Dissertation

Molecular Etiopathogenesis of Marginal Zone B-cell Lymphomas

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Mag. rer. nat. Alexander Deutsch

Mat.Nr.: 9913488

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Prof. Dr. Peter Neumeister

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Eidesstattliche Erklärung

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Zusammenfassung

Studien der letzten Jahre haben gezeigt, dass ein neuer molekularer Mechanismus für genetische Instabilität, der aberrant somatische Hypermutation (ASHM) genannt wird, eine essentielle Rolle in der Entstehung von B-zelligen Non-Hodgkin Lymphomen spielt. Während ASHM durch Mutationen bekannter Proto-Onkogene für den Transformationsprozess wichtig ist, scheint das Chemokinrezeptorprofil der Tumorzelle mit ihren Liganden für den primären Manifestationsort und die Dissemination essentiell zu sein. Um nachzuweisen, ob der Mechanismus der ASHM in Marginalzonen-B-Zelllymphomen der Haut (PCMZL) auftritt, und welche Chemokinrezeptoren in extragastralen MALT-Lymphomen exprimiert werden, wurde eine umfassende Studie der Mutationsprofile von *PAX5, RhoH/TTF, cMYC* und *PIM1* in PCMZL und des Chemokinrezeptor-Expressionsmusters in der Parotis, Parotis mit Sjögren Syndrom (SS), extragastrale und gastrale MALT-Lymphome, extranodal diffus großzellig B Zelllymphome (eDLBCL), die aus MALT-Lymphomen hervorgingen (sogenannte transformierte MALT-Lymphome) durchgeführt. In den PCMZL wurden 18 Mutationen in 8 der 11 analysierten PCMZL-Fälle gefunden. Alle Mutationen wiesen die charaktistischen Merkmale der ASHM auf. Weiters führten 2 Mutationen, jeweils eine im *PIM1*- und eine im *cMYC*-Lokus, zum Austausch von Aminosäuren mit potentiellen funktionellen Konsequenzen. Das Chemokinrezeptor-Expressionsmuster wies signifikante Unterschiede zwischen extragastralen MALT-Lymphomen und ihren transformierten Formen (eDLBCL) auf mit differentieller Expression multipler Chemokinrezeptoren, die zur Aufrechterhaltung der B-zell Homöostase and Aktivierung notwendig sind. CCR4 konnte nur in MALT-Lymphomen, welche eine Trisomie 3 aufwiesen, detektiert werden. Der Vergleich des Chemokinerezeptor-Expressionsmusters zwischen gastralen und extragastralen MALT-Lymphomen zeigte, dass CXCR1 und CXCR2 in gastralen MALT-Lymphomen signifikant stärker exprimiert waren, währenddessen CCR8, CX3CR1 und XCR1 eine verminderte oder fehlende Expression zeigten. Somit scheint diesen Chemokinrezeptoren eine wesentliche Rolle für den primären Manifestationsort der MALT-Lymphome zuzukommen. Zusammenfassend belegen diese Daten, dass der Mechanismus der ASHM mit der Entstehung von PCMZLs assoziiert ist. Durch die Mutationen von Proto-Onkogenen könnte dieser Mechanismus wesentlich zu Entstehung dieser Lymphome beitragen. Das veränderte Expressionsniveau von unterschiedlichen Chemokinrezeptoren könnte an dem stufenweise Entstehungsprozess von extragastralen MALT-Lymphomen beteiligt sein.

Abstract

Several recent studies revealed that a novel mechanism introducing genetic instability termed aberrant somatic hypermutation (ASHM) contributes to the development of Non-Hodgkin lymphomas. Whereas ASHM is involved in lymphoma initiation, the site of origin and dissemination is mainly determined by individual chemokine receptor profiles and their cognate ligands. To investigate whether ASHM also occurs in primary cutaneous marginal zone B cell lymphoma (PCMZL) we studied the mutational profile of the proto-oncogenes *PAX5, RhoH/TTF, cMYC and PIM1* in PCMZLs, all known to be involved in lymphomagenesis. Further, in order to characterize the dissemination and homing pattern of extragastric MALT lymphomas, the expression profile of 19 chemokine receptors in parotid glands, parotid glands affected by Sjoegren Syndrome, extragastric MALT lymphoma and extranodal diffuse large B cell lymphoma (eDLBCL) originating from MALT lymphoma was analyzed. A total of 18 sequence variants were found in 8 out of 11 primary cutaneous marginal zone B cell lymphoma cases (72.7%) and displayed the molecular features typical for the ASHM. Two mutations, one mutation in *PIM1* and one in *cMYC,* led to amino acid substitution which may have potential functional consequences. By investigating the expression of chemokine receptors by semi quantitative real time PCR and by immunohistochemistry we show that the expression profiles of extragastric MALT lymphomas differ substantially compared to their transformed counterparts originating at the same site (eDLBCL) with differential expression of multiple chemokine receptors involved in B-cell homeostasis and activation dependent chemokine receptors. Expression of CCR4 was just detected in trisomy 3 positive MALT lymphoma cases. Comparing gastric to extragastric MALT lymphomas up-regulation of CXCR1 and CXCR2 accompanied by down-regulation of CCR8 and CX3CR1 and loss of XCR1 expression in extragastric MALT lymphomas appear to be key determinants for the site of origin of MALT lymphomagenesis. These data indicate that aberrant SHM is associated with the development of PCMZLs. By mutating regulatory and coding sequences of the targeted genes ASHM may represent a major contributor to their pathogenesis. Our chemokine receptor expression profile support a model of a stepwise progression of extragastric MALT lymphoma from a non neoplastic event to Sjoegren Syndrome, to MALT lymphoma and finally overt eDLBCL lymphoma guided by differentially expressed B cell homeostatic and activation dependent chemokine receptors.

Publications

This thesis is based on the following publications:

Deutsch, AJ; Frühwirth, M; Aigelsreiter, A; Cerroni, L; Neumeister, P (2009): Primary cutaneous marginal zone B-cell lymphomas are targeted by aberrant somatic hypermutation. J Invest Dermatol. 2009; 129(2):476-479

Deutsch, A; Aigelsreiter, A; Steinbauer, E; Frühwirth, M; Kerl, H; Beham-Schmid, C; Schaider, H; Neumeister, P (2008): Distinct signatures of B-cell homeostatic and activation-dependent chemokine receptors in the development and progression of extragastric MALT lymphomas. J Pathol. 2008; 215(4):431-444

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Introduction

Approximately 8% of all B-cell Non-Hodgkin lymphomas (B-NHL) are extranodal marginal zone B cell lymphomas of mucosa associated lymphoid tissue (MALT), also known as MALT lymphoma, which was first described in 1983 by Isaacson and Wright.[1,2] MALT-lymphoma preferentially arises in the mucosa associated lymphoid tissue of the gastrointestinal tract, but it may affect virtually every organ in the body. The histological feature of MALT lymphoma is its infiltration around B cell follicles in the region corresponding to the Peyer's patch marginal zone, spreading diffusely into the surrounding tissue. MALT lymphoma cells share the same cytological features and immunophenotype (CD20+, CD21+, CD35+, IgM+, IgD-) like the marginal zone B cells. The development of MALT lymphomas is preceded by acquisition of inflammatory reactive lymphoid tissue. Hence, long-lasting chronic inflammation of Sjoegren Syndrome, Hashimoto thyroiditis and *Helicobacter pylori (H. pylori)* gastritis provide the pathogenetic background at these sites.[3] Primary cutaneous marginal zone B cell lymphoma (PCMZL), one of the most frequent types of cutaneous B cell lymphomas, is considered as part of the group of MALT lymphomas [4]. PCMZL shares some histological and clinical features with MALT lymphoma of extracutaneous origin but differences in biology with respect to dissemination, association to chronic inflammation and treatment modalities are found between PCMZL and extracutaneous MALT lymphomas suggesting that they should be considered as separate entity [5-8].

Recently, a novel mechanism has been identified to crucially contribute to development of hematological disorders: Aberrant somatic hypermutation (ASHM), which was first described in diffuse large B cell lymphomas (DLBCL). In DLBCL the physiological process of the somatic hypermutation, occurring in the rearranged *V* genes to generate antibody diversity of germinal-centre B cells and of all germinal-centre-derived B-cell tumours[9,10], aberrantly targets the 5′ sequences of several proto-oncogenes relevant to lymphomagenesis, including *PIM1*, *PAX5*, *RhoH/TTF* and *cMYC*. This phenomenon occurs in >50% of DLBCL, but is rare in indolent lymphomas like MALT lymphoma[11-16] and it has not been investigated for PCMZL. The pathogenesis of most B-NHLs is associated with distinct genetic lesions, including chromosomal translocations and aberrant somatic hypermutation (ASHM), which arise from mistakes during of class switch recombination (CSR) and SHM occurring in the germinal centre[9,10,17,18]. Activation-induced cytidine deaminase (AID) is an enzyme required for SHM and CSR, mistargeting of AID to known proto-oncogenes linked to B-cell tumorigenesis in

germinal centre B-cells combined with a breakdown of protective high fidelity repair mechanism has been shown to be a principal contributor to the pathogenesis of B-NHL[17,18].
Another mechanism involved in B-cell homing and dissemination is the interaction of chemokines with their chemokine receptors. Patterns of chemokine receptor expression have been identified in different B cell malignancies playing a role in malignant B-cell circulation leading to emergence of monoclonal B cells, and, eventually to transformation[19-22]. Chemokines, also known as proinflammatory, chemotactic cytokines, represent a large superfamily of peptides with diverse biological functions. Chemokines interact with a target cell by binding to the chemokine receptors. There exist numerous chemokines and chemokine receptors but no single chemokine is assigned to a single receptor. Chemokine signalling can coordinate cell movement during inflammation, as well as the homeostatic transport of haematopoietic stem cell, lymphocytes and dendritic cells.[23-25] The homeostatic transport of precursor B cell to secondary lymphoid tissue is essential for B cell development. CCR6, CCR7, CXCR3, CXCR4 and CXCR5 play a crucial role in this homing process, therefore the group of these five chemokine receptors is called B cell homeostatic chemokine receptors.[26-28]
The group of activation dependent chemokine receptors, which are expressed on effector leukocytes (including activated effector/memory T cells) play an essential role in inflammation processes responsible for migration towards chemokines produces by inflamed cells[23].
The aim of the present study was to investigate the role of the ASHM in PCMZL and to identify expression patterns of 19 chemokine receptors of parotid glands, of parotid glands affected by Sjoegren Syndrome, of parotid and other extragastric MALT lymphomas and of extranodal diffuse large B cell lymphoma (eDLBCL) arising from MALT lymphoma of the parotid glands.

