High Level of Glutathione-S-Transferase \( \pi \) Expression in Mantle Cell Lymphomas

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ABSTRACT

Purpose: Prognosis of mantle cell lymphoma (MCL) remains poor. Patients who achieve a response to first line therapy usually relapse, and the probability of cure remains low. Glutathione-S-transferase \( \pi \) (GST-\( \pi \)) overexpression has been associated with alkylating agents and anthracycline resistance. GST-\( \pi \) gene is located in 11q13 and is coamplified along with CCND1 gene in some human solid tumors.

Experimental Design: We performed immunohistochemical analysis of GST-\( \pi \) expression in 24 consecutive MCLs, 12 follicular lymphomas (FLs), and 69 diffuse large B-cell lymphomas (DLBCLs). Cases were classified in three groups: high GST-\( \pi \) expression (> 50% of cells were stained), moderate (5 to 50% cells were stained), or absent (< 5% cells were stained). GST-\( \pi \) and CCND1 mRNA levels were also assessed by real-time reverse transcription-PCR analysis.

Results: All MCLs exhibit high GST-\( \pi \) protein expression, compared with 29% of the DLBCLs and none of the FLs. MCLs expressed high levels of GST-\( \pi \) and CCND1 mRNAs compared with DLBCLs and FLs. There was a strong relation between GST-\( \pi \) and CCND1 mRNAs transcript levels in MCLs but not in DLBCLs. In conclusion, protein and mRNA GST-\( \pi \) expression is high in MCL compared with FL and DLBCL.

Conclusions: Overexpression of CCND1 in MCL is associated with transcriptional up-regulation of the GST-\( \pi \) gene. Our results suggest that the glutathione system could play a role in drug resistance in MCL.

INTRODUCTION

Mantle cell lymphoma (MCL) is one of the most frequent of the newly recognized non-Hodgkin’s lymphoma entity in the Revised European American Lymphoma classification. Despite their morphological heterogeneity, these lymphomas are characterized by their immunophenotype (CD5+, CD23−, CD10−, sigM+, sigD+) and the t(11;14)(q13;q22) chromosomal translocation resulting in overexpression of the cyclin D1 gene (CCND1; Ref. 1). MCL appears to represent one of the worst prognostic categories of non-Hodgkin’s lymphomas. MCL lacks both the long survival of the indolent lymphomas and the curative potential of aggressive lymphomas. With conventional dose combination chemotherapy usually active in lymphomas, complete remission are difficult to achieve and median survival is only 36 months (2). First-line intensive high-dose chemotherapy seems to give better results in terms of complete remission and event-free survival, but the probability of cure remain low (3–6).

Glutathione-S-transferases (GSTs) are a complex multigene family of enzymes that may detoxify electrophilic xenobiotics such as alkylating agents (7–12). GST-\( \pi \) isoenzyme has been shown to catalyze the conjugation of glutathione to chlorambucil and thiota (13, 14). GST-\( \pi \) overexpression has been associated with resistance to alkylating agents and anthracyclines (7). Furthermore, transfection of GST-\( \pi \) antisense cDNA increased the sensitivity of a cancer cell line to various antitumor agents including Adriamycin, etoposide, and melphalan (15). GST-\( \pi \) isoenzyme is the main GST isoenzyme in lymphoid tissues, and GST-\( \pi \) expression has been recently correlated with efficacy of alkylating agent-based chemotherapy in head and neck carcinomas, acute myelogenous leukemia, and non-small cell lung carcinomas (8–12, 16–18).

The GST-\( \pi \) gene in located at 11q13 and is coamplified with the cyclin D1 gene (in the same amplicon) in some human solid tumors (breast carcinoma, squamous cell lung carcinoma, bladder carcinomas, and esophageal carcinoma; Ref. 19). The unusual resistance to chemotherapy in MCL compared with other lymphoma entities and the presence of both GST-\( \pi \) and cyclin D1 on the same 11q13 amplicon in some solid tumors led us to evaluate GST-\( \pi \) expression at the protein and mRNA levels in these lymphomas.

PATIENTS AND METHODS

Patients. Twenty-four consecutive untreated MCLs, 12 follicular lymphomas (FLs), and 69 diffuse large B-cell lymphomas (DLBCLs) with enough histopathological material were studied. Initial characteristics of the patients are summarized in Table 1. Routine stainings including H&E-safran and Giemsa, immunohistochemical study using a panel of monoclonal antibodies (L26/CD20, CD79a, CD3, CD5, CD23, CD10, CD43, CNA42, BcIII) was performed for diagnosis. All of the FLs included in this study had IgH-BcII (MBR-mcr) transcript and

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Note: A. Bennacere-Griscelli and J. Bosq contributed equally to this work.

