in the EORTC classification [21,24]. The clinicopathological significance of this group of primary CBCL is currently debated. In fact, all the major investigators and groups coworking in the EORTC Cutaneous Lymphoma Task Force agree that CBCL, characterised by a rapid growth of skin lesions; large “round” cell histology (i.e., centroblast- and

immunoblast-like), high proliferation rate, and *bcl-2* protein expression at presentation, do occur more rarely. Their possible predilection for the elderly (> 70 years of age) and the selected skin sites (lower legs) and, more importantly, their worse prognosis have been investigated in a larger series in a EORTC multicentre study. The results of this study suggest that *de novo* large “round” cell histology, age > 70 years, and location on the legs are significantly associated with a worse prognosis [25]. However, in the WHO classification, diffuse large cell lymphomas (DLCL) of the B-cell origin are grouped together, without any distinction with regard to the specific site of onset on the skin [20]. In this paper, we will refer to CBCL with *de novo* large round cell histology as “high grade CBCL”.

By the combined use of different methods, including immunohistochemistry, 2-colour immunofluorescence, and molecular analysis (reverse transcriptase-polymerase chain reaction (RT-PCR) and *in situ* hybridisation), we have analysed the phenotypic and genotypic expression of BCA-1 and CXCR5 in normal skin and different cutaneous lymphoproliferative disorders—CBCL, cutaneous B-cell pseudolymphoma (PCBCL, defined as reactive, polyclonal lymphoid hyperplasia with a prominent B-cell component), and cutaneous T-cell lymphoma (CTCL), in order to investigate the possible involvement of these molecules in the pathogenesis of B-cell disorders of the skin. In addition, we examined the relevance of their possible differential expression in the different subcategories (PCBCL versus low grade CBCL versus high grade CBCL).

2. Materials and methods

2.1. Patients and skin samples

The 17 lesional skin samples, consisting of 6-mm punch biopsies, were obtained under local anaesthesia from: 3 patients with PCBCL (insect bite-related); 10 patients with CBCL (7 low grade, classified as MZL according to the EORTC and extranodal MZL according to the WHO classification; 3 high grade, DLCL, two of which were located on the legs and one on the back), and 4 patients with CTCL (*Mycosis Fungoides*). Control, healthy skin samples were also obtained from 5 patients who underwent cosmetic surgery.

Each tissue specimen was in part formalin-fixed and paraplast-embedded for routine histological examination; in part embedded in O.T.C. (“Tissue Tek”, Miles Scientific, Naperville, IL), snap frozen and stored at -80 °C until sectioning for use in immunohistochemistry (IHC); 2-colour immunofluorescence; (2c-IF), and *in situ* hybridisation (ISH); and in part prepared for molecular analysis (Reverse Transcriptase-Polymerase Chain Reaction, RT-PCR). The specimens were put in a

RNAse-free microfuge, and immediately frozen in liquid nitrogen until RNA extraction.

2.2. Immunohistochemistry (IHC)

Immunohistochemical staining was performed on 7 µm cryostat sections by using the Alkaline phosphatase and anti-alkaline phosphatase (APAAP) method, as previously described in Ref. [26]. The monoclonal antibodies used were mouse anti-human CD3, CD4, CD8, CD19, CD22, CD35, CD45RO, kappa and lambda light chains (Dako A/S Glostrup, Denmark), and BLC/BCA-1/CXCI13, CXCR5/BLR-1 (R&D Systems, MN, USA). Normal human lymph nodes were stained in parallel as positive controls. Sections incubated with an irrelevant, isotype-matched mouse antibody were used as negative controls. The step section method was used to evaluate the results; serial sections of each tissue specimen were carefully and blindly evaluated by two of the authors. For quantitative analysis, the stained cells in the dermal infiltrate were counted in five consecutive microscopic fields ($\times 250$). The results were expressed as the mean number of stained cells per 100 cells observed. Only cells whose nuclei were contained in the plane of the section were considered. The results were scored independently by two observers, and the resulting figures were averaged.

