From the tumor cell to the cure of Hodgkin's Disease

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In 1832 Thomas Hodgkin was not sure, whether the tumours, he observed macroscopically, were reactive, infectious or malignant proliferations. At the beginning of the 20th century Sternberg and Reed described for the first time the peculiar cells, which determined thereafter the cellular face of this entity. In 1978 the Reed Sternberg Hodgkin cells were for the first time isolated and cultured in vitro to open the exiting era of research, that elucidated the character and nature of the cell and gave inside views on the intertalk between the tumour cell and its environment. Only in 1992 it became evident that the Sternberg Reed cells were genetically crippled mononuclear germinal center B-lymphocytes, unable to produce immunoglobulins and undergo apoptosis. There are great expectations for a broader and deeper understanding of the molecular mechanisms, which inhibit programmed cell death and more over the search has started to detect the "master-switch" for malignant transformation by new micro-array techniques and gene biotechnology.

Parallel to this fascinating events in the laboratory the fate of patients diagnosed with Hodgkin's disease experienced a remarkable advent of increasingly curative treatment options with decreasing short- and long term toxicity. Firstly, new radiation qualities and techniques in the sixties of the last century offered high cure rates in localised stages of HD but only the development of the MOPP poly chemo therapy opened a new avenue for cure, even for advanced stages.

A rather frustrating lag phase of stagnation came during the end of the last century, when ABVD, alternating MOPP/ABVD and hybrid constructs of these drug combinations did not add to tumour free survival and cure rates in advanced stages.

Only recently, new principals of tailoring the old combinations in a time and dose escalated schedule offered new hope not only for better tumour control but also for higher survival rates even in the worst prognostic groups. Furthermore, more sensitive diagnostic tools like PET might open the possibility of response adapted therapy, decreasing possible over treatment with the chance of unwanted sequelles.

Radio- immune therapy trials have begun to target antigens and molecules in the hope to open up a new era of selective and hopefully less harmful treatment for this young group of patients with the chance of getting again a normal life expectancy.
Oral Presentations
1. Molecular Heterogeneity and New Treatments in CLL

**CHRONIC LYMPHOCYTIC LEUKEMIA: CHALLENGING PARADIGMS**
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Important progress has recently been made in the diagnosis, prognostic assessment, and treatment of chronic lymphocytic leukemia (CLL), thus challenging classical paradigms in this disease. Concerning diagnosis, the immunophenotype of the neoplastic cells is a key-feature that allows the diagnosis of CLL and its separation from other chronic lymphoproliferative disorders. The study of the genomic signature of CLL indicates that this is a single disease with different clinical forms. Among staging maneuvers, it is likely that imaging studies (e.g., CT scans) will find a place in the evaluation of lymphnode territories involved in the disease. The assessment of prognostic in patients with CLL has been revolutionized. Thus, besides classical clinical parameters a number of biological features (i.e., CD38 expression on neoplastic cells, serum markers such as b2-microglobulin, thyminid kinase, and soluble CD38, as well as cytogenetic abnormalities, and IgV mutational status) have shown to correlate with prognosis and to add prognostic value to Rai's and Binet's clinical stages. In addition, progressive and smoldering forms of the disease can now be separated more accurately.

The treatment of the disease has also dramatically changed, from an approach primarily based on a primum non nocere concept to risk-adapted treatments. Experimental therapies such as purine analogs combined with other cytotoxic agents and monoclonal antibodies, stem cell transplants (including non-myeloablative allotransplants), and immunotherapy offer hope for a longer survival and even the cure of the disease in a fraction of cases. As a result of all these advances the median survival of patients with CLL has increased from 5-6 years in series reported two decades ago to about 10 years at present, and will hopefully continue increasing in the future.

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**CLL Clinical Forms Detected by Genetics**
H. Döhner

Important aspects of CLL biology have been elucidated which relate to its stage of differentiation and to its transforming events. There are two variants of CLL arising at different stages of B-cell differentiation as reflected by the mutational status of the immunoglobulin variable (IgV) genes. Furthermore, using the modern molecular cytogenetic techniques genomic aberrations can now be diagnosed in approximately 80% of CLL cases. The genomic regions recurrently affected by chromosomal deletions (6q21, 11q22-23, 13q14, 17p13), trisomies (+3q26, +8q24, +12q13), and, less frequently, by translocations (14q32) contain mostly as yet unknown tumor suppressor genes and oncogenes. Both the IgV mutation status and the pattern of genomic aberrations have been shown to have a high predictive value for disease progression and survival in CLL patients. The prognostic information from these new genetic markers is independent from that obtained by the conventional clinical markers. It is now necessary to investigate these markers prospectively in the large clinical trials of the cooperative study groups. In addition, it will be important to assess the efficacy of specific therapeutic agents in the biologically defined subgroups of CLL.

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**Automated Genomic Profiling for Recurrent Chromosomal Aberrations in B-CLL using Microarray Based Comparative Genomic Hybridization (Matrix-CGH)**
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Molecular cytogenetic analyses of chronic lymphocytic leukemia of B-cell type (B-CLL) has significantly contributed to the identification of genomic imbalances with prognostic relevance. Screening for such genomic gains and losses in a single experiment can be achieved by a recently developed microarray based technique, termed Matrix-CGH. In this approach, comparative genomic hybridization is applied to defined DNA targets, spotted on glass slides, allowing a fully automated evaluation of copy number changes of hundreds of defined regions. Initially, we constructed a B-cell lymphoma chip, containing 495 PAC- and BAC-DNA fragments. PACs and BACs were selected for frequently altered chromosomal regions in CLL (3q26, 6q21, 8q24, 10q24, 11q22-23, 12q13, 13q14, 17p13, 19q13), as well as for 60 oncogenes and 29 tumor suppressor genes. DNA samples of 20 CLL patients were analyzed and the results were compared to data obtained by Fluorescence in Situ Hybridization (FISH). In 17/20 patients (85%) all aberrations revealed by FISH were confirmed by Matrix-CGH. In one patient additional genomic aberrations were identified on 11q22, 7q31 and 2q23. To enhance the sensitivity of our microarray we have increased the number of targeted DNA fragments per CLL specific region. At present, we evaluate the sensitivity achieved by this optimization in a series of B-CLL patients in a blind fashion. The current status of this study will be presented. Our results underline the potential of Matrix-CGH as a powerful technique for the simultaneous identification of chromosomal prognostic markers in a single experiment. Automated analyses in B-CLL patients for a subclassification resulting in risk-adapted therapy decisions can be envisioned.

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**CLL: A SINGLE DISEASE WITH CLINICALLY IMPORTANT MOLECULAR SUBTYPES**
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Some CLL patients have a progressive course requiring early treatment whereas other patients have a relatively stable course requiring treatment only after many years, if at all. Recently, the presence of somatic mutations in the immunoglobulin (Ig) genes of CLL leukemic cells was associated with the more indolent form of CLL whereas the absence of Ig gene mutations was associated with the progressive form of the disease. This observation raised the hypothesis that CLL may represent more than one disease entity. This hypothesis was tested by gene expression profiling of purified CLL cells from untreated patients. All cases, irrespective of the Ig mutational status, shared a common gene expression signature that distinguished CLL from other normal and malignant B-cell types. This finding suggests that all cases of CLL may share a common mechanism of oncogenic transformation and/or arise from a common normal precursor cell type, and support a view of CLL as a single disease. The CLL signature genes included known CLL markers such as CD5, but also many unexpected genes such as War-1. Since War-1 can promote the proliferation of B cell precursors, its potential functional role in CLL should be investigated. The CLL signature genes also included genes encoding cell surface proteins such as Bort and MIRC-02X that might be targeted for monoclonal antibody therapy.

Despite the overall similarity of all CLL cases, Ig-somatized CLL and Ig-mutated CLL differed in the expression of several hundred genes. These genes were combined into an Ig mutational status predictor that correctly assigned 96% of the CLL cases. Interestingly, the most predictive gene, ZAP70, encodes a tyrosine kinase that was previously thought to be restricted to expression in T lymphocytes. ZAP70 mRNA was 4-5 fold more highly expressed in Ig-mutated than in Ig-mutated CLL, a finding that can be easily translated into a clinical test that should prove useful in the management of CLL patients.
CELLULAR INTERACTIONS IN CLL
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The capacity of malignant cells to respond to selected microenvironmental signals confers a growth advantage and an extended survival to chronic lymphoid malignancies of B-cell type. CLL, characterized by the absence of B lymphocytes, is a paradigmatic example as malignant cells aggregate in secondary lymphoid organs, bone marrow (BM) and peripheral blood (PB) not only because of genetic lesions but also thanks to their interactions with non-tumoral bystander cells. T lymphocytes and accessory cells, like BM stromal cells and follicular dendritic cells (FDC), appear to play a critical role in the malignant cell/microenvironment crosstalk. CLL cells and CD4+ T cells are involved in a reciprocal dialogue via CD40/CD40L signaling (L) interactions. A link has been established between the CD40/CD40L pathway and the expression and modulation of Survivin, a prominent member of the family of Inhibitor of Apoptosis Proteins (IAP) that integrates apoptosis and proliferation. The expression of Survivin is absent in resting PB CLL cells but can be induced in vitro by CD40 stimulation. Survivin’s cells acquire an extended survival and an increased proliferative rate. In vivo Survivin’s cells are localized in lymph node (LN) pseudofollicles and in BM clusters of proliferating CD5+ B cells that are interspersed with T cells, essentially of the CD4+ type and frequently CD40L+ indicating the in vivo availability of this signal to leukemic cells.