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High Level of GST-π Expression

**Table 1** Phenotype and cytogenetic analysis of the 24 mantle cell lymphomas

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<tr>
<th>Patients</th>
<th>Cyogenetic</th>
<th>CCnD1</th>
<th>CD5</th>
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<td>nd</td>
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<td>CD20+</td>
<td>CD23−</td>
<td>BCL2+</td>
<td>ND</td>
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* ND, not done.

A t(14;18) detected by PCR and cytogenetic analysis, respectively. Molecular characterization of the 24 MCLs are presented in the “Results” section. Staging of the disease included complete physical examination with assessment of the performance status, blood counts, lactate dehydrogenase level, computed tomography scans of the chest and abdomen, gastrointestinal colonoscopy when appropriate, a bone marrow biopsy, and a lumbar puncture. The disease was then classified according to Ann Arbor classification and to the International Prognostic Index (IPI). Response to chemotherapy was assessed using recently reported guidelines (20).

Initial characteristics of the 24 MCL patients are presented in Table 2. Lymph node biopsy specimens were obtained in all cases. Although the number of antibodies used varied from case to case, all of the cases fulfilled the diagnosis criteria for MCL (Table 1). All of the 24 MCL cases showed similar results of CD5+CD20+CD23-CD10-BclII+ phenotype pattern. All MCLs except five were studied for t(11;14) and/or cyclin D1 overexpression using classical cytogenetic analysis or mRNA cyclin D1 overexpression, and all had t(1;14) and/or cyclin D1 overexpression assessed, as reported previously (21). Phenotype and cytogenetic and/or molecular analysis results are summarized in Table 1. Median age was 56 years (range, 28–73 years), and male/female was 3.2. Twenty-one had stage III/IV disease (Ann Arbor stage); lactate dehydrogenase serum levels were elevated in 7 (29%) patients; performance status (according to the WHO classification) was 0/1 in 23 patients. Eleven (46%) patients had an IPI score of 0 to 1, and 13 (54%) patients had an IPI score of 2–4.

Patients with DLBCL had a median age of 58 (range, 20–95) and male/female of 1:1. Forty-one (59%) patients had stage I/II disease; lactate dehydrogenase serum levels were elevated in 25 (36%) patients; performance status (according to the WHO classification) was 0/1 in 64 patients (93%). Forty-five (65%) patients had an IPI score of 0 to 1, and 24 (35%) patients had an IPI score of 2–4. Most (10 of 12) of the patients with FL had stage IV disease, a good performance status (PS 0–1 in 11 of 12 cases), and a low IPI score (IPI 0–1 in 9 of 12 cases).

**GST-π Protein Expression Analysis.** Initial biopsy samples of all of the cases were fixed in formaldehyde (10%), AFA (alcohol, formaldehyde, acetic acid), or Bouin fluid and were embedded in paraffin. GST-π expression analysis was performed with the Envision rabbit method for GST-π (DAKO K 400) using two commercially polyclonal antibodies [NCL

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<table>
<thead>
<tr>
<th>Table 2</th>
<th>Patients’ characteristics</th>
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<td></td>
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<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>39</td>
</tr>
<tr>
<td>≥60</td>
<td>30</td>
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<td>LDH</td>
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<tr>
<td>&gt;N</td>
<td>44</td>
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<tr>
<td>≤N</td>
<td>25</td>
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<tr>
<td>Performance status</td>
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<tr>
<td>&gt;1</td>
<td>28</td>
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<tr>
<td>Stage</td>
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<tr>
<td>I-I1</td>
<td>41</td>
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<tr>
<td>III-IV</td>
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<td>0–1</td>
<td>45</td>
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<tr>
<td>&gt;1</td>
<td>24</td>
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</table>

* DLBCL, diffuse large B-cell lymphoma; MCL, mantle cell lymphoma; FL, follicular lymphoma; LDH, lactate dehydrogenase; IPI, International Prognostic Index.
GST pi Novocastra, Newcastle, United Kingdom, and from DAKO (A36000). GST-π expression, defined by localized staining in the cytoplasm or the nucleus of the tumor cells, was classified according to the percentage of tumor cells stained. Briefly, it was defined as high when >50% of the tumor cells were stained, moderate when 5–50% tumor cells were stained, and absent when <5% tumor cells were stained.