2.3. Two-colour immunofluorescence (2c-IF)

Surface expression of BCA-1, CXCR5, CD22, and CD35 was analysed by 2c-IF method using 5 different experimental protocols: (1) phycoerythrin (PE)-conjugated MoAb mouse anti-human CXCR5, biotin-conjugated MoAb mouse anti-human BCA-1 and fluorescein isothiocyanate (FITC)-conjugated avidin; (2) MoAb mouse anti-human CD22, clone IgG1, PE-conjugated goat anti-mouse IgG1 polyclonal antibody, biotin-conjugated MoAb mouse anti-human BCA-1 and FITC-conjugated avidin; (3) MoAb mouse anti-human CD22, clone IgG1, FITC-conjugated goat anti-mouse IgG1 polyclonal antibody and PE-conjugated MoAb mouse anti-human CXCR5; (4) MoAb mouse anti-human CD35, clone IgG1, PE-conjugated goat anti-mouse IgG1 polyclonal antibody, biotin-conjugated MoAb mouse anti-human BCA-1 and FITC-conjugated avidin; (5) MoAb mouse anti-human CD35, clone IgG1, FITC-conjugated goat anti-mouse IgG1 polyclonal antibody and PE-conjugated MoAb mouse anti-human CXCR5. Negative control stains were performed by replacing the primary antibody with an irrelevant isotype-matched mouse MoAb and with chromogen only.

2.4. RNA extraction and RT-PCR

Total RNA was extracted from the skin biopsies. Frozen tissues were ground to a powder with a pestle

and mortar in liquid nitrogen before being added to a RNAwiz (Ambion, Austin, TX) for homogenisation. All samples used in these experiments clearly showed 18S and 28S bands in a 0.8% agarose gel, indicating the integrity of RNA. Following extraction, 1 µg of total RNA was reverse-transcribed using M-MLV reverse transcriptase (GIBCO-BRL, Gaithersberg, MD) according to the manufacturer's instructions. All cDNAs were amplified with primers for β -actin [27]: sense 5'-CCT CGC CTT TGC CGA TCC; antisense 3'-GGA TCT TCA TGA GGT AGT CAG TC) to test the quality of cDNAs. PCR products were electrophoresed in an agarose gel and measured by image analysis. Different amounts of cDNA for each sample (corresponding to the same amount of β -actin) were co-amplified with primers for β -actin (626 bp) and for BCA-1 (368 bp) and CXCR5 (318 bp) in the same reaction and distinguished using gel electrophoresis by the differences in the product length. PCR amplification were performed using the primers for BCA-1: sense 5'-GGG CTGCAGCTC-CAGACAGAACATGAAGTTC, antisense 3'-GGA CTC GAG TGG AAA TAT CAG CAT CAG G [28] and for CXCR5: sense 5' ACG CTG GAA ATG GAC CTC GAG AAC CTG GAG; antisense 3' AGA AGT AGA ACG GGA AAC GGC ACC GG. Primers for CXCR5 were selected using the Oligo Primer Analysis Software Version 5.0 (National Bioscience, Inc. Plymouth, MN, USA). All other primers were purchased from MWG Biotech AG (Ebersberg, Germany). In all PCR reactions, a positive control and 2 negative controls (one omitting the reverse transcription without the RT enzyme, another without cDNA as a template to exclude contamination) were included. We used human genomic DNA as a template in the PCR amplification to confirm that the product was not due to pseudogene sequences. Each sample was subjected to 25 cycles of amplification using 10 pM of each primer with 0.25 units of Taq DNA in 25 µl final volume of reaction mix. The cycling conditions were 94 °C for 30 s (denaturation), 64 °C for 30 s (annealing) and 72 °C for 40 s (elongation). Before the first cycle, samples were denatured at 94 °C for 2 min and, after the last cycle, samples were subjected to a final elongation step of 72 °C for 2 min. PCR products were electrophoresed on a 2% agarose gel and visualised by ethidium bromide staining. The size of the amplified products was evaluated by comparison with molecular weight markers run in parallel lanes.

2.5. Image analysis of PCR products

The intensity of the bands obtained by PCR amplification were measured by a CCD video camera C3077/01 Hamamatsu Photonics (Japan) connected with the video frame grabber M4476 (Hamamatsu Photonics Japan), in a computer Macintosh IIxi (Apple, USA). Acquisition of the image was obtained with Imagequest

IQBase software by Hamamatsu Photonics (Japan). Image processing and analysis was performed with the free software IMAGE by Wayne Rasband, National Institutes of Health Research Services Branch NIMH, version 1.28.