Material and Methods

Material

Lymphoma entities were classified according to the WHO classification of lymphoid neoplasms[29] and according to the WHO-EORTC classification for cutaneous lymphomas [4]. Methylene-stained sections of formalin-fixed, paraffin-embedded (FFPE) lymphoma tissue containing >80% lymphoma cells were manually macrodissected by one experienced pathologist using a disposable, sterile, 30-gauge needle under direct light microscopic visualization and were further processed for RNA isolation. Attention was made, that cases of eDLBCL comprised at least – established by morphology and immunohistochemistry - one

focus of a low grade lymphoma component considering these lymphomas as "transformed MALT lymphomas"[30].

The determination of the chemokine receptors mRNA expression profiles was performed on 16 extragastric MALT lymphomas (11 parotid gland, 2 skin, 2 orbita and 1 thyroid), on 5 gastric MALT lymphomas, on eight eDLBCL of the parotid gland, five parotid glands affected by Sjoegren Syndrome (pSS), 10 non neoplastic parotid glands and on 5 samples of peripheral blood B cells (pCD19+ cells) as shown in supplementary table S1. For immunohistochemical analysis of CCR1, CCR5, CXCR6 and XCR1 11 extragastric MALT lymphomas of the parotid gland, 8 eDLBCL, 5 pSS and 10 non neoplastic parotid glands were used and for XCR1 immunohistochemical analysis five gastric MALT lymphomas were included. To gain knowledge about the expression of these four chemokine receptors on B cells of the marginal zone four samples of healthy donors were immunohistochemical analysed in this study. For FISH analyses 15 MALT lymphoma [five of gastric origin and 10 of extragastric origin (6 parotid gland, 2 orbita and 2 skin)] were used.

For investigating the role of the ASHM in PCMZLs 11 PCMZL specimens, 3 *Borrelia burgdorferi* infected skin lesions and 4 normal controls, including 3 non-neoplastic tissues samples of selected lymphoma cases (corresponding to case 3, 10 and 11) and 1 peripheral blood mononuclear cells (MNC) were included.

Ethical approval was obtained from local Hospital Ethics Committee in accordance with Declaration of Helsinki Principles.

ASHM in PCMZLs

DNA extraction and Sequencing analysis of PAX5, RhoH/TTF, c-MYC, and PIM1

For investagting the role of the ASHM in PCMZLs DNA from macrodissected paraffin embedded of PCMZL specimens, *Borrelia burgdorferi* infected skin lesions and normal controls was extracted using the DNA Mini Kit (Qiagen, Valencia, CA).

Mutational profile by direct DNA sequencing of *PAX-5* [AF386790S2 (exon 1B):630-1450] *RhoH/TTF* [AF386789 (exon 1): 265-1009]*, c-MYC* [X00364 (exon 1 through 2): 2289-3626 and 4486–5068] and *PIM1* [AF386792 (exon 1 through 4): 780-2080] was generated on selected regions previously described to contain more than 90% of mutations ("mutational hot spots") found in DLBCL [11]. The initial PCR was performed using the HotStarTaq DNA Polymerase (Qiagen, Hilden, Germany), and all oligonucleotides were also used for sequencing (available in Table S1). PCR products were purified and sequenced from both sides using the BigDye terminator chemistry 3.1 (Applied Biosystems, Foster City, CA).

Sequences were run on an ABI3730 automated sequencer (Applied Biosystems). Sequences variants were confirmed by 2 independent PCR reactions. Nucleotide changes corresponding to previously published polymorphism were excluded from analysis. Further, all changes occurring more than once in separate cases were considered as polymorphic variants and were disregarded.

Chemokine receptor expression profiling of MALT lymphomas and eDLBCLs

RNA-Isolation and cDNA synthesis

The peripheral B cells were isolated from blood of 5 healthy donors with CD19 Micro Beads (Miltenyi Biotec, Bergisch Gladbach, Germany) and LS Columns (Miltenyi Biotec, Bergisch Gladbach, Germany) in accordance to the manufacturers' protocol. Total RNA of peripheral B cells and fresh frozen tissue sample was extracted using the Trizol method (Invitrogen, Carlsbad, CA). For total RNA isolation of formalin fixed paraffin embedded (FFPE) tissue a different protocol was used as previously described.[31]

For cDNA synthesis total RNA was treated for DNA digestion with DNase I (10 units/µl, Roche) in the presence of RNase Inhibitor (40 units/µl, Roche, Switzerland) and 25mM $MgCl_2$ for one hour at 37°C. Some of the samples were tested for DNA contamination with a Bioanalyzer (Agilent Technologies, Palo Alto, CA). Total RNA was reversed transcribed into cDNA using 0.5µg random hexamer primers and 200 U of reverse RevertAid™ H Minus M-MuLV RT (Fermentas, St. Leon-Rot, Germany) according to the manufactures' protocol.

Real-time PCR

The real-time PCR was performed as previously reported by Seidl et al. [32] The nucleotide acid sequences for the primers and probes, which were used for the determination of the expression levels of 19 chemokine receptors are shown in supplementary table S2.

The expression levels were calculated based on the $2^{-\Delta\Delta CT}$ method. [33]

Immunohistochemical analyses for CCR1, CCR5, CXCR6 and XCR1

Immunohistochemical analyses were performed in collaboration with the research group of Prof. Beham-Schmid, Institute of Pathology. Formalin-fixed paraffin embedded tissue was stained after pre-treatment with Target Retrieval Solution (Dako, Glostrup, Denmark) using the DakoCytomation® (Glostrup, Denmark) automated immuno-stainer and iView detection system (Ventana Medical System, Tucson, AZ). Primary antibody to CCR1 (1:300) and CCR5

(1:300) were purchased from Novus Biologicals (Littleton, CO, USA), CXCR6 (1:100) from MBL (Woburn, MA, USA), CD3 (1:50), CD20 (1:200), CD68 (1:200), and Vs38C (1:100) from Dako (Glostrup, Denmark), and XCR1 from Acris (1:1000) (Hiddenhausen, Germany). For control purposes, tissues known to contain the respective antigens were included (positive controls). Replacement of the primary antibody by normal serum always revealed negative results (negative controls). Scoring of tissue slides was performed independently by 2 investigators, the percentage of positive cells and the intensity of staining was graded from 0 to 2+: 0, no staining; 1+, weak positive staining; 2+, moderate to strong positive staining. An immunoreactive score (IRS) was obtained by multiplying the percentage of positive cells with staining intensity divided by 10, according to Zhuang *et al*.[34]

FISH analyses

For reliable interpretation of hybridization signals, the analysis of single-cell suspension of thin sections were performed as previously described by Streubel et al [35]. In 15 MALT lymphoma cases FISH was performed on interphases using LSI® IGH/MALT1 Dual Color - Dual Fusion Translocation Probe t(14;18) (q32;q21), LSI® BCL6 Dual Color - Break Apart Rearrangement Probe (3q27), LSI® IGH/BCL2 Dual Color - Dual Fusion Translocation Probe t(14;18)(q32;q21), and LSI® API2/MALT1 Dual Color - Dual Fusion Translocation Probe t(11;18) (q21;q21) (Vysis, Downer's Grove, IL).

Statistical analysis

Statistical analysis was performed by using SPSS 15.0 (SPSS Inc., Chicago, IL, USA).

The nonparametric Kruskal Wallis test was used to analyze differences in chemokine and chemokine receptor expression levels among non neoplastic parotid glands, pSS, extragastric MALT lymphomas and eDLBCL. The chemokine receptor expression level with significant differences in their expression were analyzed using the Mann-Whitney *U* test, all significant associations were further corrected for multiple testing by applying a Bonferroni correction dividing the significance level by the number of tested variables. Spearman correlation test was performed to examine a correlation of mRNA levels with immunohistochemistry of CCR1, CCR5 and CXCR6. For the comparison of the chemokine receptor expression profile between extragastric and gastric MALT lymphomas the *Student's T-Test* or the *Mann-Whitney U Test* was performed. When p values was lower than 0.05 a significant value was reached. Expression levels of chemokine receptor are presented as mean values ± standard deviation (SD). To calculate differences between the dataset of PCMZLs and the previously published mutational profile of extracutaneous MALT lymphomas [12] *Student's T-Test* was used.

Results

ASHM in PCMZLs

11 samples of PCMZLs were subjected to DNA sequence analysis of *PAX5, RhoH/TTF, cMYC and PIM1* (table 1). 8 of 11 (72.7%) lymphoma samples showed somatic mutation in at least one of the four analyzed genes. Each of the 4 genes investigated was altered in a significant fraction (table 2): *PAX5* was mutated in 2 of 11 (18.2%) samples, *RhoH/TTF* in 3 of 11 (27.3%), *cMYC* in 4 of 11 (36.4%) and *PIM1* in 5 of 11 (45.5%) samples. To confirm somatic nature of these mutations corresponding germ line DNA was sequenced in selected cases (corresponding to case 3, 10 and 11) demonstrating the tumour specific origin. In order to rule out the occurrence of mutations in benign B cells, *Borrelia burgdorferi* infected skin lesions which are rich in inflammatory B cells were included showing no mutations.

Table 1: Mutational analysis of *PAX5, RhoH/TTF, cMYC* and *PIM1*

	PAX5	RhoH/TTF	cMYC	PIM1
Case 1				A 1414 G
Case 2				
Case 3			C 4655 T	**A 1237 C**
Case 4		T 415 C A 449 G		
Case 5				
Case 6	G 882 A			C 1303 T
Case 7			T 3493 C	C 1306 T
Case 8				
Case 9		T 936 C		
Case 10			**A 4608 G** C 4686 T	G 1938 A
Case 11	C 1104 T	C 945 T G 981 A	C 4693 T	

bold characters : missense mutations

The detailed characterization of the mutational profile is summarized in Table 1 and their features in Table 2. In total 17 sequence variants were found. These mutations were

exclusively single base pair substitutions. The average frequency of mutations, calculated taking into account only mutated cases, ranged from 0.02 x 10^{-2}/bp in the case of *cMYC* to 0.13 x 10^{-2}/bp in the case of *RhoH/TTF*; taking all four genes together the average frequency of mutation was 0.07 x 10^{-2}/bp (table 2). 15 of the 17 mutations were transitions and two were transversions corresponding to a transition-to-transversion ratio of 7.5 (expected 0.5, if each nucleotide is targeted at identical rate). In addition the ratio of G + C to A + T was shifted to a clear predominance of G + C mutations. Four of five mutations in *PIM1* and four of five mutations in *cMYC* were located in the coding region, one mutation (substitution of Thr with Pro for *PIM1* and Asn with Asp for *cMYC*) in each gene led to amino acid substitution with potential functional consequences (table 1).

