Published in final edited form as:

Leuk Lymphoma. 2008 July ; 49(7): 1403–1406. doi:10.1080/10428190802094245.

Comprehensive analysis of GST- π expression in B-cell lymphomas: Correlation with histological subtypes and survival

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Keywords

GST- π ; mantle cell lymphoma; marginal zone lymphoma; t(11;14) translocation

The current non-Hodgkin's lymphoma (NHL) classification defines disease entities using a variety of parameters. In some lymphomas, a specific immunophenotype or a given genetic abnormality is an important diagnostic criterion. The t(11;14) (q13;q32) leading to the overexpression of cyclin D1 is the genetic hallmark of mantle cell lymphoma (MCL)[1–4], but is also described in other hematological malignancies particularly in multiple myeloma. Furthermore, it has been recently reported that MCL cases lacking the t(11;14) (q13;q32) also fail to express cyclin D1[5,6]. A misdiagnosis is therefore possible, particularly between MCL and MZL[7]. Novel markers are then required in B-cells lymphomas and particularly among the non-germinal center B-cells lymphomas such as MCL and MZL following very different clinical course. Patients with MCL have a very poor overall and failure-free survival, the disease becoming rapidly refractory to chemotherapy, whereas patients with MZL, particularly in the splenic subtype, have one of the highest survivals of any lymphoma subtypes. This will implicate very different therapeutic strategies for each subtype.

The glutathione S-transferases (GSTs) comprise a complex superfamily of genes whose products are phase II detoxifying enzymes, catalysing the conjugation of reactive intermediates with glutathione. GST enzymes appear to be expressed in most, if not all, life forms, a finding

that suggest their importance in the protection of cells from harmful chemicals, such as environmental pollutants, carcinogens, products of oxidative stress, as well as antitumor drugs. The overexpression of GST- π has been associated with resistance to alkylating agents and anthracyclines[8].

Recent gene expression profiling analysis in B-cell lymphomas including mantle cell lymphomas, marginal zone lymphomas, and small lymphocytic lymphomas has shown that *GSTP1* transcript is one of the strongest predictor gene in the predictive model for the diagnosis of MCL, with a constant overexpression in MCL[9]. Furthermore it has been reported by immunochemistry that cyclin D1 overexpression in MCL is associated with a high level of GST- π expression[10]. These results prompted us to look at the specificity of GST- π expression in various subtypes of B-cell lymphomas, particularly in MCL in their typical and variant forms, with or without t(11;14). We further looked for an impact in the survival of patients considering GST- π expression.

The cohort was assembled retrospectively from 136 untreated B-cell non-Hodgkin's lymphomas cases (Table 1) after complete morphological, cytological, immunological, and cytogenetic analysis in order to assess the diagnosis of MCL including 6 cases without t(11;14) previously described [6], typical MZL (splenic, extranodal or nodal types), small lymphocytic lymphoma (SLL), follicular lymphoma (FL) or diffuse large B-cell lymphoma (DLBCL). All patients had signed informed consent for biopsy analysis. One third of these cases have been studied by gene expression analysis (MZL: n=20, 14%; MCL: n=14, 10%; SLL: n=10, 7%; DLBCL: n=0; FL: n=0)[6,9].

Paraffin-embedded biopsies were obtained from formalin- or Bouin-fluid-fixed tissues and Hematoxylin & Eosin counterstained. The 5- μ m sections were deparaffinized and stained with anti-GST- π monoclonal antibody (clone LW29, Novocastra, Tebu International, Le Perray en Yveline, France) at the recommended concentration (1/200). The sections were subjected to microwave antigen retrieval for 30 minutes in 0.01M sodium citrate buffer (pH 6.0) then exposed to 3% hydrogen peroxide diluted in water for 20 minutes at room temperature. Immunohistochemistry analysis was performed using a Ventana NEXES system according to the manufacturer's instructions. Normal lymphoid tissue (normal tonsil) was used as a control and GST- π was found to be expressed in the cytoplasm of dendritic cells, macrophages and plasma cells (Figure 1a). Samples were considered as highly positive if GST- π was expressed in more than 50% of the lymphoma cells, intermediate if GST- π was expressed in 5 to 50% of the lymphoma cells, and negative if GST- π was expressed in less than 5% of the lymphoma cells. Cyclin D1 immunostaining (Clone AM29 Zymed, 1/50) was performed in all MCL patients, with or without t(11;14), in 12 of the 31 MZL patients and in 8 of the SLL cases.

Fisher's exact test was used to examine differences in patient distribution according to GST- π expression. Overall and disease-free survival curves were estimated using the Kaplan-Meier product limit method. For overall survival (OS), the primary end point was death from any cause, and for disease free survival (DFS) progression or relapse during or after the treatment. Differences in the survival curves were evaluated with the log-rank test (statistica 6.0 software).