2.6. In situ hybridisation (ISH)

Probes for *BCA-1* (368bp) and *CXCR5* (312bp) were obtained by PCR amplification, and sequenced. DNA

fragments were subcloned using the pGEM-T Vector system (Promega Co. Madison, WI) according to the manufacturer's instruction. Sequences of the subcloned products were obtained using the Cy5™Auto-read™Sequencing kit (Pharmacia Biotech, Uppsala, Sweden) and automatically sequenced (Pharmacia Biotech, Uppsala, Sweden). Probes were labelled using the BrightStar Psoralen-Biotin labelling kit (Ambion, Austin, TX) and ISH were performed using DAKO GenPoint (Dako A/S Glostrup, Denmark) according to

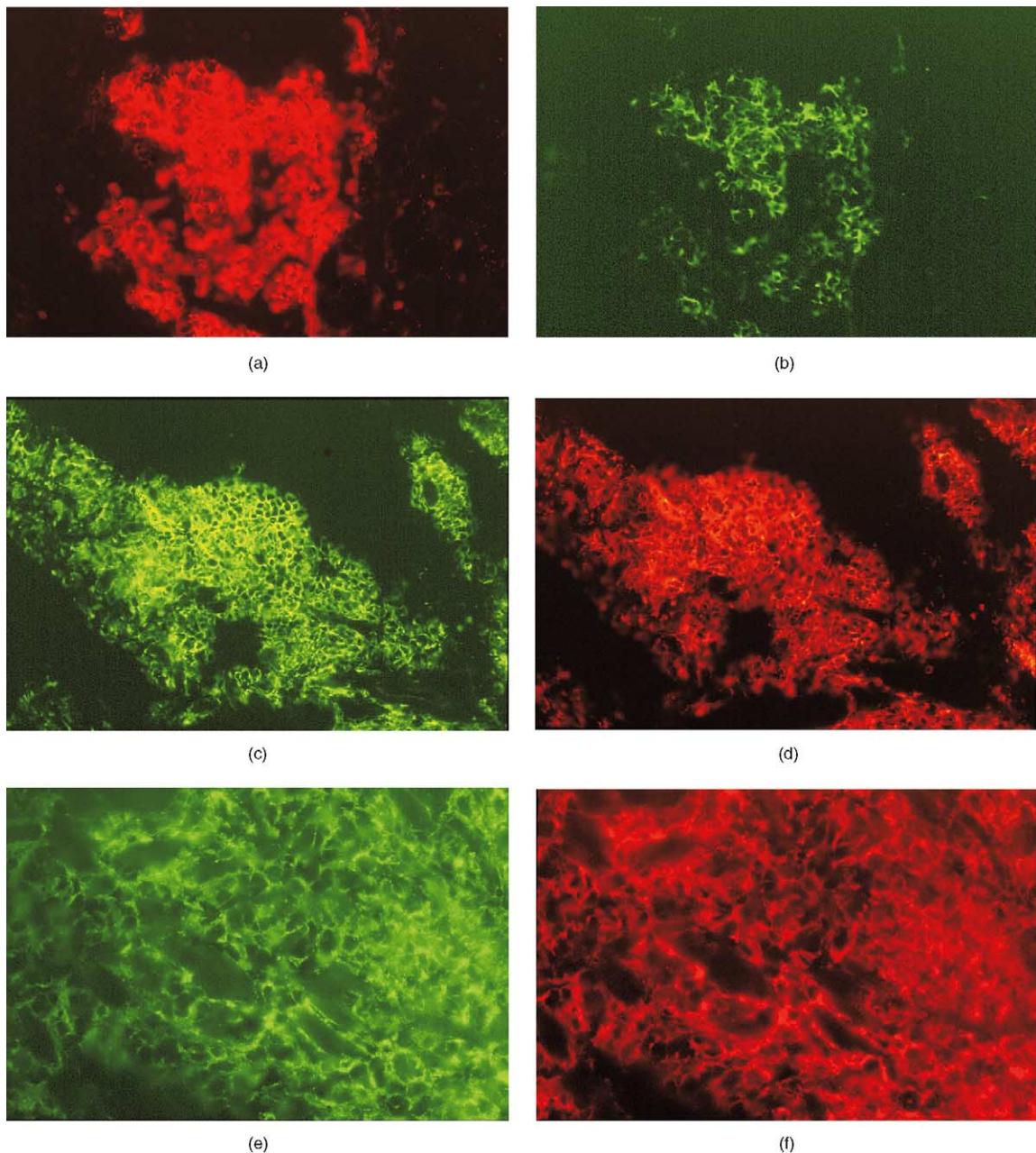


Fig. 1. Two-colour immunofluorescence detection of BCA-1, CXCR5, CD22 and CD35 expression in PCBL, low grade CBCL and high grade CBCL. PCBL (original magnification $\times 100$): heavy CD35 $^{+}$ staining of dendritic cells and, to a lesser extent, lymphoid cells within follicles (1a, top left); dendritic cells coexpress BCA-1 (1b, top right). Low grade CBCL (original magnification $\times 100$): BCA-1 expression (1c, middle left) is restricted to clustered CD22 $^{+}$ B-cells (1d, middle right). High grade CBCL (original magnification $\times 400$): large neoplastic cells show substantial expression of BCA-1 (1e, bottom left) and CXCR5 (1f, bottom right).

the manufacturer's instructions. Negative control stains were performed by replacing the labelled probe with hybridisation buffer.