CLL cells purified from involved LN and BM, but not from PB, constitutively express mRNA for the T-cell attracting chemokines CCL17 and CCL22. CD40-crosslinking of PB CLL cells induces the expression of both chemokines at RNA level. CCL22 is also released and is capable of attracting CD4+ CD40L+ T cells. These findings indicate that the stimulation of malignant cells via a physiological signal present in the tumor microenvironment endows CLL cells with the chemotactic attracting capacity for CD4+ T cells, and it is responsible for their survival signals to tumor cells. Further studies aimed to identify which molecular interactions link malignant B cells, T and accessory cells providing the signals important for the extended survival and proliferation of CLL cells. We have led us to focus upon CD190 (recently renamed Semaphorin4D) a transmembrane protein belonging to the fourth group of the semaphorin family and its receptors, CD72 and Plexin-B1.

All these observations lead to the view that CLL cells retain the capacity to respond to proliferative and anti-apoptotic microenvironmental signals provided by bystander cells through cellular contacts. This scenario gives a conceptual framework to the development of new treatment modalities aimed at interrupting the interactions between malignant B cells and the microenvironment.

MOVING TO CELLULAR THERAPY IN THE TREATMENT OF CHRONIC LYMPHOCYTIC LEUKEMIA
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As conventional cytotoxic treatment has only limited efficacy in chronic lymphocytic leukemia (CLL), efforts to develop curative treatment strategies for this disease have focussed on cellular therapy during recent years. Two basic forms of cellular therapy can be distinguished: Whereas in the supportive setting cellular components are merely used to compensate for the toxicity of cytotoxic therapy (i.e. autologous stem cell transplantation (SCT)), in the therapeutic setting antineoplastic activity is mediated by the cellular effectors themselves (i.e. immunotherapy). To date, the only clinically established form of cellular immunotherapy is allogeneic stem cell transplantation. This presentation will summarize background, current status, and perspectives of allogeneic SCT in the treatment of CLL including dose-reduced conditioning approaches. In addition, secondary immunotherapeutic manoeuvres such as antibodies and vaccination strategies will be briefly discussed.

Chemotherapy with Fludarabine, Cyclophosphamide, and Rituximab (FCR) Achieves a High True Complete Remission Rate as Initial Treatment for Chronic Lymphocytic Leukemia (CLL).
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Front-line treatment of CLL with fludarabine (F) achieves complete remission (CR) in 33% of patients. Addition of cyclophosphamide (C) increases the CR rate to 43% and median time to progression is increased by more than a year. Rituximab (R), a humanized mouse monoclonal antibody against CD20, has different mechanisms of actions than cytoxan/fluorouracil and to decrease-related activity in CLL. To further improve the CR rate, R has been combined with FC. Six cycles of FCR (25 mg/m²day and C 250 mg/m²/day on days 2-4 of cycle 1 and days 1-3 of cycle 2 and R 375 mg/m² on day 1 of cycle 1-6) was evaluated as front-line treatment for CLL. Enrolment began July 1999 and to date 135 enrolled patients (91 males, 44 females) can be evaluated for response by NCI-WG criteria. The median patient age was 57 yrs (36-86), WBC is 98.1 Kjöller (18.4-19.7), hemoglobin is 12.1 g/dl (6-18.7), platelet count is 156 Kjöller (17.367) and median 82 μm-mg/kg is 39.9 μg/ml (1.8-16.4) and 50/135 (37%) patients were in Rai stage III or IV at enrolment. Treatment was well tolerated with 100/135 (74%) patients completing 6 cycles and only 6/135 (4%) completing less than 3 cycles of treatment. CR was demonstrated in 90/135 (67%), nodular partial remission in 10/135 (14%), partial remission in 19/135 (14%). Fifty patients experienced fever and chills with first R infusion and a minority of patients experienced hypotension (7%), nausea (5%), or dyspnea (4%). Toxicity related to R infusion was very uncommon with cycles 2-4. Neutropenia (ANC<500) was observed in 31% of cycles, thrombocytopenia, grade 3 or 4 in 6%, major infection (sepsis 1% or pneumonia 2%) in 2% of cycles and minor infection (FUO 5%, herpes 2%, and soft tissue infection 12% of cycles). Molecular remission, defined by polymerase chain reaction (PCR) negativity for IgH, was documented in 30/37 (83%) CR’s tested. 125 of 135 patients are alive with a median follow-up of 27 months. All responders except six remain in remission. Seventeen of the CR patients evaluated by FCR 6-12 months after treatment and 11 remain FCR negative in CR. In conclusion, FCR is well tolerated and produces the highest complete remission rate in front-line treatment of CLL of any other regimen tested thus far. Furthermore, molecular remissions are demonstrated in a significant number of complete responders.

COMBINATION REGIMEN OF CAMPATH-1H AND RITUXIMAB IN LYMPHOID MALIGNANCIES: CLINICAL EFFICACY AND SAFETY
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Monoclonal antibodies (MoAbs) are active in the treatment of lymphocytic malignancies. Although response rates are high, their use as single agents is limited by short response durations and disease recurrence. Combinations of MoAbs with other active agents are therefore investigated. Here, we report results of the safety and efficacy of the combination of CamPATH-1H(C) and Rituximab (R) in patients (pts) with relapsed lymphoid malignancies. R is given at 375 mg/m² i.v. once weekly for 4 weeks and C at an escalating dose of 3, 10, and 30 mg/m² i.v., during week 1 and at 30 mg i.v. on days 3 and 5 of weeks 2, 3, and 4, respectively. All patients received the prophylactic antibiotics TMP/SMX and valacyclovir. The duration of therapy was 4 weeks. Cycles could be repeated if considered beneficial. 38 pts have been enrolled and 31 are evaluable for response (18 CLL, 8 CLL/PL, 2 Richter transformation, 3 mantle cell lymphoma). All pts received prior therapy (Rx) with a median of 4 regimens (range 1-9). Median age 61 yrs (42-79), median WBC 20.7 (4-436), median platelet count 72 (4-542). The median time to progression was 2 months (1-17). Response rates were mostly limited and higher seen in the peripheral blood (96%), followed by the liver (80%), lymph node (52%) and bone marrow (31%). The resistant higher were in rituximab-sensitve pts (7/12 vs. 6/17) and in patients not exposed to prior antibody Rx (5/8 vs. 9/23). Toxicities were mainly infusion-related and were usually not exceeding grade 2 by NCI criteria. 13/32 (40%) pts developed grade 3 neutropenia and 9/32 (28%) pts developed grade 3 thrombocytopenia. Infections occurred in 15 patients and included CMV antigenemia (5 pts), Pneumonia (5), FUO (7) and skin infections (1). The combination of C and R is in as safe as single antibody therapy. Responses are seen in all anatomic compartments indicating encouraging activity of this combination in a heavily pretreated and poor-prognosis group of patients.
ENHANCEMENT OF RITUXIMAB ACTIVITY IN CLL AND INDOLENT LYMPHOMA BY BCL-2 ANTISENSE (GENASENSE)

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Introduction: CLL and indolent lymphomas are characterised by a high level of expression of the Bcl-2 anti-apoptotic protein. Bcl-2 protein blocks the apoptotic death machinery at the mitochondrial level by maintaining the permeability transition pore (PTP) in the closed position. Generally, the response of CLL cells to Rituximab is low and reflects the lower level of expression of CD20 by the malignant lymphocytes. This study aimed at down-regulating the Bcl-2 protein in primary CLL cells by Bcl-2 phosphorodiamidate morpholino oligonucleotide (G3109) (ASO) (Genasense = Genta, USA), and investigating sensitisation to Rituximab and standard chemotherapy.