Table 2: Distribution and features of *PAX5, RhoH/TTF, cMYC* and *PIM1* mutations in PCMZL

Locus	Mutated cases/tested (%)	Mutation frequency per 100bp (range)*	Single base pair substitution	G+C//A+T	Transitions over Transversions
PAX5	2/11 (18.2%)	0.097 (0.096-0.098)	2	2//0	2//0
RhoH/TTF	3/11 (27.3%)	0.12 (0.10-0.13)	5	2//3	5//0
cMYC	4/11 (36.4%)	0.025 (0.02-0.04)	5	3//2	4//1
PIM1	5/11 (45.5%)	0.05 (0.04-0.06)	5	3//2	4//1
All Genes	8/11 (72.7%)	0.07	17	10//7	15//2

* Mutation frequencies were calculated on the entire region analyzed and on mutated cases only, considering 2 alleles/gene/case

Chemokine receptor expression profiling of MALT lymphomas and eDLBCLs

Chemokine receptor expression profiles in pCD19+ cells, in non neoplastic parotid gland, in primary Sjoegren Syndrome, in extragastric MALT lymphoma and in eDLBCL

The occurrence of mRNA transcripts for CCR1, CCR5, CCR6, CCR7, CCR9, CCR10, CXCR1, CXCR3, CXCR5, CXCR7, CX3CR1 and XCR1 in pCD19+ cells was consistent with previously published data [36,37].

The chemokine receptor profile of extragastric MALT lymphomas of parotid glands did not significantly differ from the chemokine receptor expression pattern of extragastric MALT lymphomas of non parotid origin (skin, thyroid, and orbita).

The percentage of T cells (CD3+), macrophages (CD68+) and plasma cells (Vs38C+) is included in table 3 to further document the morphological variation within each analyzed category of tissue. Correlating the percentage of CD3+, VS38c+ and CD68+ cells of the extragastric MALT lymphoma and gastric MALT samples with the expression levels of all chemokine receptors no significant correlation could be observed demonstrating that the expression pattern was determined by lymphoma cells and not by intratumoral inflammatory cells.

Table 3: Immunhistochemical analyses of CD3, CD68, Vs38c, CCR1, CCR5 CXCR6, and XCR1 in non neoplastic parotid glands, parotid glands affected by Sjoegren Syndrome, parotid MALT lymphomas and extranodal DLBCL

	CD3	CD20	CD68	Vs38C	CCR1				CCR5				CXCR6				XCR1			
	%	%	%	%	0	+	++	IRS	0	+	++	IRS	0	+	++	IRS	0	+	++	IRS
Parotid gland	< 5 †	< 5 †	< 5 †	< 5†	-	8 / 11 80%	2 / 10 20%	0.83	-	10 / 10 100%	-	0.92	-	10/10 100%		1.00	10/10 100%			0
Sjoegren Syndrome	60 50 - 70	30 20 - 40	< 5 †	< 5 †	-	6 / 6 100%	-	0.62	-	3 / 6 50%	3 / 6 50%	2.50•	-	5 / 6 83%	1 / 6 17%	3.05	5 / 6 100%			0
MALT lymphoma	< 5† <5 - 10	85 80 – 97	12.30 10-20	20.90 15-30	-	11/ 11 100%	-	2.09*	1 / 11 (9%)	7 / 11 63%	3 / 11 28%	1.34	-	1 / 11 9%	10/11 91%	3.05*	-	5 / 11 46%	6 / 11 54%	12.09*
eDLBCL	< 5† <5 - 5	85 80 - 95	13.60 10-15	17.10 10 - 25	-	7 / 8 88%	1 / 8 12%	3.25 ◦	-	7 / 8 88%	1 / 8 12%	2.63 ◦	-	6 / 8 75%	2 / 8 25%	2.37	-	7 / 8 88%	1 / 8 12%	4.33 ◦

† only single cells were positive

• $p<0.05$ versus non neoplastic parotid gland

* $p<0.05$ versus benign precursor of extragastric MALT lymphoma, pSS

◦ $p<0.05$ versus MALT lymphoma

Trisomy 3 leads to CCR4 deregulation in MALT lymphoma

To study the effect of genetic alterations on the chemokine receptor expression profile interphases FISH analysis on 15 MALT lymphomas were done. In total, eight [two of gastric origin and six of extragastric origin (one skin, four parotid glands and one orbita)] of the 15 MALT lymphomas showed genetic alterations. Of these eight MALT lymphomas with genetic alterations seven [two gastric- and five of extragastric origin (four parotid glands and one orbita)] cases were positive for trisomy 3. Additionally in two (one gastric- and one of parotid origin) of these seven cases a rearranged MALT1 locus was found. Another MALT lymphoma (skin) sample exclusively exhibited a rearranged IGH.

Comparing the chemokine receptors mRNA expression profile between MALT lymphomas exhibiting trisomy 3 and MALT lymphomas without this genetic aberration it was obvious that CCR4 - a chemokine receptor genomically located on chromosome 3 (3p24) - was highly expressed in all MALT lymphoma samples with trisomy 3 whereas transcripts for this chemokine receptors were just found in one of the eight lymphoma samples with a normal chromosomal pattern at very low level (figure 1a; $p=0.014$). In contrast, the comparison of all other chemokine receptors located on chromosome 3 (CCR1, CCR2, CCR3, CCR5, CCR8, CXCR6, and CX3CR1) resulted in equal expression of trisomy 3+ and trisomy 3- MALT lymphomas.

Differences in the chemokine receptor expression profile between extragastric and gastric MALT lymphoma.

To find a distinct signature of chemokine receptor expression the pattern of 16 extragastric MALT lymphomas was compared to 5 MALT lymphomas of gastric origin. Expression of CXCR1 and CXCR2 was 466 and 7315 times higher in gastric MALT lymphomas, whereas CCR8 and CX3CR1 were 460 and 1000 times higher in extragastric MALT lymphomas. XCR1 transcripts were found in extragastric MALT lymphomas only ($p<0.05$; figure 1b). Immunohistochemical analysis of XCR1 showed a four times higher expression in extragastric MALT lymphomas compared to gastric MALT lymphomas ($p<0.05$; figure 1c).

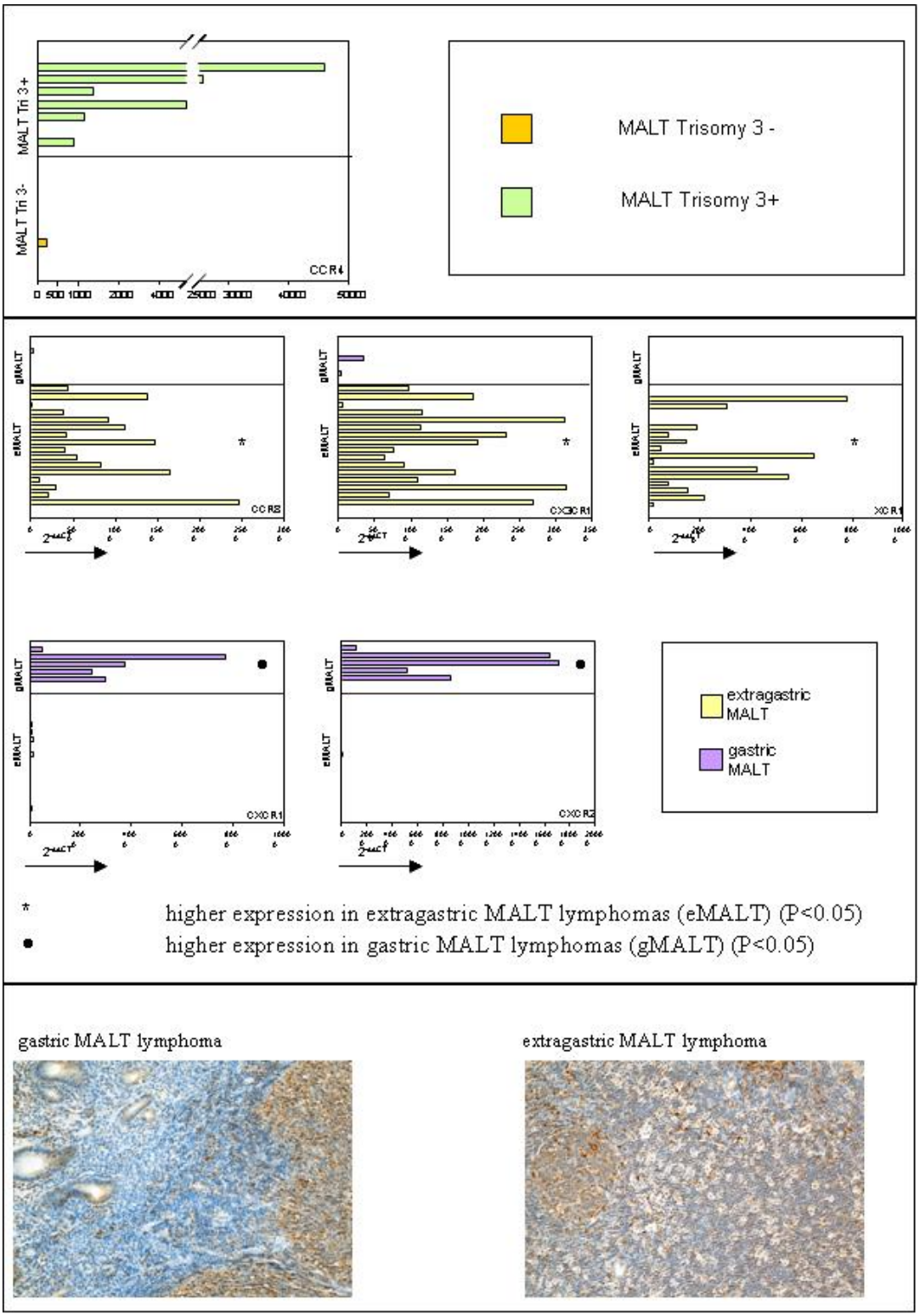

Figure 1: **Chemokine receptor expression analyses in MALT lymphomas**

a **Expression of CCR4 in trisomy 3 + and trisomy 3 – MALT lymphomas.** Each bar represents a specimen. Values of gene expression are calculated as relative expression in comparison to peripheral mononucleated cells serving as calibrator. CCR4 was higher expressed in Trisomy 3 + MALT lymphomas**.**

b **Differences in the chemokine receptor expression in extragastric and gastric MALT lymphomas.** Each bar represents a specimen. Values of gene expression are calculated as relative expression in comparison to peripheral mononucleated cells serving as calibrator.

c **Comparison of XCR1 immunohistochemical analyses between extragstic and gastric MALT lymphomas.** XCR1 was significantly higher expressed in extragastric MALT lymphomas. . All immunohistochemical pictures were kindly provided by Prof. Beham-Schmid

CC chemokine receptor expression

Expression profiles of CC chemokine receptors (figure 2 and table 4) demonstrated a homogeneous expression of CCR1, CCR5, CCR6 and CCR7 in all pCD19+ cells, in all extragastric MALT lymphoma specimens and in all eDLBCLs with the exception of CCR7, which was expressed in the majority (6 out of 8) of eDLBCLs. In contrast, the mRNA expression patterns of these receptors in the group of pSS and non neoplastic parotid glands were heterogeneously distributed (table 4).