3. Results

3.1. IHC and 2c-IF

BCA-1 and CXCR5 expression was not detected in either clinically healthy skin or CTCL. In contrast, BCA-1 and CXCR5 expression was found in all of the biopsies from PCBCL (3/3) and CBCL (10/10).

By both IHC (according to the step-section analysis) and 2c-IF, a different pattern and intensity of expression was observed among the PCBCL, low grade CBCL and high grade CBCL samples. In PCBCL, that were constantly characterised by a polyclonal B-cell infiltrate that was often arranged in well structured lymphoid follicles, BCA-1 and CXCR5 expression was clear cut and restricted to CD22+ B-cells; BCA-1 was also expressed by CD35+ dendritic cells within follicles (Fig. 1a and b). In low grade CBCL, BCA-1 expression was also clear cut and restricted to CD22+ B-cells, either clustered (Fig. 1c and d) or sparsely distributed within the often strongly T-cell reactive infiltrate. No dendritic staining pattern of BCA-1 was observed in neoplastic, follicle-like B-cell clusters. In these latter cells, CXCR5 expression was less than in the reactive follicles found in PCBCL. In all high grade CBCL, that were virtually composed of monoclonal large cells only, BCA-1 and CXCR5 were highly expressed (Fig. 1e and f).

3.2. RT-PCR and quantitative analysis

BCA-1 and/or CXCR5 were never detected by RT-PCR in either clinically healthy skin or CTCL samples. On the contrary, BCA-1 and CXCR5 signals were detected in all of the CBCL and PCBCL samples. The image analysis of the intensity of bands of β -actin, BCA-1 and CXCR5 obtained with RT-PCR showed a different level of expression for BCA-1 and CXCR5: the intensity of bands from high grade CBCL was clearly higher than that from low grade CBCL and PCBCL samples (Figs. 2 and 3).

3.3. In situ hybridisation

No *in situ* hybridisation of BCA-1 and CXCR5 was found in either the clinically healthy skin or CTCL samples. On the contrary, BCA-1 and CXCR5 staining was observed in pseudoCBCL and CBCL (Fig. 4). In particular, the expression was more evident in the high grade CBCL.

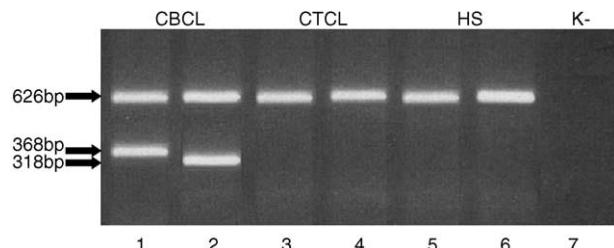


Fig. 2. Reverse transcriptase-polymerase chain reaction (RT-PCR) for β -actin, BCA-1 and CXCR5. Lane 1: co-amplification of β -actin (upper band: 626 bp) and BCA-1 (lower band: 368 bp) in a representative case of CBCL. Lane 2: co-amplification of β -actin (upper band: 626 bp) and CXCR5 (lower band: 318 bp) in the same CBCL case. No expression of BCA-1 (Lanes 3 and 5) and CXCR5 (Lanes 4 and 6) in CTCL and healthy skin (HS), respectively. K- : negative control for primers. bp: base pair.

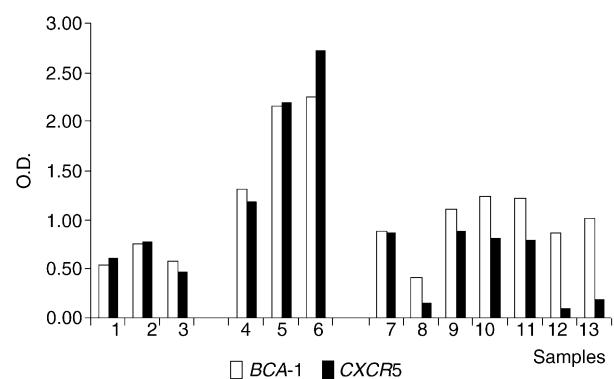


Fig. 3. Image quantitative analysis of RT-PCR products: diagram of BCA-1 and CXCR5 expression in pseudoCBCL (samples 1–3), low grade CBCL (samples 7–13), and high grade CBCL (samples 4–6). O.D., Optical Density.