Method: CD19 selected cells from 18 patients with CLL and 1 lymphoma cell line (SKD-4 and DoHH2) were treated for 72 hours with ASO (0.5-μM), or control sense or nonsense oligonucleotides. Various doses of Rituximab (0-50μg/ml) were added and the cells tested for a further 24 hours. Assessment of Bcl-2 expression, the mitochondrial PTP response (DIOC6) and apoptotic pathway (caspase 3 activity, cychronous C, cell membrane MC540) were then performed by flow analysis. RNA from the cells were hybridised to Affymetrix arrays (Human U95) and protein extracts were analysed by 2D gel electrophoresis and mass spectrometry.

Results: CLL cells treated with Genasense alone showed marked and highly significant apoptotic responses which were dose dependent and maximal at 5μM (G3109, untreated cells 240.0 μM 420.0, 5μM 489.9 μM 0.005). This was more marked than responses seen in lymphoma cell lines, (DoHH2 and SKD4). Control oligonucleotide-treated cells remained unaffected. Cells subsequently treated in combination with Rituximab showed enhanced response in combination with ASO in a dose response relationship (MC540 at 24 hours: Genasense alone 15, ASO alone 40, ASO/Rituximab 100). Array and protein mass confirmed downregulation of Bcl-2.

Summary: Downregulation of Bcl-2 protein by Genasense ASO alone shows apoptotic effect in primary CLL cells in a concentration easily achieved in vivo without toxicity. Genasense enhances the activity of Rituximab in both CLL and lymphoma. Enhanced clinical response in patients with CLL and indolent lymphomas may be achieved with Genasense alone or in combination with current therapies.
2. Waldenström’s Macroglobulinemia

RESULTS OF A MULTICENTER RANDOMIZED STUDY COMPARING THE EFFICACY OF FLUDARABINE (FAMP) TO THAT OF CYCLOPHOSPHAMIDE, DOXORURICIN, AND PREDNISONE (CAP) IN PATIENTS IN FIRST RELAPSE OR PRIMARY RECURRENT DISEASE WITH WALDENSTRÖM’S MACROGLOSSINEMIA (WM).

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Few reports are available on the treatment of patients with WM who develop primary or secondary resistance to alkylating-based regimens. Purine analogs are effective therapy for patients (pts) primarily resistant or relapsing after alkylating agents. We report the results of a multicenter randomized study in 92 pts with WM comparing the efficacy of CAP to that of FAMP (Blood 2001; 98: 2640-44). From December 1993 through December 1999, 92 pts with WM resistant to first-line therapy (42 pts) or in first relapse (50 pts) after alkylating agents were randomized to receive FAMP (25 mg/m2 D1-D5) or CAP (cyclophosphamide 750 mg/m2 D1, doxorubicin 25 mg/m2 D1, prednisone 40 mg/m2 D1-D5) in 39 French centers. Response was evaluated after 6 courses. Response was defined as a sustained decrease in the percentage ratio of IgM greater than 50%, with a greater than 30% reduction in all other involved organ. Forty five pts received CAP and 45 pts received FAMP. Two pts died before the first course of chemotherapy. No statistical differences were observed between both arms concerning the hematologic toxicity and infectious complications. Mucoedema and alopecia occurred significantly more often in pts treated with CAP. Partial responses were obtained in 14 pts (30%) treated with FAMP and 5 pts (11%) treated with CAP (p=0.19). Responses were more durable in pts treated with FAMP (19 months vs 3 months), and the event-free survival rate was significantly higher in this group (p<0.01). Forty-four patients died in the FAMP group and 22 in the CAP group. There was a benefit in terms of quality-adjusted survival in favor of the FAMP group although there was no statistical difference in the overall survival rate in the 2 study arms (Leukemia 2001; 15: 1467-70). In this randomized study, FAMP is more active than CAP as salvage therapy in WM and should be proposed as first-line therapy in a randomized comparison with alkylating agents.

ROLE OF THALIDOMIDE IN WALDENSTRÖM’S MACROGLOSSINEMIA

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Thalidomide is an oral agent with immunomodulatory and antiangiogenic properties which has shown activity in approximately 30% of patients with refractory multiple myeloma. Based on this experience, we performed a phase II study in order to assess the activity of thalidomide in patients with Waldenström’s macroglobulinemia. Twenty patients with WM were treated with thalidomide at a starting dose of 200 mg PO daily with dose escalation in 200 mg increments every 14 days as tolerated to a maximum of 800 mg. Patients’ median age was 74 years and 10 patients were refractory to treatment. On an intention-to-treat basis five of 20 patients (25%) achieved a partial response after treatment. Time to response was short ranging between 0.8 months to 2.8 months. Adverse effects were common and consisted primarily of constipation, somnolence, fatigue and mood changes. Seven patients interrupted thalidomide within 2 months because of intolerance. The median time to progression for all patients was 5 months and the median duration of response was 11 months.

Based on this modest activity of thalidomide we are conducting a study in which previously treated patients with WM are being treated with clarithromycin 500 mg PO twice a day. Twenty 300 mg PO every day for 21 days. Thalidomide 0 mg PO every week. Eight patients have been enrolled and 2 of 5 evaluable patients so far are responding to this treatment. Patient’s accrual is ongoing.

EXTENDED RITUXIMAB THERAPY IS HIGHLY ACTIVE IN LYMPHOPLASTOMENCYTIC LYMPHOMA (WALDENSTRÖM’S MACROGLOSSINEMIA, WM) INDEPENDENT OF COMPLEMENT RESISTANCE ANTIGEN EXPRESSION. Tobias G. Gernaud, C. Kim, D. Orisher A, Preffer F, Freeman A, Fernando D, Ainsley S, Mitisidis C, Mitisidis N, Anderson KC, and Frankel SR, Harvard Medical School, Dana Farber Cancer Institute, Massachusetts General Hospital, Boston MA USA, UCLA Medical Center, Los Angeles CA USA, Karolinska Institute, Stockholm, Sweden. and Greenbaum Cancer Center, Baltimore MD USA.

Recently, we reported that rituximab was active in WM and facilitated hematological recovery (J Immunother 24-272). Moreover, pts who received 28 vs. ≤ 4 infusions of rituximab had greater increase in hematocrit (HCT) and platelets (PLT) (p<0.01). Herein, we examined extended rituximab therapy in WM. 29 pts with a median age of 65 (range 43-90) yrs, and median prior treatments of 1 (range 0-2) received 4 infusions of rituximab (375 mg/m2 weekly), followed by 4 additional infusions of rituximab at week 12, if they demonstrated a response or had stable disease (SD) following first course of therapy. Overall, treatment was well tolerated. One pt died of sepsis felt unrelated to protocol treatment after 4 doses of rituximab. Best overall responses following rituximab for 22 evaluable pts are: partial response (≥ 50% decline in IgM) in 11 pts (50%) and minor response (≥ 25% decline in IgM) in 5 pts (22%). The overall response rate was 72% and 3 (13.6%) pts demonstrated SD. The median time to treatment failure for responding has not been reached with a median follow-up of 12 (range 3-21) 5 months. None of the responding or SD pts have progressed to date. Improvements in hematological function were also observed. Prior to therapy, anemia (HCT ≤30) and thrombocytopenia (PLT ≤100,000) were present in 10 (34.5%) and 8 (27.6%) pts, respectively. Following rituximab therapy, anemia and thrombocytopenia were present in only 1 (4.5%), and 2 (9.0%) of the 22 evaluable pts, respectively. We also examined the role of complement resistance antigen (CD59, CD55, CD46) expression in modulating clinical responses to rituximab, since we and others recently demonstrated that these antigens block rituximab induced complement lysis in vitro (J Immunother 24-263). These studies demonstrated that CD59, CD55, and CD46 expression on tumor cells投行 most WM pts, that their expression as well as level of expression did not correlate with clinical responses, nor significantly change following therapy. These studies therefore demonstrate that extended rituximab therapy is highly active and facilitates hematological recovery in WM, independent of CD59, CD55, and CD46 complement resistance antigen expression.

HIGH DOSE CHEMOTHERAPY WITH STEM CELL TRANSPLANTATION FOR WALDENSTRÖM’S MACROGLOSSINEMIA (WM) Nikhil C. Munshi, Dana Farber Cancer Institute, Boston MA 02115.