CCR8 expression was lacking in pCD19+ cells. All analyzed extragastric MALT lymphoma cases and six of the eight eDLBCL samples showed CCR8 mRNA transcripts. The highest expression levels of this receptor were detected in pSS specimens (4 out of 5) but it was only found in a small number of non neoplastic parotid glands tissue samples (2 out of 10).

CCR10 expression was constantly detected in pCD19+ cells and in 7 of the 16 extragastric MALT lymphoma samples but in only one of the eight eDLBCL. Remarkably, it was constantly high in pSS, but none of the non neoplastic parotid gland specimens expressed this receptor.

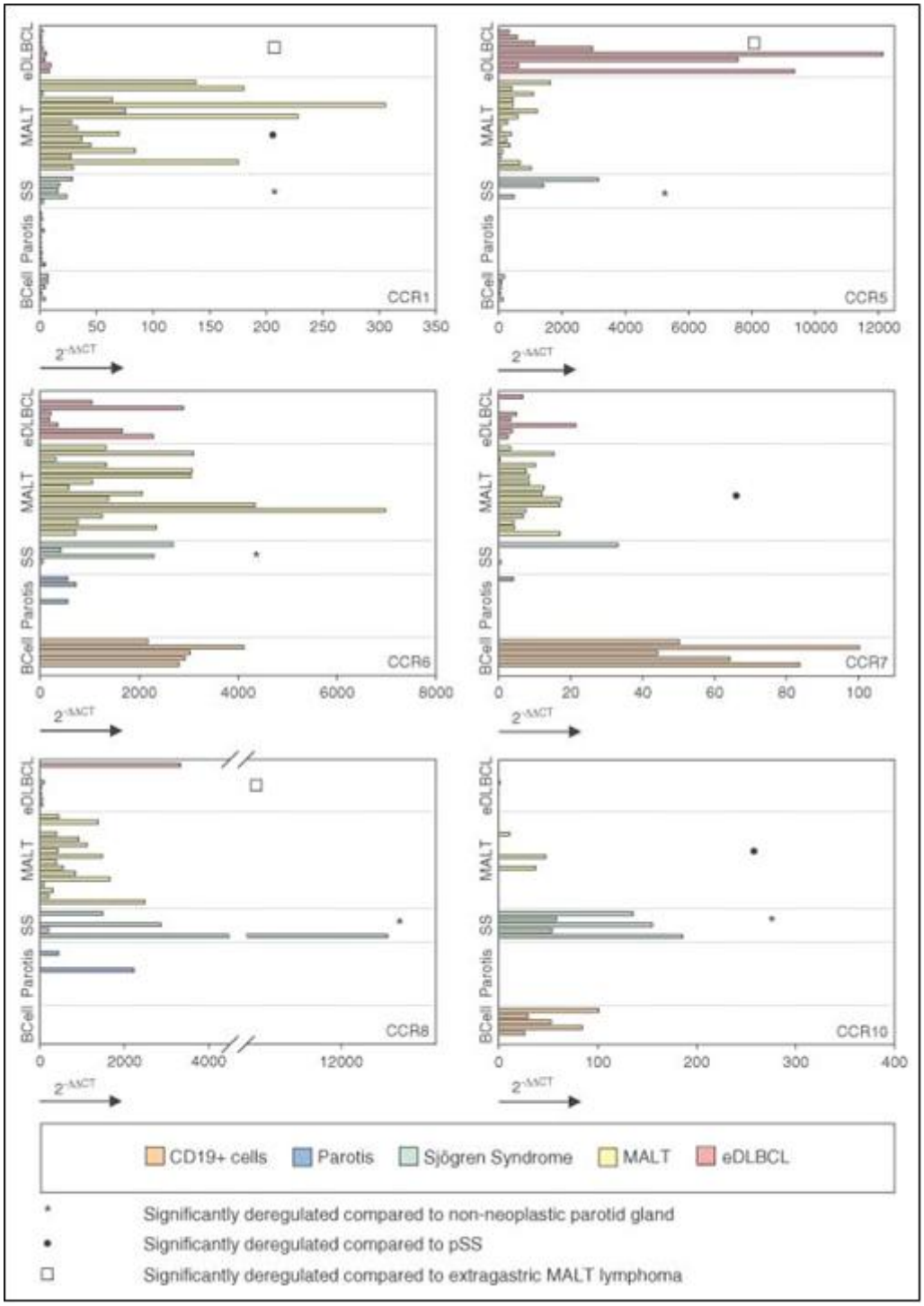

Figure 2: **CC chemokine receptor mRNA profile of pCD19+ cells, non neoplastic parotid gland, primary Sjoegren Syndrome, parotid MALT lymphoma and extranodal DLBCL.** Each bar represents a specimen. Values of gene expression are calculated as relative expression in comparison to peripheral mononucleated cells serving as calibrator.

Expression profiles of CCR4 and CCR9 were heterogeneously expressed and no significant difference was observed within the groups.

Expression of CCR2 and CCR3 was undetectable in all samples.

CXC, CX3CR1 and XCR1 chemokine receptor expression

Analyses of CXC chemokine receptors expression pattern (figure 3 and table 4) revealed a similar chemokine receptor expression profile of CXCR3, CXCR5 and CXCR6: all three receptors were broadly expressed in extragastric MALT lymphomas and in eDLBCLs, whereas in pCD19+ cells only transcripts for CXCR3 and CXCR5 were detectable. The expression profiles of CXCR3 and CXCR5 were consistent with previously reported data in non-Hodgkin's lymphoma [19,20]. No expression of CXCR3, CXCR5 and CXCR6 was found in non neoplastic parotid glands and in pSSs.

CX3CR1 and XCR1 expression was detected in all pCD19+ cell samples, in all specimens of extragastric MALT, but only in a few eDLBCL cases (two of eight for CX3CR1 and in one of eight for XCR1). No expression of these two receptors was detected in non neoplastic parotid glands and in pSSs.

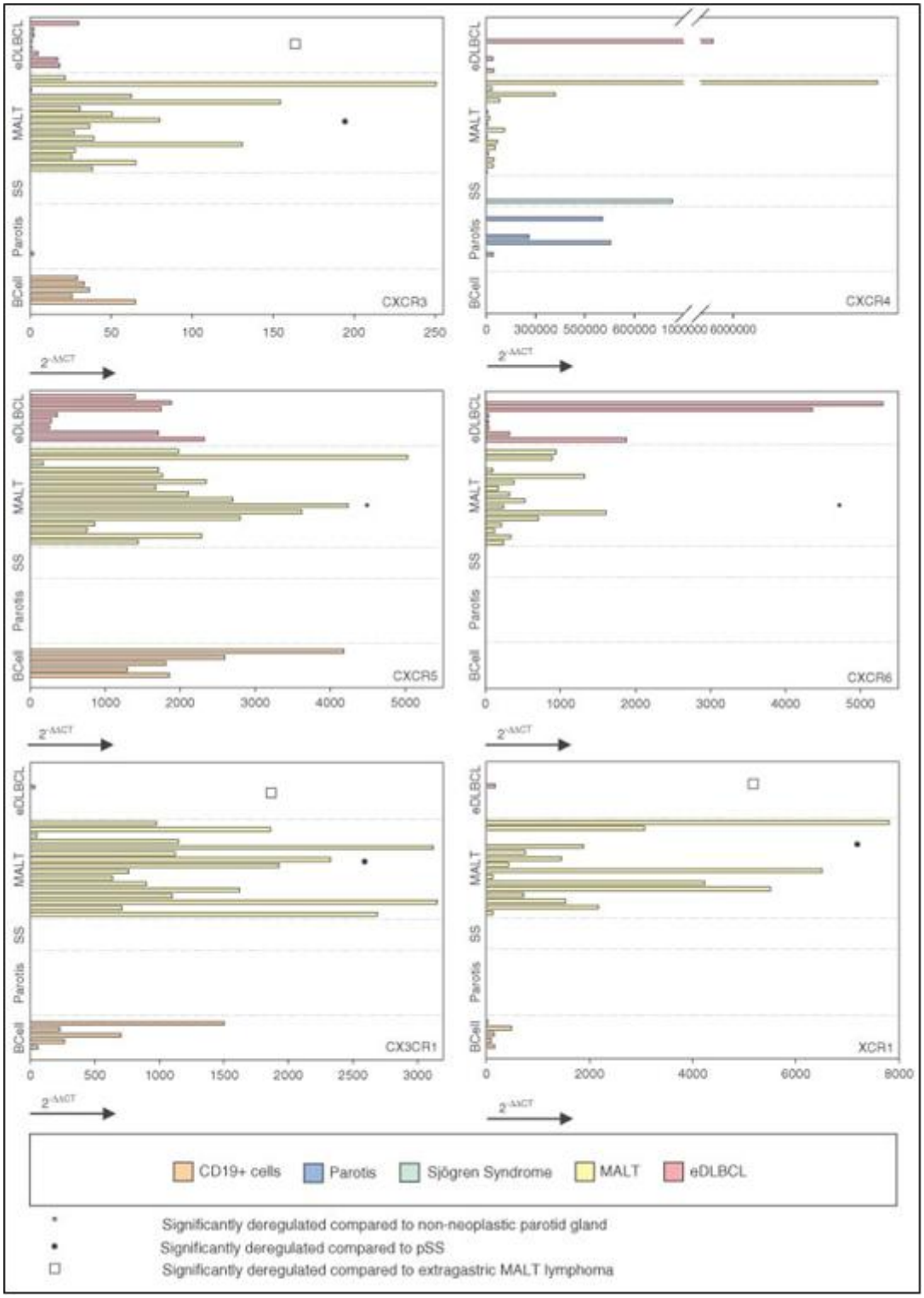

Figure 3: **CXC, CX3C1 and XCR1 chemokine receptor mRNA profile of pCD19+ cells, non neoplastic parotid glands, pSS, parotid MALT lymphoma and extranodal DLBCL.** Each bar represents a specimen. Values of gene expression are calculated as relative expression in comparison to peripheral mononucleated cells serving as calibrator.