**CCND1 (Cyclin D1) Gene and GST-π Transcripts**

Expression of CCND1 mRNA was quantified by real-time reverse transcription-PCR in a MCL established cell line (22) and was compared with the CCND1 gene expression in tumor samples from 11 patients with a DLBCL, 10 patients with a FL, and 12 patients with a MCL. Peripheral blood samples from nine healthy donors were also assessed as control. The relative kinetic method was applied using a standard curve constructed with 6-fold serial dilutions of total RNA from normal peripheral blood lymphocytes (PBLs) and from the MCL cell line.

Briefly, total RNA was extracted by RNA-B (Q-Biogen, Illkirch, France) and 1 µg was reverse transcribed using MuLV reverse transcriptase (Perkin-Elmer, Applied Biosystems). RNA quality and quantity were assessed by quantification of 18S samples with computed tomography values above 25 (threshold, 0.07) considered uninterpretable. Real-time quantification of CCND1 mRNA was performed using the specific target kit from Applied Biosystems according to manufacturer’s instructions. We used as endogenous control gene, the control kit 18S from Applied Biosystems. PCR reactions were performed on the ABI Prism 7700 Sequence Detection system (Perkin-Elmer Applied Biosystems, Foster City, CA), with thermal cycling conditions such as 50°C for 2 min, 95°C for 10 min, and 40 cycles at 95°C for 15 s and 60°C for 1 min.

The same cDNA sample was used for GST-π analysis and was amplified using the following primers and probes: Forward, 5'-CAT CTC CCT CAT CTA CAC CAA CTA TGA-3'; Reverse, 5'-GTC TTG CCT CCC TGG TTC TG-3'; probe, 5'-CGG GCA AGG ATG ACT ATG TGA AGG CA-3'. Sequences of primers and probes for the GST-π gene were designed using the primer express software program (Applied Biosystems).

We used the ddCt calculation method to determine the relative quantitation of both the CCND1 and the GST-π gene, because the efficiencies of the target and the reference 18S amplification were similar.

**Statistical Analysis.** CCND1 and GST-π expressions are described by the median Q25 (value under which are 25% of the values) and Q75 (value above which are 25% of the values) for each lymphoma type. Differences in CCND1 and GST-π expression between the different lymphoma entities were tested with nonparametric Wilcoxon tests.

The study of the relationship between CCND1 and GST-π was performed on the logarithms of relative expressions. It was performed at a global level (without making any distinction among the lymphomas) and separately for each of the different lymphoma entities. We focused on comparisons of the slopes of the curves because these slopes may be related to a coregulation of the two genes, and because differences in the slopes of the relationships might point out differences in the metabolic pathways involved in the regulation of CCND1 and GST-π.

**Table 3** Median [25% quantile to 75% quantile] CCND1 and GST-π expression in the different lymphoma entities

<table>
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<th>Lymphoma entity</th>
<th>n</th>
<th>CCND1</th>
<th>GST-π</th>
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<tr>
<td>MCL*</td>
<td>12</td>
<td>1906 [220–7145]</td>
<td>4.00 [2.68–15.3]</td>
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<tr>
<td>DLBCL</td>
<td>10</td>
<td>3.90 [1.65–18.4]</td>
<td>0.67 [0.50–0.88]</td>
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<tr>
<td>FL</td>
<td>9</td>
<td>4.60 [3.20–12.9]</td>
<td>0.24 [0.17–0.49]</td>
</tr>
<tr>
<td>PBL</td>
<td>8</td>
<td>0.58 [0.36–3.10]</td>
<td>1.07 [0.43–1.66]</td>
</tr>
</tbody>
</table>

* MCL, mantle cell lymphoma; DLBCL, diffuse large B-cell lymphoma; FL, follicular lymphoma; PBL, normal peripheral blood lymphocytes.

**RESULTS**

**GST-π Protein Expression by Immunohistochemical Analysis.** For the first 32 cases analyzed in this study (12 MCLs, 10 FLs, and 10 DLBCLs), the two polyclonal antibodies [NCL GST pi Novocastra, Newcastle, United Kingdom, and from DAKO (A36000)] were evaluated on the same lymphomas. No discrepancy between these two antibodies was observed. Therefore, only one polyclonal antibody (NCL GST pi) was used in the 74 other cases that were studied further.