Despite effectiveness of standard chemotherapeutic regimens, complete responses are infrequent in WM and there are no cures. Since WM shares certain biological and clinical features with myeloma, including responsiveness to alkylating agents, evaluation of high-dose therapy with transplant, which is effective in myeloma, is an obvious next step in an effort to achieve high response rates and improve survival. Due to the indolent nature of the disease and older patients (pts) with comorbidity, such an approach is not feasible. A phase I-II trial of high dose melphalan 200 mg/m2, followed by autologous stem cell transplantation using a total body irradiation (TBI) and high dose melphalan 140 mg/m2, followed by hematopoietic cell transplantation was undertaken. Despite earlier reports, we observed that high-dose melphalan plus TBI and stem cell transplantation was feasible. Ten pts received a median of 2 prior lines of therapy, 7 of the 9 evaluable pts achieved a complete response. However, 2 pts relapsed later and 2 pts died of salvage therapy. In the remaining 7 pts, the median time to progression was 14.5 months and 2 pts have achieved a complete response. Based on these encouraging observations, a randomized phase III trial comparing high-dose melphalan with or without high-dose melphalan plus TBI is currently ongoing. The results of this trial will be pivotal in determining the role of high-dose therapy with transplantation in WM.
3. Biology of Marginal Zone B-Cell Lymphomas

An introduction to the marginal zone lymphoma concept

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A marginal zone of IgM⁺, IgD⁻ (or weak⁺) B-cells surrounding follicles has long been recognized in the spleen. Isolated marginal zone cells are said to be present in peripheral lymph nodes but well-defined marginal zones are only seen in mesenteric lymph nodes. In reactive hyperplasia of lymph nodes accumulations of so-called mononuclear B-cells, that bear certain similarities to marginal zone cells, sometimes occur within the sinuses and in the perisinusoidal area. When the MALT lymphoma concept was originally proposed it was thought that its normal cell counterpart was the follicle centre cell. Later, when marginal zone cells were identified as a component of Peyer’s patches, it was appreciated that the normal cell counterpart of MALT lymphoma was the marginal zone B-cell and these lymphomas were characterised as extranodal marginal zone lymphomas. They are the third most common lymphoma type accounting for 7.5% of all lymphomas. When MALT lymphomas disseminate to lymph nodes they localise in the marginal zone resulting in an appearance indistinguishable from primary nodal marginal zone lymphoma (MALT). The normal cell counterpart of this rare type of lymphoma is possibly the isolated marginal zone cell said to be present in lymph nodes but in fact is unclear. Splenic marginal zone lymphoma, despite its name, differs from MALT lymphoma and nodal MZL histologically, immunophenotypically and genotypically casting some doubt as to its precise normal cell counterpart.

PATHWAYS OF RECRUITMENT INTO THE MARGINAL ZONE

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The term marginal zone lymphoma (MZL) includes three lymphoma subtypes originating in extranodal, mainly mucosa associated, lymphoid tissue (MALT type), lymph nodes, and spleen. The pathological features of the primary extranodal and nodal MZL are relatively similar and are characterized by a proliferation of mature B-cells with centrocytic/mononuclear morphology and frequent plasma cell differentiation. These cells proliferate in the marginal zone of reactive follicles with follicle colonization of the germinal centers and invasion of adjacent epithelial structures in mucosal sites. In spite of similar morphological and phenotypic features, these lymphoma subtypes seem to have different pathogenetic mechanisms and clinical behavior. Extranodal MZL of MALT type emerge from a background of chronic inflammatory disorders of infectious or autoimmune origin with tumor cells showing frequent IgH gene somatic hypermutations and evidence of antigen selection. Derogulation of apoptotic mechanisms including APO-D-MALT1, bcl-10, and Fas genes play an important role in the evolution of the disease. Tumor progression involve at least two mutually exclusive pathways. Tumors with the t(11;18) are karyotypically stable and do not evolve into large B-cell lymphomas (LBCL), whereas extranodal MZL lacking this translocation show frequent chromosomal instability and transformation into LBCL. Contrary to extranodal tumors, nodal MZL are always negative for the t(11;18), may originate from both naive and memory B-cells with or without intranodal variation, and have a more aggressive clinical course. Splenic marginal zone lymphomas are characterized by a biphasic proliferation of mature IgD⁺ B-cells with frequent leukemic and bone marrow involvement. The cell of origin is controversial but may include naive and post-germinal B-cells that seem to be associated with different cytagenic alterations including 7q deletions.
CHROMOSOMAL ANALYSIS OF MARGINAL ZONE B-CELL LYMPHOMAS MAY DELINEATE CYTOGENETIC PROFILES CORRELATED WITH CLINICAL CHARACTERISTICS AND OUTCOME: A STUDY OF 141 CASES.


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Introduction: Marginal zone B-cell lymphomas (MZL) include extranodal MALT lymphoma, splenic lymphoma with or without villous lymphocytes and nodal lymphoma. Although a spectrum of chromosomal abnormalities, including +3, +18 and the t(11;18) translocation characterize MZL cytogenetic criteria to distinguish clinicopathologic subtypes are still lacking and the prognostic significance of chromosomal changes remains unknown.

Methods: Based on the cytogenetic study of 141 MZL patients, we correlated the cytogenetic findings with clinical characteristics and outcome.

Results: In addition to +3, +12, +18, we identified five additional recurrent abnormalities: 1q43 deletion, 6q21-25 deletions, +7, 7q21-36 aberrations and i17q. The distribution of those changes was closely related to a distinct clinicopathologic profile. The outcome with splenic involvement (P = .044); +7 and 6q21-25 deletion with nodal involvement (P = .014 and P = .018, respectively); i17q with thymic presentation (P = .003); t(12;12) changes with disseminated disease (P = .011), and t(11;18)(q21;q21) with extra-nodal presentation. The risk of disease progression was significantly higher in patients with +3 (P = .006), +7 (P = .001), +12 (P = .003), 1q43 deletion (P = .004), 8;10-24 (P = .009) involvement and i17q (P = .003). Moreover, in patients with a splenic presentation, additional aberrations such as -3q (P = .032), +12 (P = .029) and 9;13-22 (P = .021) changes were associated with a shorter time to progression.

Conclusions: These findings underline that MZL represents an heterogeneous disease that can be distinguished based on the occurrence of those chromosomal changes which have different significance on clinical presentation and outcome.

IRTA1: A NEW MOLECULE ASSOCIATED WITH NORMAL AND NEOPLASTIC MARGINAL ZONE B CELLS

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Introduction: Abnormalities of band 1q21-q23 are frequent genetic lesions in B-NHL and MM. The cloning of 1q21(1q21-32) in the FR4 MM cell line led to the identification of the human IRTA1 (Immune Receptor Translocation Associated 1) gene, a new member of the immunoglobulin superfamily. Methods: Specific monoclonal (named M-IRTA1) and polyclonal antibodies were generated against the IRTA1 protein to investigate by immunohistochemistry and flow cytometry its expression in normal and neoplastic lymphoid tissues and to study by PCR the IgV gene mutations in microablated single IRTA1+ tonsil B cells.

Results: In normal and reactive lymphoid tissues, IRTA1 was selectively expressed by a subset of B cells that usually showed a dendritic morphology and homed to the marginal zone (MZ) or their analogical equivalents, e.g. the sub- and intrinsic epithelial areas of the tonsil, the external border of mantle zones and the marginal sinus of reactive lymph nodes, and, less consistently, the MZ of the spleen. Monocyto B cells of toxoplasmic lymphadenitis were also strongly IRTA1+. Double staining of immune cells and cell suspension revealed that tonsil IRTA1+ cells consistently expressed C217 (a memory B-cell marker) in the absence of other mantle cell-, germinal center- and plasma cell-associated molecules. PCR analysis of single tonsil IRTA1+ cells proved that they consist of a mixed population of mutated (antigen-experienced) and unmutated B-cells (representing either virgin B cells or, most likely, cells driven by antigens that did not induce or select for IgV gene mutation). IRTA1 was strongly expressed in about 60% of nodal and extranodal MZ lymphomas and only rarely in other lymphoma subtypes. In MALT lymphomas, the anti-IRTA1 antibodies mainly labelled the neoplastic cells participating to the formation of the so-called lymphoepithelial lesions, thus mimicking the epithelioepithelial of the normal IRTA1+ cells in the tonsil.

Conclusions: Antibodies against IRTA1 represents the first marker for MZ-associated B cells and are expected to be a useful tool for the study of this B-cell subset and for diagnosis of MZ lymphomas.
4. What's New in Mantle Cell Lymphoma?