CXCR1, CXCR2, CXCR4 and CXCR7 were heterogeneously expressed showing no significant difference within the 5 groups.

Table 4: **Expression of CC and CXC receptors in pCD19+ cells, non neoplastic parotid glands, parotid glands affected by Sjoegren syndrome, extragastric MALT lymphoma and eDLBCLs of the parotid gland**

	CCR1	CCR5	CCR6	CCR7	CCR9	CCR10	CXCR3	CXCR4	CXCR5	CXCR6	CX3CR1	XCR1
peripheral B cell	5/5	5/5	5/5	5/5	0/5	5/5	5/5	0/5	5/5	0/5	5/5	5/5
Parotid gland	10/10	8/10	3/10	1/10	2/10	0/10	0/10	4/10	0/10	0/10	0/10	0/10
Sjoegren Syndrome	5/5	3/5	4/5	2/5	4/5	5/5	0/5	1/6	0/5	0/5	0/5	0/5
MALT lymphoma	16/16	16/16	16/16	16/16	16/16	7/16	15/16	15/16	15/16	16/16	16/16	14/16
extranodal DLBCL	8/8	8/8	8/8	6/8	6/8	1/8	7/8	4/10	7/8	7/8	2/8	1/8

Immunohistochemistry of CCR1, CCR5, CXCR6 and XCR1

Because of their highest differences in mRNA expression levels in all specimens of extragastric MALT lymphomas and eDLBCL compared to pCD19+ cells, immunohistochemical analysis of CCR1, CCR5, CXCR6 and XCR1 was performed. To our knowledge none of these four receptors has ever been reported to be expressed in MALT lymphoma. Four spleens of healthy donors were included to assess the chemokine receptors expression of marginal zone B cells.

Comparing protein levels to mRNA expression, only CXCR6 and XCR1 showed a significant positive correlation ($p<0.01$; correlation coefficient (Spearmann Rho) = 0.594 for CXCR6 and 0.798 for XCR1).

In contrast to CXCR6 and XCR1 expression, which was weakly found on approximately 10% of B cells for CXCR6 and on 5 to 15% of B cells for XCR1 of the marginal zone in spleens of healthy donors, CCR1 and CCR5 were not expressed (data not shown). CCR1, CCR5 and CXCR6 were moderately to strongly expressed on cells of excretory ducts (figure 4(i)). On inflammatory T cells of pSS, CCR1 and CCR5 were moderately expressed, whereas CXCR6 showed a strong surface expression on T cells in all samples. XCR1 was not expressed in any specimen (figure 4(i) b,f,j,n).

In the majority of extragastric MALT lymphomas approximately 25% of the malignant cells showed moderate CCR1 expression, however, considerable expression was also evident on germinal centre centroblasts in reactive lymph follicles (IRS average: 2.09) (figure 4(i)c and table 3). CCR5 immunohistochemical reactivity was apparent in only a minority of malignant B-cells in MALT lymphomas (figure 4(i)g and table 3). As opposed to CCR5, 13% of MALT lymphoma cells and surrounding centroblasts of reactive lymphatic follicles strongly expressed CXCR6 (IRS on average: 3.04). It was also obvious that blasts in the lymphomas showed a

stronger CXCR6 expression (figure 4(i)k) than small neoplastic lymphocytes. XCR1 was strongly expressed on approximately 78% of malignant cells and on surrounding centroblasts of reactive lymphatic follicles in all extragastric MALT lymphomas (IRS average: 12.1) (figure 4(i)o).

All analyzed eDLBCLs moderately expressed CCR1 on approximately 50% of the malignant blasts (IRS on average: 3.25) (figure 4(i)d), whereas only a weak expression of CCR5 was found on approximately 6% of the malignant blasts (IRS on average: 2.63) in 4 of 9 specimens (figure 4(i)h). All eDLBCLs exhibited a strong CXCR6 expression on approximately 14% of lymphoma cells (IRS on average: 2.375) (figure 4(i)l). Moderate XCR1 expression was found on approximately 39% of lymphoma cells in all eDLBCLs (IRS on average: 4.33) (figure 4(i)p).

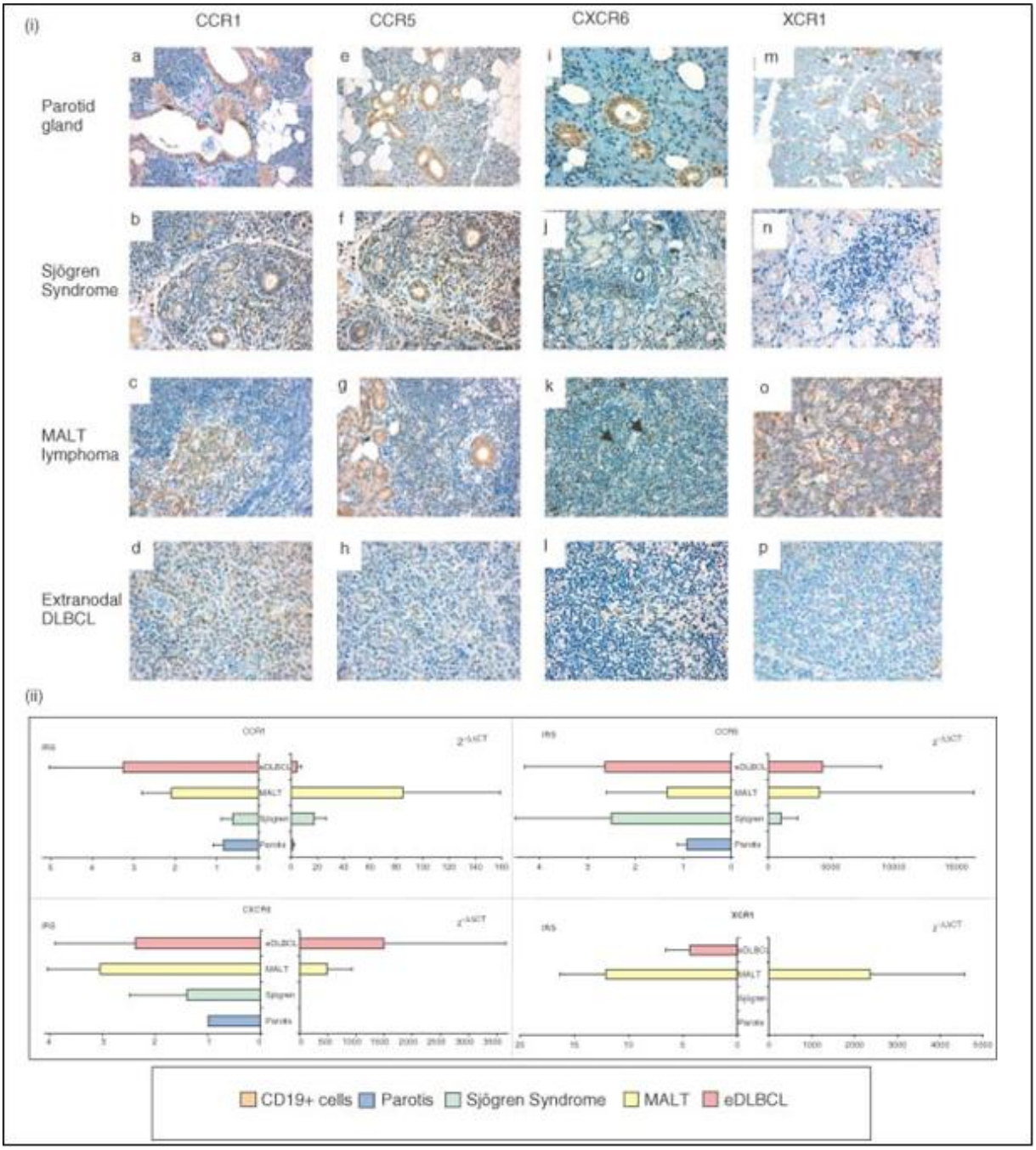

Figure 4: **Immohistochemistry of CCR1, CCR5, CXCR6 and XCR1 in parotid glands, parotid glands affected by Sjoegren Syndrome, MALT lymphoma and extranodal DLBCL (i) and their correlation to RNA expression (ii).**

(i) a, e, i and m: immunohistochemistry for CCR1, CCR5, CXCR6 and XCR1 in non neoplastic parotid glands demonstrating expression of CCR1, CCR5 and CXCR6 on excretory ducts cells.

b, f, j and n: in parotid glands affected by Sjoegren Syndrome inflammatory T cells stain also positive for CCR1, CCR5 and CXCR6.

c, g, k and o: extragastric MALT lymphoma; all four receptors were expressed at varying degrees at MALT lymphomas cells. It is worth mentioning that blasts in the lymphomas showed a stronger CXCR6 expression (indicated by arrows).

d, h, l and p: extranodal DLBCL: All analyzed eDLBCLs moderately expressed CCR1 and XCR1 on approximately half of the malignant blasts and a strong CXCR6 expression on approximately 14% of lymphoma cells.

All immunohistochemical pictures were kindly provided by Prof. Beham-Schmid.

(ii) RNA expression levels of chemokine receptors CCR1, CCR5, CXCR6 and XCR1 are presented as mean values ± standard deviation on the right and at protein levels on the left; comparing protein levels to mRNA expression, a significant positive correlation for CXCR6 and XCR1 could be observed.