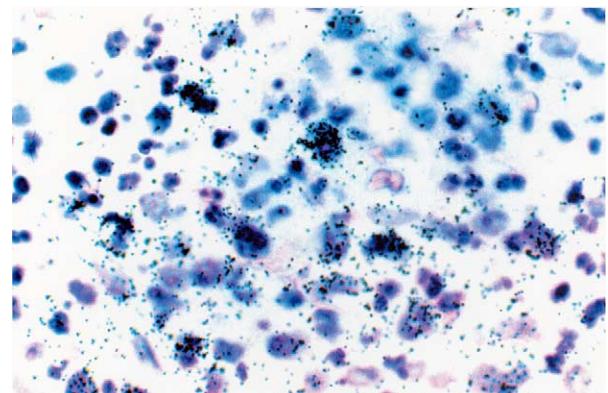


Fig. 4. *In situ* hybridisation (ISH, see Materials and Methods section for technical details). Representative picture: BCA-1 staining in CBCL, low grade. Original magnification, $\times 160$.

4. Discussion

In this study, we have analysed the phenotypic and genotypic expression of BCA-1 and CXCR5 in CBCL and PCBCL, in order to investigate the possible involvement of these molecules in the pathogenesis of these

characteristic disorders. We also wanted to examine the relevance of their possible differential expression in the different subcategories (PCBCL versus low grade CBCL versus high grade CBCL).

No expression of BCA-1 and CXCR5 was found at the immunohistochemical and molecular level in biopsies from either clinically healthy skin or CTCL, thus confirming the B-cell restricted specificity of these chemokines.

Interestingly, significant differences were found among the PCBCL, low grade CBCL and high grade CBCL. In fact, although the expression of CXCR5 and BCA-1 was restricted to CD22+ B-cells in all of the examined specimens, CD35+/BCA-1+ dendritic cells were found by 2c-IF in lymphoid follicles of PCBCL only. In low grade CBCL, conversely, virtually no CD35+ dendritic cells coexpressed BCA-1, and the intensity of CXCR5 expression on CD22+ cells was lower than that of BCA-1. In high grade CBCL, both BCA-1 and CXCR5 were strongly expressed by neoplastic CD22+ B-cells. In particular, the intensity of CXCR5 was much higher than that observed in low grade CBCL.

The above findings, although only in a small series of patients, are in keeping with those evidenced in *Helicobacter pylori* (HP)-induced MALT (gastritis) and gastric lymphoma, MALT type [14]. In fact, the distribution and intensity of BCA-1 and CXCR5 in (secondary) lymphoid follicles of PCBCL parallel those found in HP-induced MALT (gastritis) [14]. On the other hand, the expression of BCA-1 and CXCR5 was virtually restricted to neoplastic B-cells in CBCL, as well as in gastric lymphomas [14]. In addition, in both high grade CBCL and high grade gastric lymphomas, BCA-1 and—even more so—CXCR5 expression by large blasts was very pronounced [14]. An interesting issue for debate is the absence of dendritic staining for BCA-1 in both low grade CBCL and low grade gastric lymphomas [14]. In this latter group, we have interpreted this finding as reflecting the absence of follicular dendritic cells from the neoplastic infiltrate (CD21- and CD23-negative staining on paraffin sections). In low grade CBCL, conversely, we usually found CD35+ follicular dendritic cells, with a stronger staining in cryostat sections, while the expression of CD21 is ± in cryostat sections and often lacking in paraffin sections. Indeed, we have previously stressed this “aberrancy” of dendritic cells in neoplastic follicle-like clusters of classic CBCL [15–18,29,30]. Therefore, it is not unlikely that immunologically aberrant follicular dendritic cells have a substantially downregulated expression of BCA-1 in both low grade CBCL and low grade gastric lymphoma of the MALT type.

Taken altogether, the findings described in this paper add to the evidence of close similarities between CBCL (primary cutaneous B-cell lymphoma) and MALT lymphomas, with chronic antigen stimulation as a possible common pathogenic denominator [14,21].

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