**BIOLOGY IN MANTEL CELL LYMPHOMA**

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Mantle-cell lymphoma is a lymphoproliferative disorder derived from a subset of naive B-cells coexpressing CD5. Two cytological variants have been identified, typical and blastic. Typical tumors show a proliferation of small to intermediate size lymphoid cells with irregular nuclei. Blastic variants include a spectrum of larger cells with blastic or pleomorphic nuclei, higher proliferative activity, and a more aggressive behavior. MCL are characterized molecularly by rearrangements of the bcl-1 region with cyclin D1 overexpression which plays an important pathogenetic role, probably deregulating cell cycle control by overcoming the suppressor effect of retinoblastoma and p27. Conventional cytogenetics and comparative genomic hybridization studies have recently demonstrated a high number of chromosomal aberrations and translocations that are more frequent in blastic variants and may have prognostic significance. These chromosomal alterations target different tumor suppressor genes and oncogenes involved in the progression of the tumors. ATM, a gene increasingly implicated in atypical and blastic MCL by truncating and missense mutations in the PI3 kinase domain, and by allelic deletions. The association between ATM inactivation and higher number of chromosomal imbalances suggests a role of these alterations in increasing chromosomal instability in these tumors. Blastic variants have additional inactivation of the ARF/p53 and p16/CDK4 pathways. Both with inactivation of both pathways by deletions of the whole INK4A/ARF locus have a worse prognosis than patients with only p53 mutations suggesting a potential cooperative effect of these two regulatory pathways in the progression of MCL. The common recurrent aberrations in other chromosomal regions, such as 1p13 and BMI-1, suggest that other genes may play an important role in the progression of these tumors.

**GENOTYPING OF MANTEL CELL LYMPHOMA IDENTIFIES A NEW TUMOR SUPPRESSOR REGION ON 1p13p33**

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Background: Mantle cell lymphoma (MCL) is a recently generally accepted subentity of non-Hodgkin's lymphoma characterized by the chromosomal translocation t(11;14). Morphologically, MCL covers a large cytological range including classical (few malignant) MCL and blastic (high malignant) variants. Secondary transformation from low to high malignant lymphoma is closely related to alteration of p53 on 1p16 and p16 on 9p.

Methods: We previously established a semi-automated alleleotyping method using fluorescence labeled microsatellite markers. 52 cases of morphologically confirmed MCL according to the WHO criteria were analyzed using a total of 20 microsatellite markers on chromosome band 1p13. Based on previous test amplifications of all primer pairs, allelic loss was diagnosed if in tumor cells the peak ratio of both alleles was altered at least by the factor 1.5 (M = 3 SD) in comparison to normal peripheral blood cells.

Results: In the 52 cases of MCL genotyped so far, loss of chromosome band 1p13 was the most frequent secondary alteration (38.5%) exceeding the previously reported frequency of p53 alterations (10 - 30%). To further delineate the tumor suppressor region, a more detailed molecular analysis of 1p13 was performed. Two distinct minimally deleted regions could be identified, one on 1p13.1 including p13 and one on 1p13.3 flanking D17S831 and D17S8145 indicating the location of another tumor suppressor gene. 1p13.1 and 1p13.3 were selectively deleted each in 19% of cases, whereas in 30% of cases, both genomic regions were involved. In concordance to the literature, rearrangements of the p53 region were detected more frequently invariable MCL. In contrast, 1p13.3 rearrangements were found in classical and variant MCL with similar frequency.

Conclusions: Our data indicate the frequent genetic rearrangement of chromosomal band 1p13.1 in MCL. Two distinct tumor suppressor regions could be identified, one on 1p13.1 including p13 and one more terminal on chromosomal band 1p13.3. Currently, additional molecular analyses are being performed (for example alternative fluorescence in situ hybridization with different YAC probes) to further characterize the two distinct tumor suppressor regions.

**PATHOLOGY OF MANTEL CELL LYMPHOMA**

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Mantle cell lymphoma (MCL) represents a tumour of pre-peripheral centre, IgH gene unmutated mantle zone B cells. Approximately 85% of the tumors have a classical cytology, but aggressive (blastic and pleomorphic) as well as rarely small cell variants do occur. K-or MIB1 staining may provide a historically more important prognostic factor than morphology. Most tumours have a classical phenotype: IgM+ and/or IgD+, CD5+, CD10-, CD20-, CD43+, CD45R, BCL2+, BCL6- and cyclin D1+. Class switching to IgG and IgA is absent. Apart from CLL and follicular lymphoma, MCL should be discerned from (CD5+ or -) marginal zone and CD5+ diffuse large B cell lymphoma. Most importantly, it is in 50-70% of MCL lack CD5 expression. Cyclin D1 immunostaining remains the most important ancillary technique to make these distinctions, however, weak expression may be easily missed. Several reliable immunohistological markers, often included in multiparameter assays, are currently available. Alternatively, quantitative RT-PCR and FISH to detect the t(11;14) can be applied. We developed an interphase FISH assay to test for this translocation. In our hands, there is an almost 100% correlation between FISH results and IHC for cyclin D1 protein in MCL. 11q13 breakpoints in the 350 kbp BCL1 region are also observed in 15-25% of multiple myelomas (MM). Most patients with prolymphocytic leukemia and pancreatic/basal-like lymphoma (both are often considered as variant MCL), few parathyroid adenomas and several renal cell oncocytesomas. In MCL, 1q11 sequences are always juxtaposed to IgH joining or diversity gene segments, whereas in MM they are often juxtaposed to IgH switch sites, indicative of an origin during IgH class switching, in renal oncocyteoma, the translocation partners have not yet been identified. Using DNA fibre FISH, we found clustering of 11q13 breakpoints at the BCL1-MTC in 40% of MCL, but not in myeloma or renal cell oncocyteoma. Other chromosomal abnormalities in MCL are loss of 9p21-22, 9p21 (p16INK4A), 13q14 (harbouring an unarranged locus called BCMS or BCMSun), 11q22-23 (ATM) and 1p13.32, 8q, and 1p13 (p53). Amplification has been observed at 3q26 or 8q. Interestingly, mono- or bi-allelic inactivating ATM mutations are associated with chromosomal imbalances, suggesting a critical role of ATM in at least half of the MCL.
PHASE II STUDY OF IDIOTYPE VACCINE FOLLOWING EPOCH-RITUXIMAB TREATMENT IN UNTREATED MANTLE CELL LYMPHOMA

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Introduction: Mantle cell lymphoma (MCL) is responsive to allogeneic CVL, and idiotype-KLH vaccine (iKLH) has been shown to induce T-cell responses in follicular lymphomas, suggesting Id-based vaccine may be active in MCL. Id-vaccine are likely to be most effective against MCL and in pts with intact cellular immunity. Effects of rituximab on Id-CR and CTL responses are unknown, but animal models suggest B-cell depletion may enhance cellular but reduce humoral responses.

Methods: Dose-adjusted EPOCH-R was administered q 6 weeks x 6, followed by 3 cycles of Id-vaccine beginning at least 12 weeks later. DA-EPOCH-R (mg/m²): 375 rituximab (IV) day 1; 200 mg esosome (IV), 1.6 vincristine and 40 mg doxorubicin (dox) CIV x 3 q 6 weeks; doxorubicin (IV); cyclophosphamide (CV) IV x 6 (day 1); and prednisone (P) QD x 6.

Results: Of 26 enrolled pts, 21 have enroled. Pt characteristics are median (range) age 56 (38-73) and PS 1 (0-3). No grade 1 toxicity was observed. 5 Pts developed grade 1 toxicity (vs 4/24 in EPOCH-R arm). Complete responses (CR) were seen in 3/21 pts (14.3%), 1 pt had a partial response (PR), and 2 pts had minimal residual disease (MRD).

Conclusion: This study shows that Id-vaccine can be administered safely and effectively following EPOCH-R treatment. Further studies are needed to evaluate the efficacy of Id-vaccine in MCL.

ALLOTRANSPLANTS FOR MANTLE CELL LYMPHOMA

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Bone marrow transplant is now accepted as the treatment of choice for young patients with chemotherapy sensitive relapse of diffuse aggressive lymphoma. Autologous transplantation has been used less widely for tumor patients with non-Hodgkin's lymphoma. The relatively high median age of patients with these lymphomas, the difficulty in finding suitable donors, and the high transplant related mortality have all been contributing factors.

Patients with mantle cell lymphoma represent a particularly difficult therapeutic problem. To date, standard chemotherapeutic regimens have rarely been reported curative. Autologous transplantation has been widely applied in the treatment of patients with mantle cell lymphoma, but a pattern of continual relapses has been the rule and only a small proportion of patients appear to have been cured. It is uncertain that this is due to an inability of the high dose regimen to eliminate lymphoma in the patient or the relapse of malignant cells in the graft or both.

Autologous transplantation offers the attractive features of a tumor free graft and the possibility of a graft-versus-lymphoma effect. The latter appears to be a real phenomenon in mantle cell lymphoma. This can be documented by patients cured with allogeneic transplantation after failing autologous transplantation.
5. Biology of Lymphomas

MULTICOLOR FUSION: A NEW TOOL FOR THE MORPHOLOGIC, IMMUNOPHENOTYPIC AND GENETIC ANALYSIS IN LYMPHOMAS

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Introduction: The analysis of phenotypic and genotypic cell features is an important aim for understanding mechanisms of lymphomagenesis as well as for the diagnosis and classification of these malignancies. Here we report a novel multicolor approach based on the FICION (Fluorescence Immunophenotyping and In situ Hybridization) technique, which allows the simultaneous detection of morphologic, immunophenotypic and genic characteristics at the single cell level.