Comparing the protein expression levels of these four receptors, CCR1 immunohistochemical reactivity was 3.3 times higher in extragastric MALT lymphomas compared to pSSs and 1.5 times higher in eDLBCLs compared to extragastric MALT lymphomas. CCR5 was 2.7 times higher expressed in pSSs than in non neoplastic parotid glands and 2 times higher in eDLBCLs than in extragastric MALT lymphomas. CXCR6 immunohistochemical reactivity was 3 times higher in extragastric MALT lymphomas than in pSSs (table 3). XCR1 was 3 times weaker expressed in eDLBCL compared to extragastric MALT lymphoma.

Chemokine receptors in the model of the MALT lymphomagenesis of the parotid gland

In the model of parotid MALT lymphoma genesis significant deregulations of CCR6, CCR7, CXCR3 and CXCR5, which are important chemokine receptors for the B cell development and B cell homing to secondary lymphoid tissues, [26-28] were detected ($p<0.001$). Moreover, five activation dependent chemokine receptors, CCR1, CCR5, CCR8, CCR10 and CXCR6, and CX3CR1 and XCR1 were also found to be significantly deregulated ($p<0.001$) (figure 5).

During the development of Sjoegren Syndrome significant mRNA up-regulation of CCR1 and CCR5, together with *de novo* expression of CCR6, CCR8 and CCR10, all representing inflammation dependent chemokine receptors, occurred ($p<0.001$). Significant up-regulation of CCR5 could be confirmed by immunohistochemistry as demonstrated in table 2 and figure 4 (ii).

During development of parotid MALT lymphoma from pSS to CCR7, CXCR3 and CXCR5, important B cell homing factors [26-28], as well as CXCR6, CX3CR1 and XCR1 were *de novo* expressed ($p<0.001$). CCR1 was two times up regulated and CCR10, an inflammation dependent chemokine receptor, lost its expression ($p<0.001$). Transformation of MALT lymphoma to extranodal DLBCL was accompanied by a 13 times down-regulation of 2 inflammation dependent chemokine receptors, CCR1 and CCR8 and of the B cell homing chemokine receptor CXCR3 ($p<0.006$). For CX3CR1 and XCR1 no transcripts were found in extranodal DLBCL ($p<0.001$).

CXCL16 mRNA, the ligand of CXCR6 [38] was expressed at the similar level in all non neoplastic parotid glands, in all parotid glands affected by Sjoegren Syndrome and in all parotid MALT lymphomas. However, CXCL16 expression of extranodal DLBCLs was significantly down regulated ($p=0.001$) compared to parotid MALT lymphoma (data not shown).

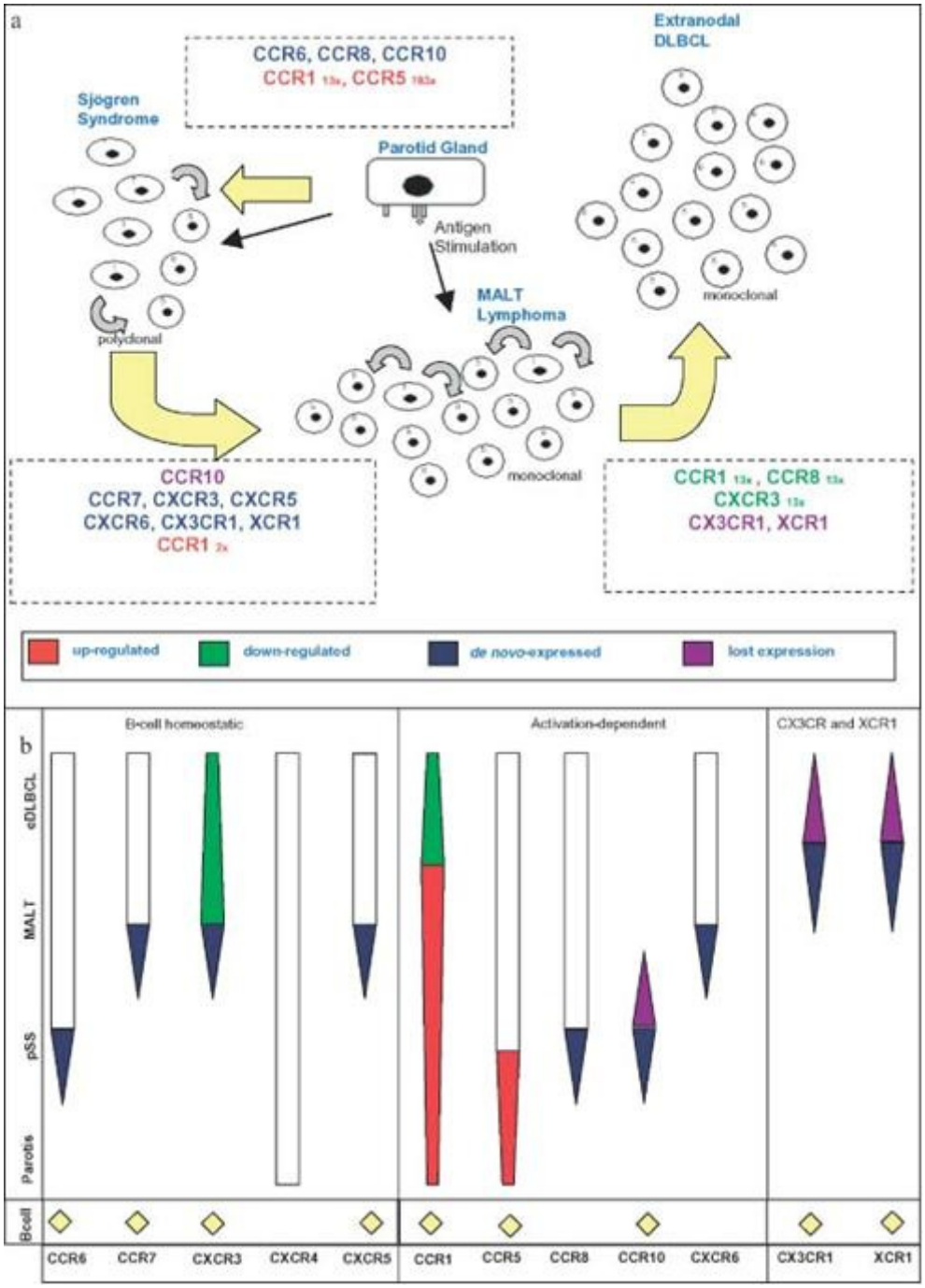

Figure 5: **Overview of significant changes in the chemokine receptor expression profile during the development of parotid MALT lymphoma for 19 chemokine receptors at mRNA levels.**
Chemokine receptors in red represent up-regulation, green receptor down-regulation, blue de novo expression and purple loss of expression.

a In the development of pSS CCR1 and CCR5 were up regulated and CCR6, CCR8 and CCR10 were *de novo* expressed; progression to parotid MALT led to CCR7, CXCR3, CXCR4, CXCR5, CXCR6, CX3CR1 and XCR1 *de novo* expression and loss of CCR10; during the transformation of MALT to eDLBCL, CCR1, CCR8 and CXCR3 were downregulated and loss of CX3CR1 and XCR1 expression (numbers indicate magnitude of changes).

b Graphical illustration of chemokine receptor expression divided into B cell homeostastic, activation dependent and CX3CR and XCR1 chemokine receptors. Yellow triangles depict expression of chemokine receptors in pCD19+ cells.

Discussion

ASHM in PCMZLs

In PCMZL molecular analyses show a number of distinct cytogenetic alterations in less than 50% of cases [39], but a molecular mechanism inducing genetic instability has not yet been described. We show that 72 % of PCMZL lymphoma samples show point mutations in at least one of the four analyzed proto-oncogenes. Whereas only a few PCMZL cases were mutated at the loci of *PAX5*, nearly 46% of cutaneous MALT lymphoma cases harboured mutations in the *PIM1* gene and more than 36% in the *cMYC* gene locus.

Several features of the molecular profile of mutations - like predominance of GC over AT mutations, elevated transition over transversion ratio, frequency of mutations and their distribution 1-2 kb downstream of the transcription initiation site- are similar to that detected in other lymphoma entities, and closely resemble the one reported for *IgV* and *BCL6*-mutations [11,12,15,40-44] indicating aberrant somatic hypermutation to be responsible for these nucleotide alterations. Further, mutations at these 4 loci have not been found in normal germinal centre B cells in DLBCL [11] or in inflammatory B cells from *Borrelia burgdorferi* infected skin lesions supporting the hypothesis that these mutations are tumor specific and result from an abnormal activity of the SHM mechanism. Besides primary cutaneous follicle centre lymphomas (PCFCL) [16], PCMZLs are the second cutaneous lymphoma entity with indolent clinical course to be aberrantly targeted in these 4 proto-oncogenes. However, although the mutational load analyzed for all genes is higher in PCFCL (mutation frequency 0.1 versuss 0.07 per 100 bp) compared to PCMZL more PCMZL cases (72% versus 53%) are affected by ASHM.

Because the genes targeted by ASHM are proto-oncogenes, introducing somatic mutations may have important functional consequences. First, mutations clustering around the 5´ regulatory regions may deregulate gene transcription as previously documented for *cMYC* [45]. Second, in the case of *cMYC* and *PIM1* in which also coding sequences are targeted causing amino acid substitutions, the mutations may alter structure and, subsequently, the function of these molecules. Mutations affecting the *cMYC* transactivation domain can alter the functional properties of *cMYC* by interfering with its phosphorylation, protein stability or repression of transactivation by the RB-related protein p107 [45,46].

In diffuse large B-cell lymphoma 17 out of 130 (13%) investigated genes were found involved in ASHM (Pasqualucci *et al* 2004), suggesting that a much higher number of genes might be affected by this mechanism and that AID-mediated mutations outside the *IgV* regions are a genome-wide phenomenon [47]. Although the mutational profile of *PAX5, RhoH/TTF, cMYC and*

PIM1 of PCMZL is similar to the mutational profile of extracutaneous MALT lymphomas [12] a different targeting of additional genes by ASHM might contribute to the different biological behaviour between PCMZL and extracutaneous MALT lymphomas [5-8].

Recently, it has been demonstrated that *Helicobacter pylori* infection - a risk factor for the development of gastric cancer- triggers aberrant expression of AID in gastric epithelium and H. pylori-mediated upregulation of AID resulted in the accumulation of nucleotide alterations in the TP53 tumor suppressor gene (Matsumoto. *et al* 2007). Since PCR analyses show the presence of *Borrelia burgdorferi* infection in roughly 20% of cases of PCMZL [39], it is tempting to speculate whether a similar mechanism is responsible for some of the mutations observed in our study.

