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DAP-kinase hypermethylation in the bone marrow of patients with follicular lymphoma

We studied whether DAP-kinase hypermethylation plays a role as a prognostic marker in patients with follicular lymphoma (FL). We found that DAP-kinase was frequently hypermethylated in bone marrow (BM) samples of 52 FL patients at diagnosis (71%) and identified patients with worse progression-free survival (p=0.06). In particular, patients with histologically proven BM infiltration and DAP-kinase hypermethylation had a poorer outcome (p=0.037). In a total of 170 BM samples obtained at diagnosis or during follow-up, DAP-kinase hypermethylation and the bcl2/IgH rearrangement gave concordant results in 67% of samples (48% both positive, 19% both negative). Both markers were independent predictors of the disease status (p<0.001).

Key words: DAP-kinase hypermethylation, follicular lymphoma, prognosis.

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Follicular lymphomas (FL) are characterized by the t(14;18) translocation which induces overexpression of the Bcl-2 protein. Yet, 10-20% of FL cases lack Bcl-2 overexpression and still exhibit inhibition of apoptosis.1 Mice overexpressing Bcl-2 under the control of an immunoglobulin H enhancer develop follicular hyperplasia, but not FL, and rare bcl2/IgH+ cells can be detected in non-malignant human lymph nodes and tonsils with follicular hyperplasia (reviewed by de Jong).2 These data suggest that additional pathogenetic mechanisms, other than Bcl-2 overexpression, exist in FL.

Death associated protein kinase (DAP-kinase) is a pro-apoptotic serine-threonine kinase involved in the extrinsic pathway of apoptosis, initiated by γ-interferon, FAS ligand and tumor necrosis factor-α.3,4 Inactivation of DAP-kinase due to promoter hypermethylation is a common event in B-cell diseases, in particular in FL, in which it occurs in about 80% of cases.5 Since DAP-kinase suppresses the transformation of primary fibroblasts by Myc, in the presence of p53,5,6 its inactivation may prevent apoptosis and could be a pathogenetic mechanism in non-Hodgkin’s lymphoma. Furthermore, it could be one of the events that determine disease progression.

The t(14;18) translocation giving rise to the bcl2/IgH rearrangement is a marker of minimal residual disease. Although the clinical significance of circulating bcl2/IgH-positive cells is still controversial, molecular monitoring has been shown to be of clinical value in patients with FL.6-10 In patients lacking a detectable bcl2/IgH rearrangement, an alternative BM marker of residual disease may be useful to evaluate treatment results and to prospectively identify patients at risk of relapse.

We investigated whether DAP-kinase hypermethylation in the BM of patients with FL could have a prognostic value and be a marker of persistent disease.

Design and Methods

Patients’ characteristics

We studied 52 patients with grade I-III follicular non-Hodgkin’s lymphoma (20 males, 32 females, median age 54 years, range 20-76 years). Tumor samples were derived from lymph nodes and BM obtained during routine diagnostic procedures. Diagnosis was based on morphology and immunophenotype and was complemented by polymerase chain reaction (PCR) analysis for the bcl2/IgH rearrangement. After initial diagnosis, 34 patients were treated with CHOP (cyclophosphamide, doxorubicin, vincristine and prednisone), 10 with fludarabine/mitoxantrone, 4 with radiotherapy alone, 1 patient with chlorambucil and 3 patients were followed with a wait and watch approach. Rituximab (375 mg/m², four cycles) was added to chemotherapy in 32 patients. Complete remission, partial remission and relapse were defined according to standard international criteria. The study was conducted according to good clinical and laboratory practice rules, and the principles of the Declaration of Helsinki.

Analysis of the bcl2/IgH rearrangement and DAP-kinase hypermethylation

BM samples harvested at the time of diagnosis were available for 45 patients.
Mononuclear cells were separated from BM aspirates, using Ficoll density centrifugation. DNA was extracted using DNAzol (Gibco BRL, Eggenstein, Germany). To study minimal residual disease, bcl2/IgH-nested PCR and BCL-2 reference PCR assays were performed.  