Methods: Multicolor in situ hybridization (MI-FISH) assays for the study of B-cell and T-cell non-Hodgkin’s lymphoma (NHL), and anaplastic large cell lymphoma (ALCL) were developed allowing the simultaneous detection of the most frequent primary genetic aberrations in these neoplasms, like 5q31.3 (6 favorable), 14q32 (7 favorable), 11q22 (2 favorable) and 1q11 in B-NHL, rearrangements of the PGR loci in T-NHL and rearrangements of the ALK gene in ALCL. In order to establish the multicolor (M) FISH technique, these assays were combined with the detection of lineage- or tumor-specific antigens, like CD20, CD3 and ALK, respectively. For evaluation of the M-FISH analyses, image acquisition by automatic sequential capturing of multiple focal planes was performed in order to obtain three-dimensional information from each color channel.

Results: Both MI-FISH and M-FISH were shown to reliably detect chromosome changes in cytogenetically well characterized B-NHL, T-NHL and ALCL samples. By M-FISH, these chromosomal aberrations were found exclusively in the CD20+, CD3+ or ALK+ cell subpopulations, respectively.

Conclusions: With the development of M-FISH we provide a new tool for the comprehensive interdisciplinary study of lymphomas. In the near future, M-FISH might take advantage of upcoming data generated by genomic and expression analyses. Thus, novel markers with discriminating diagnostic and prognostic predictive value could be simultaneously visualized at the single cell level to establish diagnostic tests with unprecedented clinical relevance. Supported by the IZKF Kiel.

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PATTERN OF IMMUNOGLOBULIN (Ig) GENES SOMATIC HYPERMUTATION AND CHROMOSOME 11q22-23 DELETION IN MANTLE CELL LYMPHOMA

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Introduction: MCL and chronic lymphocytic leukemia (CLL) share immunophenotypic and karyotypic similarities. CLL appears to comprise two different entities with differing prognosis based upon the Ig genes somatic mutation status, the IgV genes family usage and the presence of 11q deletion. This study evaluated the relevance of these characteristics in a series of histologically reviewed classical MCL cases to determine a putative prognostic impact.

Methods: The Ig genes CD22 and CD73 regions were amplified, cloned and sequenced. Sequences were compared with the germine Ig genes to define the hypermutation rate. Chromosome 11q22-23 deletion was evaluated by genomic real-time PCR using a series of primers.

Results: Thirty-two cases with proven monotypic underwent clonal and sequencing of the Ig genes. The most frequently expressed Ig genes families were: IVS-21 in 100% (31%), IVS-17, IVS-13, IVS-11, IVS-7, IVS-5, IVS-4 and IVS-3 in 37% (8%) each. Somatic Vκ deletions were detected in 37% cases (29%), with a deviation from the closest germline sequence ranging from 3% to 13% in Chromosome 11q22.2, harboring the ATM gene, and 1q23.2 regions were deleted in 1547 cases (32%) and 1646 cases (33%), respectively. Detection of both deletions was present in 1145 cases (24%). Twenty-five cases were analysed for both Ig and 11q status. Loss of 1q22-23 was found in 871 cases (47%) and in 718 cases (37%) with IgV genes mutated and mutated genes, respectively.

Conclusions: MCL exhibits distinct Ig genes and 11q status compared to CLL, reflecting the different pathogenesis of the two diseases. The possible prognostic impact of each individual status will be further investigated.

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CHARACTERISTIC PATTERNS OF CHROMOSOMAL ABERRATIONS IN DIFFERENT B-CELL NEOPLASMS: OMMIC HYBRIDIZATION (CGH) ANALYSIS OF 523 B-CELL NEOPLASIA

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Cytogenetic data have gained increasing importance for the classification of lymphomas. In this study we analyzed the results of 523 lymphomas of patients with B-cell neoplasias examined by CGH, 174 diffuse large cell lymphomas (DCLL), 128 follicular lymphomas (FL), 43 primary mediastinal B-cell lymphomas (PML), 36 chronic lymphocytic leukemias (CLM), 34 Burkitt lymphomas (BL) and 28 aggressive lymphomas of the gastrointestinal tract (GILT), 32 mantle cell lymphomas (MCL) and 46 multiple myelomas (MM). We found chromosomal imbalances in 70.4% of all cases. Some specific chromosomal changes were found frequent in all types of B-cell neoplasia, particularly losses on chromosome arm 8q (17/13%) and 16q (16/12%), gains on 12q (18/10%), 7p (11/18%) and 18q (12/12%). However, we found substantial differences between different entities. The complexity of karyotype (defined as number of imbalances per cases) was high in aggressive lymphomas (DCLL 2.9, PML 5.0, MCL 4.3, BL 2.6), in contrast to indolent lymphomas (FL 1.9, CLM 0.7, p<0.0001). Fisher's exact test, in some cases, chromosomal imbalances seem to be characteristic for specific lymphoma types: in CLL, 11q losses were more frequent than in other lymphomas (15.8% vs. 1.4%, p<0.0003 Fisher's exact test). Gains on 6p were associated with PML (48.9% vs. 3.5%, p<0.0001), but also gains on 2p (23.2% vs. 6.3%, p=0.0005), 12p (20.4% vs. 5.2%, p=0.0008) and Xp (32.6% vs. 10.6%, p=0.002). In MCL, losses on 11q (12.5% vs. 1.8%, p=0.006), 13q (62.5% vs. 13.8%, p<0.0001) and 8p (25.0% vs. 3.9%, p=0.0001) were characteristic, as well as gains on 8q (31.5% vs. 7.1%, p=0.0001). In MM, losses on 13q (36.9% vs. 14.8%, p=0.0004) and 8p (13.0% vs. 5.0%, p=0.038), as well as gains on 9q (15.2% vs. 6.1%, p=0.021) and 15 (10.8% vs. 2.5%, p=0.008) were characteristic. In FL, we found a higher amount of 7p gains (16.4% vs. 6.3%, p=0.003). Further 8p gains were higher in DCLL (17.2% vs. 8.3%, p=0.004). These data indicate, that in addition to chromosomal translocations, chromosomal imbalances are highly characteristic for some lymphomas entities.
ATM, TP53 and NBS1 MUTATIONS IN DIFFUSE LARGE B-CELL LYMPHOMA

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Introduction: The ATM serine-threonine kinase plays a central role in the cellular response to DNA damage. Germ-line mutations in the ATM gene cause ataxia-telangiectasia (A-T), a multisystem disorder associated with predisposition to lymphoma and acute leukemia, and somatic ATM mutations have been identified in T-cell prolymphocytic leukemia, mantle cell lymphoma, and B-cell chronic lymphocytic leukemia. TP53 and Nibrin (encoded by NBS1) are both downstream effectors of ATM.

Methods: The entire coding sequences of ATM and TP53 were examined by PCR, denaturing gradient gel electrophoresis and direct sequencing of genomic DNA from 12 lymphoma neoplasms. Similarly, NBS1 mutation analysis was performed in 45 diffuse large B-cell lymphomas (DLBCL).

Results: Novel mutations and mutations implicated in cancer and/or A-T were found in 9 of 45 (20%) DLBCL. 2 of 24 (8%) follicular center lymphomas, and 1 of 27 (4%) adult T-cell lymphomas, whereas no such mutations were detected among 24 peripheral T-cell lymphomas, unspecified. Most of these mutations were associated with loss or mutation of the paired ATM allele. A novel TP53 mutation was observed in DLBCL (P = 0.015). One mutation that has been identified in tissue from apparently healthy individuals and thus previously considered as a rare normal variant was found to be 5.6 times more frequent in DLBCL than in random individuals (P = 0.026), suggesting that it may predispose to B-cell lymphoma. A novel nonsense mutation in the Nijmegen breakage syndrome gene, NBS1, was found in 2 DLBCLs.

Conclusions: Our data suggest that ATM mutations contribute to the pathogenesis of DLBCL, and that the combined loss of ATM and TP53 may cooperate in the development of some aggressive B-cell lymphomas.