In conclusion, we demonstrate that in the majority of PCMZLs the proto-oncogenes *PAX5, RhoH/TTF, c-MYC* and *PIM1* are targeted by aberrant somatic hypermutation. These molecular changes may be of functional relevance in the development of PCMZLs. By mutating regulatory and coding sequences of the targeted genes ASHM may represent a major contributor to their pathogenesis.

Chemokine receptor expression profiling of MALT lymphomas and eDLBCLs

There is ample evidence that chemokine receptor expression is induced or modulated through factors imposed by the microenvironment like hypoxia and growth factors, or regulated through epigenetic modifications [48-51]. Expression of chemokine receptors or their ligands is advantageous to lymphoma cells in many aspects of their biology as they promote survival and stimulate cell motility [52-56]. The composition of the immune response is also dependent on the complex interactions of the chemokine network [57,58].

We report about a restricted pattern of expression, employing a semi quantitative real time PCR approach and identified some differentially regulated receptors, which may be interesting candidates for further investigations. Selective expression of certain chemokine receptors in extragastric MALT lymphoma and eDLBCL (=transformed MALT lymphoma still exhibiting a low grade component), like CCR6, CCR7, CXCR3, CXCR4 and CXCR5 are responsible for B cell homing to secondary lymphoid tissue. These chemokine receptors have already been identified in MALT lymphoma - and DLBCL cells likewise [20-22,59-63]. In our study CXCR1 and CXCR2 were significantly higher expressed in gastric MALT lymphomas in comparison to extragastric MALT lymphomas. Both receptors are activated by binding CXCL8, CXCL1 and CXCL2 [64]. It was reported that *H. pylori* positive gastritis, the benign precursor lesion of gastric

MALT lymphoma, induces CXCL1 and CXCL8 [65,66]. In accordance, these two receptors are highly expressed in gastric MALT lymphoma but not in extragastric MALT lymphomas. CX3CR1 and CCR8 were significantly higher expressed and XCR1was *de novo* expressed in extragastric MALT lymphomas in comparison to MALT lymphoma of gastric origin. Also in immunohistochemical analysis XCR1 was significantly higher expressed in extragastric MALT lymphomas. All three chemokine receptors are usually expressed on T cells [67-69], and XCR1 also on B lymphocytes [37]. Receptor activation by their ligands is able to induce cell proliferation and survival pathways [70-72]. Although we cannot entirely exclude that the expression pattern might be influenced by the presence of intratumoral T cells in the lymphoma tissues, we hypothesize that activation of these receptors might enhance proliferation and survival of malignant B cells in extragastric MALT lymphoma.

In MALT lymphomas exhibiting trisomy 3, transcripts of CCR4 were found to be significantly higher expressed compared to trisomy 3 negative MALT lymphomas. CCR4 is usually expressed on tonsillar B cells and on IL-2 stimulated T cells [73] and correlates with advanced disease stage and poor prognosis [74]. We hypothesize that the aberrant CCR4 expression of MALT lymphoma cells might be responsible for this aggressive clinical behaviour.

In the stepwise progression during parotid MALT lymphomagenesis deregulation of four out of 5 B cell homeostatic and of five inflammation dependent chemokine receptor occurs accompanied by deregulation of XCR1 and CX3CR1.

In pSS CCL19, CCL18, CXCL9, CXCL10 (IP10), and especially CXCL13 (BCA-1) - a B cell attracting chemokine described as transformation marker from pSS to parotid MALT lymphoma - were expressed by inflamed glands leading to an accumulation of TH1 cells and B cells [75-79]. Besides mRNA transcripts for chemokine receptors of TH1 cells [80], we found expression of CCR1, CCR4, CCR6, CCR8 and CCR10 receptors known to be expressed on different subsets of T cells but have not yet been described to be expressed in pSS [67,73,81,82].

During the development of parotid MALT lymphomas from pSS CCR1 mRNA levels was up-regulated whereas CCR5 mRNA expression did not change. Immunohistochemistry underscored the de novo expression of CCR1 and CCR5 on extragastric MALT lymphoma cells in comparison to marginal B cells of healthy donors. CCL3, the ligand of CCR1 and CCR5, was observed to induce the highest migration activity on malignant B lymphocytes [83,84] supporting our observation that parotid MALT lymphoma cells express functional CCR1 and CCR5. In the development of parotid MALT lymphomas *de novo* expression of CCR7, CXCR3 and CXCR5, important homing factors for B cells [26-28], was detected. It was reported that CCL19 together with CCR7 lead to activation of prosurvival pathways in head and neck cancer cells [85]. Thus, the

expression of CCR7 on the surface of extragastric MALT lymphoma cells together with the reported production of CCL19 in pSS [78] may induce prosurvival signals in lymphoma cells.

CXCR6 is expressed on a subset of T cells, natural killer T cells and B cells [86-89]. CXCL16 treatment of CXCR6+ cells was reported to induce proliferation through NF-kappa B activation [90,91]. Previous studies showed that CXCR6 has an impact on metastasis of nasopharyngeal cancer and melanomas [32,92]. Immunohistochemistry showed occurrence of CXCR6 on extragastric MALT lymphoma cells with strongest expression on lymphatic blasts. Thus, a complex interaction between different chemokine receptors might be responsible for the attraction of MALT lymphoma cells to parotid glands affected by Sjoegren Syndrome and may also facilitate proliferation and possibly progression of MALT lymphoma to eDLBCL.

During transformation of MALT lymphomas to eDLBCL the expression of CCR8, an inflammation dependent chemokine receptor, together with the B cell homing chemokine CXCR3 became weaker, CX3CR1 and XCR1 lost their expression at mRNA levels and XCR1 protein was also reduced. Increased protein expression of CCR1 on eDLBCL cells by immunohistochemical analyses is consistent with a previous report of Mahadeva showing expression of CCR1 and its ligand CCL5 in nodal DLBCL led to a potential autocrine stimulus for replication [93].

In summary, our data suggest that CCR8, CXCR1, CXCR2, CX3CR1 and XCR1 play an essential role in the site of origin of MALT lymphoma. The development of extragastric MALT lymphoma is associated with increased expression of the inflammation dependent chemokine receptors CCR1, CCR8 and CXCR6 and additionally of CX3CR1 and XCR1. The high grade transformation process to eDLBCL is accompanied by increased CCR1 expression and loss of CX3CR1 and XCR1. Thus, a complex of dynamic interacting signals in the progression from a non-neoplastic event to Sjoegren Syndrome, or MALT lymphoma and finally to overt eDLBCL mediated by various chemokine receptors and their ligands indicate a pathogenetic role in MALT lymphomagenesis and seems to determine the origin and fate of these lymphomas.

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Curriculum Vitae

Personal Data

Name:	Alexander Deutsch
Date / Place of Birth:	May 17th 1979, Graz, Austria
Citizenship:	Austria
Steady Address:	Am Arlandgrund 1/1/8, 8045 Graz, Austria,
Email:	*alexander.deutsch@meduni-graz.at*

Education

Since 06/2007	***Medical University Graz, Austria*** Doctoral thesis at the Department of clinical Hematology
05/2005 – 01/2007	***Karl Franzens University Graz, Austria*** Master thesis of molecular microbiology at the Department of clinical Hematology Master Thesis: Molecular Etiopathogenesis of extragastric MALT lymphoma
03/2001 – 05/2005	***Karl Franzens University Graz, Austria.*** Bachelor degree
10/1997 – 09/2000	***Academy for Medical Technician Graz, Austria***

Work Experience

Since 06/2007	**Activities during doctoral thesis at Medical University Graz** *Participation in different research projects of Univ. Prof. Dr. Peter Neumeister at the Department of clinical Hematology*
09/2004 – 01/2007	**Activities during master thesis at *Medical University Graz*** *Participation in different research projects of Univ. Prof. Dr. Peter Neumeister at the Department of clinical Hematology*
06/2003 – 08/2004	**LIFECORD/ECCOCELL Graz** *Technician*
05/2002 – 06/2003	**ORIDIS BIOMED Graz** *Technician*
10/2000 – 04/2003	**Institute of Pathology, MedicalUniversity Graz** *Technician in research projects of Univ. Prof. Dr. Ratschek, Univ. Prof. Dr. Moinfar and Univ. Prof. Dr. Lax*

Awards

2008:	**Sanofi Aventis Prize**
2007:	**Carl von Rokitansky-Prize**

Publications

- Deutsch, AJ; Frühwirth, M; Aigelsreiter, A; Cerroni, L; Neumeister, P (2008): **Primary Cutaneous Marginal Zone B-Cell Lymphomas Are Targeted by Aberrant Somatic Hypermutation.**
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Conference Proceedings and Abstracts

Posters

- ***„Chemokine receptor expression in nongastric mucosa-associated lymphoid tissue (MALT)-type lymphoma****“ A Aigelsreiter, A Deutsch, M Stefan, C Beham-Schmid, A Beham, G Lanzer, E Stelzl, W Linkesch , H Schaider , and P Neumeister.* Eurpean Congress of Patholgy in Paris 2005

- ***"Analysis of 16 chemokine receptors in Sjögren's Syndrome and nongastric mucosa-associated lympoid tissue (MALT)-type lymphomas of the parotid gland"*** *A Deutsch, A Aigelsreiter, A Barounig, C Beham-Schmid, A Beham, W Linkesch, H Schaider, P Neumeister.* Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaften für Hämatologie und Onkologie in Hannover 2005
- ***"Association between Chlamydia psittaci infection and extranodal marginal zone B-cell lymphoma of mucosa associated lymphoid tissue (MALT)-lymphomas."*** *A. Aigelsreiter, E. Stelzl, A. Deutsch, C. Beham-Schmid, A. Beham, G. Lanzer, W. Linkesch, H. Kessler, P. Neumeister* American Society of Clinical Oncology annual meeting in Atlanta 2006
- ***"Extranodal marginal Zone Be-cell lymphoma of mucosa associated lymphoid tissue (MALT-)-lymphomas and Sjögren's Syndrome are associated with Chlamydia psittaci infection"*** *A. Aigelsreiter, E. Stelzl, E. Leitner, A. Deutsch, M. Frühwirth, C. Beham-Schmid, A. Beham, W. Linkesch, N. Borel, A. Pospischil, H. Kessler, P. Neumeister* Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaft für Hämatologie und Onkologie in Leipzig 2006