A high GST-π expression (>50% cells stained) was observed in the 24 cases of MCL. None of the FLs tested expressed GST-π in more than 5% of the cells. High expression, (i.e., in less than 50% lymphoid tumor cells), was present in 20 DLBCLs (29%) and moderate expression (i.e., in 5–50% lymphoid tumor cells), was present in 22 DLBCLs (32%).

**Expression of CCND1 mRNA and GST-π mRNA by Real-Time Reverse Transcription-PCR.** The relative quantitation of GST-π and CCND1 mRNAs was performed by real-time reverse transcription-PCR in tumor samples from 12 MCLs, 11 DLBCLs, and 10 FLs and in 9 normal PBLs.

GST-π mRNA levels were significantly higher in MCLs compared with DLBCLs, FLs, and PBLs (P = 0.006, P = 0.002, and P = 0.007, respectively) (Table 3). GST-π mRNA expression did not differ among DLBCLs, FLs, and PBLs.

CCND1 mRNA levels were higher in MCLs compared with DLBCLs, FLs, and PBLs (Table 3); P = 0.002, P = 0.0002, and P = 0.0002, respectively). No difference was observed between DLBCLs and FLs, but both expressed higher levels of CCND1 mRNA compared with PBLs (P = 0.05 and P = 0.01, respectively; Table 3).

There was a significant relation between CCND1 mRNA and GST-π mRNA expression when all of the lymphomas were analyzed together (P < 0.0001). However, when the different lymphoma entities were analyzed separately, different patterns were present. There was a strong relation between CCND1 mRNA and GST-π mRNA level in MCL with a high expression for both genes (P = 0.003). The slope of the relation curve is 1.8 (Fig. 1). No relation between these two genes was observed in DLBCL (P = 0.1). In FL and PBLs, there was a relation between CCND1 mRNA and GST-π mRNA expressions (P = 0.001 and P = 0.04, respectively). However, CCND1 mRNA and GST-π mRNA were expressed at a low level in FL and PBLs, and the slope of the relation curve was less abrupt and equal to 0.8 (Fig. 1). Eleven DLBCLs had GST-π expression studied at both protein and mRNA levels. There was no significant relation between the GST-π protein expression level and
the amount of GST-\(\pi\) mRNA \((P = 0.1)\). The mean GST-\(\pi\) mRNA level was 0.78 in DLBCLs that expressed a high amount of GST-\(\pi\) protein (>50% tumor cells stained) compared with 2.61 in those that expressed a low amount of GST-\(\pi\) protein.

**DISCUSSION**

Since the revised European-American lymphoma classification, MCL is now a recognized lymphoma entity. MCL originates from the mantle zone surrounding lymph-node germinal centers, and tumor cells characteristically express CD5, CD19, CD20, FMC7, monotypic surface IgM and IgD, but rarely CD10 and CD23. The common MCL molecular basis is the t(11;14). This molecular event is not always identified because of the technical limitations of conventional cytogenetics, PCR, and Northern blot analysis (2).

MCL is one of the most chemotherapy-drug-resistant lymphomas. When patients are treated with classical cyclophosphamide-Adriamycin-vincristine-prednisone (CHOP) chemotherapy, the complete remission rate is low compared with other lymphoma categories, and salvage chemotherapy regimens, even with high-dose therapy with stem cell reinfusion, are not curative in the vast majority of patients (2). The probability of cure seems to be very low (2).

The 24 MCL cases were diagnosed on histological and phenotype analysis. Furthermore, all had cyclin D1 overexpression and/or (11;14)(q13;q22) translocation. Clinical characteristics showed that there was a male predominance, most of the MCL were stage III/IV according to the Ann Arbor classification, and one-half had a high serum lactate dehydrogenase level, as usually observed in MCL series (2). As in other MCL series, a high relapse rate and few complete remissions were observed when patients were initially treated with CHOP chemotherapy without high-dose chemotherapy (2).