INACTIVATION OF THE INK4A GENE CLUSTER IN MANTLE CELL LYMPHOMA – DIFFERENTIAL METHYLATION PATTERNS OF CELL CYCLE REGULATORS

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Introduction: Mantle cell lymphomas (MCL) is characterized by the chromosomal translocation t(11;14)(3q23;1q24) resulting in the overexpression of cyclin D1. However, additional genetic alterations are detectable in the vast majority of cases. Thus, deletions of the INK4A gene cluster (p16⁰⁰⁰⁰, p15⁰⁰⁰⁰, and p19⁰⁰⁰⁰⁰) on chromosome band 9p21 are one of the most frequently detected secondary alterations in MCL. So far, little is known on the differential effect of the p53⁰⁰⁰⁰ and p16⁰⁰⁰⁰⁰ on cyclin D1 expression and in the p53⁰⁰⁰⁰ and p19⁰⁰⁰⁰⁰ on NFκB dependent gene expression in MCL.

Methods: We have established a comprehensive microarray approach to detect additional genetic alterations specific for MCL. We have done this with the help of the microarray gene expression platform of the Institute of Pathology, University of Würzburg.

Results: In an initial series of 5 hematological cell lines, the methylation patterns of p16⁰⁰⁰⁰⁰ and p19⁰⁰⁰⁰⁰ could be confirmed and characterized in more detail. A complete map of the promoter methylation patterns covering approximately 150 kb of the p16⁰⁰⁰⁰⁰ and p19⁰⁰⁰⁰⁰ promoter region was obtained. A complete methylation analysis of 52 MCL previously characterized for INK4A gene cluster deletion and p53 alteration (mutation, expression) was performed to more accurately define the differential impact of p16 methylation patterns on MCL.

Results: In a total of 49 samples (53%) analyzed by PCR were methylated in the p16⁰⁰⁰⁰⁰ promoter region and confirmed by sequence analysis. In contrast, in 20 out of 47 samples (43%) mutations-specific PCR of the p16 promoter was false positive due to incomplete bisulfite treatment and could not be confirmed by direct sequencing. Currently, methylation analysis of the p16⁰⁰⁰⁰⁰ promoter region is being investigated.

Conclusion: In summary, the INK4A promoter regions are differently methylated in MCL. p16⁰⁰⁰⁰° is frequently methylated whereas methylation of the p15⁰⁰⁰⁰⁰ promoter could be detected in none of the MCL cases. In addition, our results suggest that promoter methylation assays have to be confirmed by sequence analysis. Finally, a multivariable analysis will be performed to identify the biological impact of differential INK4A methylation.

ACQUISITION OF N-GLYOSYATION SITES IN IMMUNOGLOBULIN VARIABLE REGIONS: INSIGHTS INTO THE BIOLOGY OF GERMLINE CENTRE LYMPHOMAS

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B-cell NHL of germinal centre origin typically have somatically mutated V genes with intraclonal variation, consistent with location in the germinal centre site. Using our own and published sequences, we have investigated the frequency of potential N-glycosylation sites introduced into functional V genes as a consequence of somatic mutation. Tumour derived V gene sequences were compared with normal memory B cells or plasma cells matched for similar levels of mutation. Strikingly, novel sites were detected in 55/70 cases of follicular lymphoma (FL, 79%), and 14/17 cases of follicular BURKITT Lymphoma (EBL, 82%) as compared to 77/5 (6%) in normal B cells (X2 = 0.001). Diffuse large B-cell lymphoma (DLCL) (13/32, 41%) and splenic Burkitt lymphoma (10/23, 43%) showed an intermediate frequency. Myeloma and the mutated subset of chronic lymphocytic leukaemia showed frequencies similar to the normal cells with 5/64 cases (8%) and 5/40 (13%) cases respectively. Surprisingly, MALT lymphoma which have ongoing somatic mutation also lie within the same range (3/36, 8%). In 3/3 random cases of FL, the Ig was expressed as recombinant single chain Fv in Pichia pastoris and N-glycosylation was demonstrated. These findings indicate that N-glycosylation of the variable region may be common in FL and BCL and in subsets of DLCL. The majority of novel sites are located in the complementarity-determining regions. Vα sequences of non-functional V genes contained few sites, arguing for positive selection. One possibility is that the added carbohydrate in the variable region common to flies to interaction with elements in the germinal center environment. This common feature of FL end EBL may be critical for tumor behavior while in MALT lymphoma growth may be stimulated by other pathways.

C-FLIP IS CONSTITUTIVELY EXPRESSED BY Hodgkin and Reed-STERNBerg cells.


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Introduction: Hodgkin Reed-Stemberg cells (HRS) cells in classical Hodgkin’s disease (cHD) derive from germinal-center B-cells (GCs) in most cases. Physiologically, GCs are selected for immunization of high-affinity surface immunoglobulins (sIgs) that do not express slg are eliminated by Fas-mediated apoptosis. HRS cells, despite harboring rearranged and mutated Igs genes, completely lack slg expression and should therefore be prone to negative selection by Fas ligation in the GC. Instead, they are resistant to Fas-mediated apoptosis and expand clonally.

c-FLIP is a recently described protein that potently inhibits Fas-mediated apoptosis and has been shown to be a key factor in germinal center B-cell survival. We therefore studied the role of c-FLIP in cHD.

Methods: Two Fas-resistant HRS cell lines were studied for expression of c-FLIP mRNA and protein. Both cell lines were treated with the protein synthesis inhibitor cycloheximide (CHX) to downregulate c-FLIP and analyzed for their susceptibility to undergo Fas-mediated apoptosis. Finally, 23 primary cases of cHD were analyzed for c-FLIP expression by immunohistochemistry.

Results and conclusion: High levels of c-FLIP protein were identified in two Fas-resistant Hodgkin-derived cell lines. In contrast to other tumor cell lines, inhibition of protein synthesis by cycloheximide (CHX) did not lead to downregulation of c-FLIP protein in these HD cell lines. Furthermore, Fas-mediated apoptosis was only partially restored suggesting that normal regulation of cFLIP was disrupted. The demonstration of significant c-FLIP expression by immunohistochemistry in 18 out of 19 evaluable cases of primary Hodgkin’s disease supports the in vivo relevance of these findings. Taken together, c-FLIP is constitutively expressed in HD and may therefore be a central mechanism responsible for Fas-resistance of HRS cells.
HEAVY CHAIN SOMATIC HYPERMUTATION ANALYSIS IN WALDENSTRÖM MACROGLOBULINEMIA AND CHRONIC LYMPHOCYTIC LEUKEMIA

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Introduction: Waldenström Macroglobulinemia (WM) and B-cell Chronic Lymphocytic Leukemia (B-CLL) are both B-cell malignancies with monoclonal Immunoglobulin rearrangements. The malignant cells from WM are likely derived from IgM expressing memory B-cells. The analysis of the immunoglobulin mutational status was reported for only few patients. IgH hypermutation analysis of B-CLL shows that malignant cells are derived either from pre- (unmutated) or postgerminal (mutated) B-cells. The mutation status was reported to be of strong prognostic significance. It was also proposed that CD38 expression of B-CLL cells could be a good surrogate marker for the mutational status, but this correlation is currently a matter of discussion.

Methods: Bone marrow or peripheral blood samples from 16 WM and 72 B-CLL patients were analysed for IgH gene rearrangements with FRH, FR2 and JH consensus primers. Electrophoresis of PCR products after heteroduplex analysis allowed detection of monoclonality and isolation of the monoclonal product. Direct sequencing of the PCR products was performed. Flowcytometric analysis using monoclonal anti-CD38 antibodies was used to detect CD38 on lymphocytes.

Results: Hypermutation was found in 16 WM patients (monoclonal range 86.7% - 94.7%). Thirty-four unmutated (96%) cases (one with germline and 38 mutated (<5%) dominance B-CLL patients were identified. A weak correlation of the CD38 expression of lymphoid cells with the mutational status was observed (Fisher exact test, p<0.03, accuracy = 62%). Consequently CD38 cannot be used as a surrogate marker to determine immunoglobulin mutational status. On the other hand, the mutational status was significantly correlated with RAI-stage, lymphocyte doubling time and cytogentic risk groups.

Conclusion: WM are indeed IgM expressing post-germinal B-cells. The analysis of IgH hypermutations of B-CLL patients at diagnosis is warranted since the CD38 expression can not be used as a surrogate marker for the mutational status of the rearranged immunoglobulin genes.

FOLLICULAR LYMPHOMA GRADE III-B INCLUDES THREE CYTOGENETICALLY DEFINED SUBGROUPS: t(14;18), IgH or JH translocations; t(11;18) and t(3;27) are mutually exclusive

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Abstract: Chromosomal translocations involving t(14;18)(q32;p21) and the chromosome 3q27 region are common in B-cell non-Hodgkin's lymphoma (NHL) of germinal center cell origin. Grade III-B follicular large cell lymphoma (FLCL), consisting almost exclusively of centroblasts, is a distinct subgroup of follicular lymphomas which has clinically more in common with the aggressive B-FLCL than with their indolent follicle center lymphoma (FCL) counterparts. We studied the cyto genetic and molecular genetic aberrations by classical cytogenetics, PCR, Southern Blot hybridization (SBH) and fluorescence in situ hybridization (FISH), with special emphasis on t(14;18), affecting bcl-2, and 3q27 rearrangement, affecting c-myc in 12 cases of FLCL grade III-B.