- ***"Aberrant Somatic Hypermutation of Follicular Lymphoma Transformed to Diffuse Large B-Cell Lymphoma"*** *Margareta Frühwirth, Alexander JA Deutsch, Philipp B Staber, Ariane Aigelsreiter, Werner Linkesch, Christine Beham-Schmid, Peter Neumeister* American Society of Hematology 48th annual meeting in Orlando 2006

- **"*Chemokine receptor expression profile in the model of parotid MALT lymphomagenesis: De novo expression of CXCR6"*** *Deutsch, AJA; Aigelsreiter, A; Steinbauer, E; Linkesch, W; Beham-Schmid, C; Schaider, H; Neumeister, P*
  American Society of Hematology 49th Annual Meeting in Atlanta, 2007

Oral presntations

- ***„Aberrant Somatic Hypermutation in Extranodal Marginal Zone B-Cell Lymphoma of MALT Type"*** *A Deutsch, A Aigelsreiter, C Beham-Schmid, A Beham, W Linkesch, P Neumeister*
  American Society of Hematology 47th annual meeting in Atlanta 2005

- ***"*Extranodal marginal zone B-cell lymphomas of MALT type are targeted by aberrant somatic hypermution"** *A Deutsch, A Aigelsreiter, C Beham-Schmid, A Beham, W Linkesch, P Neumeister*
  Gemeinsame Jahrestagung der Deutschen, Österreichischen und Schweizerischen Gesellschaft für Hämatologie und Onkologie in Leipzig 2006

Supplements

Supplemental Table S1: Primer-sequences for *cMyc, Pim1, PAX5* and *RhoH/TTF*

Gene		Primer sequence	PCR product, bp
c-MYC	m1	CCTCTCTCGCTAATCTCCGCC	242
	m1	CTGGAATTACTACAGCGAGTTAGA	
	m2	AAAGAACGGAGGGAGGGATCG	253
	m2	TCGTGGATGCGGCAAGGGTTG	
	m3	TTCGCCTCTGGCCCAGCC	171
	m3	CCCAAATGGGCAGAATAGCCTCC	
	m4	CGAGCAAGGACGCGACTC	261
	m4	AAAGTGTCAATAGCGCAGGAA	
	m5	GCTGAGATGAGTCGAATGCCTAA	312
	m5	GCCCACCGCAAAGCAACCC	
	m6	GGAGAGGAGAAGGCAGAGGGAAA	304
	m6	CTGTCAGAAATGCGGTGAGCCG	
	m7	GCAGCCGCTGACTTGTCCC	205
	m7	TAACACCTCTAGAGACCCAGGTT	
	m8	ATTCCAACCCGCCCTGATCCTTT	237
	m8	GAGGACACCCGAGGGCGG	
	m9a	CTTTAACTCAAGACTGCCTCCCG	168
	m9a	TGCTGCTGCTGCTGGTAGAAGTT	
	m9b	TCGGTGCAGCCGTATTTCTACTG	160
	m9b	AGCAGAGCCCGGAGCGGC	
	m10	TCGAGCTGCTGCCCACCC	182
	m10	CTGGTTCACCATGTCTCCTCCCA	
	m11	TCTCCACGGCCGACCAGC	255
	m11	GGTACAAGCTGGAGGTGGAGCA	
	m12	CGCCGCCAAGCTCGTCTC	203
	m12	AGGACTTGGGCGAGCTGCTG	
PIM1	p1a	ACAGCCGCAACGCCACCC	168
	p1a	GCAGGTGGGCAAGCGAGTTGATT	
	p1b	CTGCGCCGACATCCTGGAG	162
	p1b	AGGAGATCCCGCCGCCGCC	
	p2a	CGCCCTGCAACGACCTGC	189
	p2a	CTGGTACTGCGACTCCAGGG	
	p2b	GCGGGCCGGCACTGAGTC	207
	p2b	AAAGCACGCCCGTGCGCC	
	p3a	GGCTCGGTCTACTCAGGCATC	185
	p3a	CCAGTCGGAAATCCGGTCCTTCT	
	p3b	CCTGGGCTTCCAGGTGGC	136
	p3b	GTTAGGGCGGGAGTCGGG	
	p4a	GGACCGGATTTCCGACTGGG	169
	p4a	CACCTTCTTCAGCAGGACCACTT	
	p4b	CCCTGCAGCCTAATGGCACTC	162
	p4b	AGTCGAAGAGATCTTGCACCGGC	
	p5a	GAGAGGCCCGACAGTTTCGTC	172
	p5a	TGTCGCGGTGGAGCACCC	
	p5b	AGCTTCTTCTGGCAGGTGCTGG	193
	p5b	TCCCTCCCGGGCCTGGCT	

PAX5	5_1a	GTTGGAGAGAGGCGGAGGG	175
	5_1a	AATCGGCCCTTGACTTCACTCAG	
	5_1b	GGAGGGACCTCAGAAGCATCG	148
	5_1b	TCCGGGACAGCCAGCAGC	
	5_2a	GCCTCCTCAAAGCTGCTCCTTC	147
	5_2a	CTCCCTGGCTGGCTCAAGCA	
	5_2b	CCTTGGCCGCAGCTACCC	149
	5_2b	TGCTGGAGACCGCCCGGA	
	5_3a	CCGGGTGCGCTAGCACCAT	156
	5_3a	GCCCAGGGCGGATAGGGA	
	5_3b	CTGAGCCCAGCTCGCACAGT	136
	5_3b	GTGCTTACAGTGTATTTCCATCGGG	
	5_4a	GCTCCCTATCCGCCCTGG	193
	5_4a	CTGACCCACGGTCCGGCA	
	5_4b	GCATAGTAAGTAGGCGGCGAGC	149
	5_4b	CCTGGGATCCCTGCACTTTGC	
	5_5	GTCAGGTACTAAGGCCGGAAGGA	224
	5_5	GTCCAAGCCAGGGTTCTCCGA	
RhoH/TTF	R1	GGCATTCTGCAACAGGTAAGGAT	287
	R1	GCATATGCATTCTCTAGAATATCTG	
	R2	CCTCACCACAGTCCTAACAAGAT	261
	R2	GAAATTCCAGATTGAAATCTACATCA	
	R3	GCTCTAAGTTGTGCAAATATGAAT	244
	R3	AGGCTACTGACTGGTACCTC	
	R4	CTTCTGAGGTGGTTTGATTTGGA	247
	R4	CACAACTACCAGATTATAGGGTCC	

Supplementary Table S2: Nucleotide acid sequence of primers and probes, used in the real-time PCR

Gene	Primer forward	Primer reverse	Probe
CCR1	CACGGACAAAGTCCCTTGGA	TGTGGTCGTGTCATAGTCCTCTGT	TGGAGTTTCCATCCCGGCTTCTCTCT
CCR2	GATGAATGGGAGTGAGGGATAGTG	GAGCCCTTTGCTTCACCTTTG	TTTGTCCAGGCTCAGCCATGCTCA
CCR3	AAAGCTGATACCAGAGCACTGATG	GTCATAATTCGGAGCCTCCTGTA	CCAAGAGGCCCACAGTGAACACCAG
CCR4	TAATATTGCAAGGCAAAGACTATTCC	GCGATTTACTCCATCAGCCAGTA	TGCAATTCCCTCTGGAGAAACCCATCA
CCR5	GATTGATTTGCACAGCTCATCTG	TGTCATAGATTGGACTTGACACTTGA	TCTCCCCGGGTGGAACAAGATGG
CCR6	CAGAGCACTGCCTGAGAGTCAC	TGGTTGTAGAAAAAGGAGTGTATGGT	TTCCTGCTACCGCTGCCTGTGAGC
CCR7	GGGCACAGCCTTCCTGTG	CCACCACCAGCACGCTTT	ACCGCCCAGAGAGCGTCATGGA
CCR8	GAAGAAGGAATTGGCAACACTGA	CACTGAGGTCAAGTGTATAATCCATCA	AGAACAAAGGCTGTCACTAAGGTCCCGCT
CCR9	GACTTCACAAGCCCTATTCCTAACA	AAGTCAAGTGAAGTTGAAGTTAACGTAGTCT	ACTATGGCTCTGAATCCACATCTTCCATGG
CCR10	GGAGGCCACAGAAGCGGTT	GGACATCGGCCTTGTAGCAA	AGGACGGCATACTCGGCTGAGCCACT
CXCR1	GCAGCTCCTACTGTTGGACACA	CATGTCCTCTTCAGTTTCAGCAA	CTGGCCGGTGCTTCAGTTAGATCAAACC
CXCR2	GCAGGTCACAGCTGCTCTTCT	TTTTTGAGGTAAACTTAAATCCTGACTG	TGTCCCCTACAGGTGAAAAGCCCAGCGA
CXCR3	ACCCAGCAGCCAGAGCAC	CATAGGAAGAGCTGAAGTTCTCCAG	CCACCAAGTGCTAAATGACGCCGAG
CXCR4	CAATGACTTGTGGGTGGTTGTG	ATGCAATAGCAGGACAGGATGA	CATGGTTGGCCTTATCCTGCCTGGTA
CXCR5	CAGCCATGAACTACCCGCTAA	CCAATCTGTCCAGTTCCCAGA	AGGTCCTCCAGGTTCTCGAGGTCCATT
CXCR6	TGGAACAAACTGGCAAAGCAT	CTGAACCCATAGTCTTCATGGTAATC	CATCAGAAGCAGACACCATGGCATGGCAGAG CAT
CXCR7	TGCAAAGTGCTCAGCACTAAGG	AGTAGTCGAAGAGATGCAGATCCA	CAGCCCGGAGGTCATTTGATTGCC
CX3R1	TGACTGGCAGATCCAGAGGTT	TTCTGTCACTGATTCAGGGAACTG	AGTCCACGCCAGGCCTTCACCA
XCR1	CCATCGTGGTGGCCTACTTC	CGCAGCTCCGGATGATCT	TCTGCAGACGCTGTTTCGGACCC
CXCL16	GCTTGTGTGGSGGCAACA	TCTGGTGGGCCACAATCC	TCAAGACAGCTCAATCAATTCCTGAACCCA
XCL1	CCCATTCTGAGGGTCCACTACT	ATGGGACAAAAAAGGGACAAGT	TGCTTCACTCAATTTTTTTCACCTCTTTGTGT
XCL2	TCAGCAGCAGTAAAGAGGAATGTCT	CCGATGGAGGCCCAGAAAATG	CAGCTAATTCAGGAGAAATG