To our knowledge, no classical drug resistance mechanisms have been evaluated in MCL. It has been shown that P53 mutation confers a poor prognosis in patients with MCL, but P53 mutation seems to occur in only 10–20% of cases (23, 24). Bcl/III is a well-known antiapoptotic protein, and its expression confers a broad spectrum of anticancer agent resistance. Bcl/II-protein expression has been shown to be a prognostic factor in DLBCL (25, 26). All of the MCLs tested expressed bcl2. High bcl2 protein expression is probably not the only factor involved in MCL drug resistance. FLs that expressed bcl2 in most cases have not the same pattern of early relapse when treated with anthracyclin and alkylating-based chemotherapy, which suggests an additive drug-resistance mechanism in MCL present at diagnosis in most, if not all, the MCL cases.

Interestingly the GST-\(\pi\) gene is located on the chromosome 11q13, close to the \(CCND1\)/Cyclin D1/BCL1 gene. All of the MCLs tested revealed a high GST-\(\pi\) protein expression that was observed in approximately one-third of the DLBCLs but in none of the FLs. We observed that there was a positive relation between \(CCND1\) and GST-\(\pi\) mRNA expression in MCL and FL but not in DLBCL. This relation was especially strong in MCL, in which these two genes were highly expressed, which was not the case in all of the others lymphomas tested. The strong \(CCND1\) mRNA expression was already largely known in MCL but not the strong GST-\(\pi\) mRNA expression. The reason why there was a relation between these two genes that are strongly expressed in MCL is currently unknown. One possibility could be the consequence of a position effect, as has been observed with c-myc expression in translocations involving IgH enhancers (27, 28).

No relationship between GST-\(\pi\) protein and mRNA level was observed in DLBCL, but only 11 cases were analyzed with both techniques and only 5 of 11 DLBCLs had a high protein GST-\(\pi\) expression. These results need to be confirmed with large numbers of DLBCLs, because we found that GST-\(\pi\) expression (at both the mRNA and protein levels) is very heterogeneous in DLBCL. One possibility could be that changes in the half-life of either mRNA or protein, or, as observed in other cell models, posttranscriptional regulation could also play a role in GST-\(\pi\) expression in DLBCL and that immunohistochemical analysis of the protein rather than an analysis mRNA should be preferred in these lymphomas (29–32). We recently observed that GST-\(\pi\) expression as assessed by immunohistochemistry is associated with a poor freedom from progression in DLBCL, but according to the IPI, is associated with survival and a good prognosis in DLBCL (IPI 0–1; Ref. 33). The results observed in DLBCL and in MCL suggest that GST-\(\pi\) expression could be a marker of sensitivity to anticancer agents in lymphoid malignancies. The precise mechanism whereby GST-\(\pi\) is responsible for resistance to anticancer agents is still a matter of debate (7, 15). Hypotheses based on the physical properties of this conjugating enzyme include glutathione conjugation to anticancer drugs, cytosolic sequestration of anticancer drugs by direct binding to GST-\(\pi\), and chemical reduction of the hydroxyl radicals formed by anthracyclins. Recently, new mechanisms underlying GST-\(\pi\)-mediated cellular protection against oxidative stress-induced cell death have
been identified. It has been shown that GST-π is involved in the coordinated regulation of stress kinase as reflected by increased nuclear factor κB activities together with suppression of JNK signaling (34–36). These observations, together with the potential importance of the nuclear factor κB pathway in DLBCL, suggest that this drug-resistance mechanism should be further studied in lymphomas (37).

The 10 patients who received sequential CHOP and cytarabine-based chemotherapy at diagnosis did not reach complete remission after CHOP alone but entered into complete remission after high-dose cytarabine (38). The modest role of the glutathione system in cytarabine resistance compared with alkylating and anthracyclin resistance could be an explanation for the high complete remission rate observed after high-dose cytarabine therapy in MCL (7, 39). It is noteworthy that the prognostic value of GST-π expression has already been reported in other human cancers such as acute myelogenous leukemia, head and neck carcinomas, breast carcinoma, and non-small cell lung carcinoma (16–18). The pleiotropic role of the glutathione system could be explained by the presence of GST-π in almost all of human tissue, and drug resistance mediated by GST-π is probably not specific of a tumor type but of anticancer agents metabolism. However, the location in the same 11q13 region of both the GST-π and the BCLI genes might explain the possible deregulation of the GST-π gene transcription in MCL.

In conclusion, MCL expressed GST-π at a higher level than did FL and DLBCL. The direct role of GST-π in in vivo drug resistance in MCL and DLBCL will have to be more precisely evaluated. The combination of both bcl2-protein expression and high GST-π expression could partly explain the broad anticancer agent resistance observed in MCL.

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