Three distinct subgroups were identified based upon the existence of breakpoint t(3;27) a translocation (t(14;18)) or the absence of both. Group I with a t(14;18) and no 3q27 aberrations (n=13); group II: without a t(14;18) and without 3q27 aberrations (n=9), but with other cytogenetic aberrations; group III: without a t(14;18) but with aberrations involving 3q27 (n=10). None of the FLCL grade III-B cases was found to harbour both a t(14;18) and 3q27 aberration. These results, in particular the finding of a mutual exclusiveness of bcl-2 and bcl-6 rearrangement, indicate at least three different pathways of oncogenesis in follicular large cell lymphoma. group I FLCL III-B with bcl-2 rearrangement only is part of the same entity as the other follicular lymphomas (grade IIIA), whereas the cases with 3q27 abnormalities or other unrelated translocations are more closely related to the majority of diffuse large cell lymphomas of germinal center cell origin.

An updated version with additional data concerning bcl2 and bcl6 expression, CD10 expression, P53 expression and mutation and enlarging of the FISH investigations will be presented.

IDENTIFICATION AND VALIDATION OF 7 GENES, AS POTENTIAL MARKERS, FOR THE LEUKEMIAL FORMS OF INDOLENT NON-FOLLICULAR B-CELL LYMPHOMAS BY CDNA ARRAYS ANALYSIS


Introduction: Indolent non-follicular B-cell non-Hodgkin’s lymphomas represent a group of lymphomas of clonal proliferations of mature B-cells. Accurate diagnosis is sometimes difficult but is essential to evaluate prognosis and determine optimal therapy. The most frequent histological subtypes are small lymphocytic lymphoma (SLL/CLL), marginal zone B-cell lymphoma (MZL), mantle cell lymphoma (MCL).

Methods: Our objective was to identify new diagnostic markers to improve the characterization of these lymphomas using macro- and microarray gene expression analysis and to validate the results by quantitative real-time PCR. Lymphoma cells were puizled from blood samples of 48 untreated patients.

Results: Comparison of the 3 lymphoma transcription profiles, performed with 14 cases (3 B- SLL/CLL, 5 MZL and 4 MCL) on CDNA macroarrays bearing 588 genes, showed that the majority of transcript levels were the same. However, 7 genes (bcl2, bcl1, mmp17, a-hype Fasligand, myh2, apar and sim1) were found to be expressed at different levels. Validation of these results was performed on 40 patients by quantitative real-time PCR. Significant differences in the expression levels of the transcripts were confirmed for all of the candidate-genes except a-hype Fasligand. Three of the genes (mmp17, a-hype, apar) are related to the interaction of the lymphoma cells with the extra-cellular matrix (ECM).

Conclusion: These results emphasize the importance of the ECM in the pathophysiology of lymphoid malignancies. We conclude that B- SLL/CLL, MZL and MCL exhibit specific transcriptional profiles that may be relevant to the pathogenesis of these histological subtypes. Transcriptional profiles provide targets that could lead to a better identification and accurate diagnosis of the heterogenous group of lymphomas.

MICRO-ARRAY ANALYSIS TO ELUCIDATE THE PATHWAYS INVOLVED IN THE TRANSFORMATION OF FOLLICULAR LYMPHOMA


Introduction: Transformation to more aggressive disease in follicular lymphoma (FL) is a common event. The prognosis after transformation to diffuse large B-cell lymphoma (DLBCL) is poor and this forms a major cause of death in FL patients. Thus far, biological insights in the factors involved in transformation are limited to few and individual genes. Microarray analysis offers the opportunity to do a genome-wide screening for relevant factors and pathways.

Material and methods: From the pathology files of 3 institutes in the Netherlands, 16 patients with transformed follicular lymphoma were available from both the indolent and transformed phase were identified; 12 cases of FL followed by DLBCL (median interval 24 mo, range 13-68 mo), 3 cases of DLBCL relapsed as FL (median interval 12 mo, range 12-50 mo) and 3 cases of synchronous FL and DLBCL at different localizations. All cases were reviewed, classified and graded according to the WHO-classification. RNA was isolated, linearly amplified and Cy5- or Cy3-labelled and co-hybridized with reference aRNA-mix to 18,000 gene CDNA arrays. All experiments were performed in duplicate.

Results: Preliminary data using unsupervised clustering with J-Express and CLUSTER showed separation into 3 major clusters of indolent tumors, transformed tumors and an intermediate group. The expression profile of the intermediate group revealed a higher degree of similarity to the transformed group than to the indolent group. However, histologically, these cases were all FL grade 3 and display signs of transformation. In the transformed group, proliferation, basic metabolism and invasion-related genes were upregulated, anti-apoptotic genes and accessory and T-cell related genes were downregulated. Additionally, MHC-regulating genes differed strikingly between the two indolent clusters. Supervised clustering will be performed on the full data set.

Conclusions: Preliminary data on pairs of indolent and transformed FL using micro-array analysis show a histologically unrecognizable group of FL with an intermediate profile between DLBCL and FL. At least two separate pathways for transformation are suggested by expression profiles.
IDENTIFICATION OF KARYOTYPIC PATHWAYS OF CLONAL EVOLUTION IN FOLLICULAR LYMPHOMAS.
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Introduction: Follicular lymphomas (FL) are characterized by t(14;18)(q32;q21) or variant in 85% of cases; 15% do not show a t(14;18) by cytogenetics or FISH. Nearly all cases show evidence of additional chromosomal alterations. These karyotypes are generally more complex than in other hematopoietic malignancies. The significance of this complexity and the sequence by which these secondary changes arise have not been determined. The inability to address these issues has largely been due to the lack of appropriate methods to analyze complex chromosomal alterations in a sufficiently large number of cases.

Methods: We have developed and adapted several statistical methods that allow the analysis and interpretation of complex karyotypes. We analyzed 336 cases of FL with and 182 cases without t(14;18). First we identified the 29 and 40 most common changes in each subtype, respectively, then each lymphoma case was assessed for the presence or absence of each karyotypic change.

Results: The chromosomal changes appear in an apparent temporal order with distinct early and late changes. We identified, by principal component analysis, four possible cytogenetic pathways in lymphomas with t(14;18) and three in lymphomas without t(14;18). These pathways converge to a common route at later stages. We found that FL with t(14;18) may be classified into cytogenetic subgroups determined by presence or absence of 6q-, +7, or del(18)(q14); and FL without t(14;18) by the presence or absence of +3, -6, or +12.

Conclusions: This type of statistical analysis reveals patterns of acquisition of secondary chromosomal alterations not apparent by standard karyotype assessment and defines distinct cytogenetic subgroups within a well defined subtype of malignant lymphoma.

PREDICTING RITUXIMAB RESPONSE OF FOLLICULAR LYMPHOMA USING CDNA MICROARRAY ANALYSIS
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Follicular lymphoma (FL) is a relatively common, indolent form of non-Hodgkin's lymphoma. FL is frequently responsive to chemotherapy and/or radiation but is rarely, if ever, curable. The recent introduction of rituximab, a chimeric monoclonal antibody targeting the B-cell surface protein CD20, has revolutionized the treatment of FL. Phase 2 trials involving patients with low-grade or follicular lymphoma relapsed after prior chemotherapy demonstrated a response rate of approximately 50% with rituximab treatment. The mechanism of rituximab action remains unclear and data from in vitro studies support various potential mechanisms, including complement-mediated lysis, antibody-dependent cellular cytotoxicity (ADCC), and induction of apoptosis.

In this study, we analyzed the expression of more than 20,000 genes in diagnostic tumor specimens of patients who subsequently received rituximab for treatment of FL to identify genes expressed differentially in rituximab responders versus nonresponders. The resulting gene expression data were analyzed using hierarchical cluster analysis to examine similarities in overall gene expression patterns and statistical techniques to evaluate the significance of differences in expression level of individual genes. To date, we have completed analysis of tumors derived from 17 patients.

Hierarchical cluster analysis of the gene expression patterns divides the tumors into two groups. One group clustered with normal lymphoid tissues from tonsil and spleen; rituximab nonresponders where found to be contained within this group. Conversely, rituximab responders were predominately found in the second group (P=0.002, Fischer's exact test). Analysis of the differentially expressed genes suggests differences in the anti-lymphoma immune response between responders and non-responders. Analysis of a larger cohort of patients and discussion of specific differences in gene expression patterns will be presented.