All typical 25 cases of MCL cases with t(11;14) and cyclin D1-positive, except 2, strongly expressed GST- π with a cytoplasmic and/or nuclear localization in more than 50% of the lymphoma cells (Figure 1b). In 2 patients, GST- π was expressed only in the nuclei without any clinical significance. Among the 6 MCL patients lacking t(11;14) and cyclin D1-negative, 5 strongly expressed GST- π with cytoplasmic localization. None of the 31 cases of low-grade splenic MZL or the 5 MALT lymphoma cases expressed GST- π . In nodal MZL, GST- π expression was absent or intermediate in 16 (84%) of the cases, whereas staining was present in more than 50% of the lymphoma cells in 3 (16%) cases. None of the FL cases expressed

GST- π , except for two grade 3 cases with intermediate intensity. High expression (> 50% cells stained) was detected in 28% of the SLL cases and in 30% of the DLBCL cases. In nodal MZL samples, correlation was noticed between GST- π expression and plasmacytic differentiation.

Among B-cell lymphomas, MCL is clinically characterized by an aggressive course with a transient response to chemotherapy due to rapid acquisition of chemoresistance. It is likely that the high level of GST- π protein expression is at least partially responsible for this chemoresistant phenotype[9,10]. We have previously shown, using gene expression analysis [9], that the MCL genomic signature is specifically characterized by 2 major functional groups, cell proliferation and drug resistance, with the latter consisting of not only *GSTP1* but also MDR/ATP-binding cassette (*ABC*) membrane proteins (*ABCG2* and *ABCC5*). Thus high GST- π expression in MCL is an intrinsic abnormality of the disease and likely to confer resistance to chemotherapy. The precise mechanisms whereby GST- π is responsible for resistance to alkylating agents and anthracyclines are still poorly defined[11], but have been related to glutathione conjugation of anticancer drugs, cytosolic sequestration of anticancer drugs by direct binding to *GSTP1*, chemical reduction of hydroxyl radicals formed by anthracyclines and, more recently, *GSTP1*-mediated cellular protection against oxidative stress-induced cell death [12]. Interestingly, the *GSTP1* gene is located on chromosome 11q13, 2.204 Mb centromeric to the *CCND1/BCL1* gene. However GST- π expression in MCL cases was independent of the presence or the absence of the t(11;14) and cyclin D1 expression. This indicates that transcription of the two genes is potentially not co-regulated.

This overexpression of GST- π likely contributes to the poor outcome of MCL patients, as has previously been reported in malignant tumors with high GST- π expression levels such as DLBCL, acute myelogenous leukaemia, head and neck carcinoma, breast carcinoma, and non-small cell lung carcinoma[13]. We effectively confirmed for the remaining lymphoma subtypes (nodal MZL, DLBCL and SLL) that the high expression was correlated with worse disease-free and overall survivals (data not shown). With a median follow-up at 5.62 years for nodal MZL, 2.86 years for DLBCL and 4.84 years for SLL, 3-year DFS was longer for patients that did not express high level of GST- π : 52.5 percent versus 0 percent in nodal MZL patients ($p = 0.01$), 77 percent versus 50 percent in DLBCL patients ($p=0.05$); and 100 percent versus 21 percent for SLL patients. Three-year OS was also longer for patients with low expression of GST- π : 93 percent of nodal MZL patients and DLBCL patients and 100 percent of SLL patients were alive, compared to 66 percent of nodal MZL patients ($p < 0.05$), 53 percent of DLBCL patients ($p < 0.05$), and 83 percent of SLL patients (p NS) highly expressing GST- π .

Finally, we conclude that GST- π is almost constantly expressed in MCL and seems to be correlated to a poor survival when overexpressed in other B-cell lymphomas. After further validation in larger series, immunohistologic analysis of GST- π protein expression may become helpful diagnostic marker to differentiate MCL to splenic MZL and could constitute an interesting therapeutic target in patients with MCL.

Acknowledgement

We thank Aurelie Penet and Frederique Rebouillat for their excellent technical help.

C.T. is supported by the Hospices civils de Lyon - Appel d'offres Jeune Chercheur AO HCL 2004 – UF 36294-, France - A.R. is supported by the Interdisciplinary Center for clinical Research (IZKF), University of Wuerzburg, Germany

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Figure 1. GST- π immunostaining

In normal lymphoid tissue (1a), GST- π is normally expressed in the cytoplasm of dendritic cells, macrophages (red arrows), and plasma cell (black arrows). In MCL cases (1b), a high GST- π expression was observed, as a strong cytoplasmic signal in more than 50% of the cells. (Microscope: LEICA DMLB; objective lenses: LEICA HCPL Fluotar $\times 20$; camera SONY 3CCD exwave HAD; acquisition software SAMBA)

Table 1

GST- π immunostaining in B-cell lymphomas

	n	GST- π Immunostaining			Cyclin D1
		GST- π < 5%	GST- π 5– 50%	GST- π > 50%	
Mantle cell lymphoma	136				
with t(11;14)	25	2	0	23	25
without t(11;14)	6	1	0	5	0
Splenic marginal zone lymphoma	31	31	0	0	0/12
Extranodal marginal zone lymphoma	5	5	0	0	0
Nodal marginal zone lymphoma	19	5	11	3	-
Small lymphocytic lymphoma	21	12	3	6	0/8
Follicular lymphoma	15	13	2	0	-
Diffuse large B-cell lymphoma	14	9	1	4	-