DAP-kinase promoter hypermethylation was determined by a methylation-specific PCR, as described by Katzenellenbogen et al., with minor modifications as previously published. The presence of a distinct methylated band on a 3% agarose gel was taken as demonstrating hypermethylation. The sensitivity of the methylation-specific PCR, studied by serially diluting Raji cells (ATCC, Middlesex, UK), which are completely methylated for DAP-kinase, into a negative control (Hela cells), was 0.02% (data not shown). The functional significance of DAP-kinase hypermethylation was assessed by induction of DAP-kinase mRNA in Raji cells, following treatment with the demethylating agent decitabine at 1 μM (data not shown).

**Results and Discussion**

**Frequency of DAP-kinase hypermethylation at diagnosis**

We examined BM and lymph node samples obtained at diagnosis from 52 patients with FL. At the time of initial diagnosis, 19 of 28 (68%) lymph node biopsies were bcl2/IgH-positive, while 20 (71%) were hypermethylated for DAP-kinase. The presence of the bcl2/IgH rearrangement did not correlate with DAP-kinase hypermethylation (p=0.69). BM samples frequently showed a DAP-kinase hypermethylated signal (32/45, 71%). PCR for bcl2/IgH rearrangement was informative in the BM aspirates of 52% of our patients with FL, which is in line with results reported by other authors. DAP-kinase hypermethylation was present at the same frequency in BM samples of both bcl2/IgH-positive and negative patients (p=0.32). When looking for associations between hypermethylation and patients’ characteristics, no such correlations were evident, with the exception of frequent DAP-kinase hypermethylation in patients with low hemoglobin levels (p=0.019).

**Prognostic role of DAP-kinase hypermethylation at diagnosis**

Following treatment, 46 of 49 patients were evaluable for response. Thirty-five patients achieved complete remission, 9 patients partial remission, and 2 patients were resistant to treatment. The three patients in the watch-and-wait regimen did not progress. Seventeen patients relapsed. At a median follow-up of 20.8 months (range 5.5-60 months), progression-free survival was 73% (95% CI: 55%-84%), and 46 patients (96%) are alive. Hypermethylation of DAP-kinase in BM samples obtained at diagnosis did not correlate with response to induction treatment (22 hypermethylated samples in 33 patients in complete remission versus 7 in 9 patients with persistent disease, p=0.2). On the other hand, DAP-kinase hypermethylation showed a trend to being a negative prognostic factor for progression-free survival (p=0.06, Figure 1A). This trend was confirmed when restricting the analysis to patients whose treatment included rituximab (p=0.06) and reached statistical significance when restricting the analysis to patients treated with CHOP (p=0.012). On the other hand, the presence of bcl2/IgH did not have any prognostic value (Figure 1B).

The presence of BM infiltration tended to be associated with a worse progression-free survival (Figure 1C, p=0.1). Analyzing the 21 patients with a positive BM biopsy at diagnosis, DAP-kinase hypermethylation identified a subgroup of patients with reduced progression-free survival (23% at 2 years, 95% CI: 4%-53%), while the 2-year progression-free survival was 100% in patients with BM infiltration who were negative for DAP-kinase methylation (Figure 2A). Also under these conditions, the presence or absence of bcl2/IgH had no prognostic impact on progression-free survival (Figure 2B).

**DAP-kinase hypermethylation and bcl2/IgH rearrangement in the BM during follow-up**

We then analyzed 173 BM samples obtained at diagnosis and during follow-up. Positivity for the bcl-2/IgH rearrangement and methylated DAP-kinase were concordant in 67% of 170 samples analyzed (48% both negative and 19% both positive). This overlap in a major proportion of samples resulted in a significant association...
between the two markers as evidenced by Fisher’s exact test ($p<0.01$). On the other hand, additional information was obtained in 11% (only bcl2/IgH positive) and 22% (only DAP-kinase hypermethylated) of samples. DAP-kinase hypermethylation in BM was a risk factor for disease activity (OR: 5.5; CI: 2.8-10.9), as was the bcl2/IgH (OR: 6.2; CI: 2.7-14) ($p<0.001$). The multiple logistic regression analysis showed that the additional information each marker provided translated into independent prediction of disease status (OR: 4.2; CI: 2.1-8.6 and OR: 4.5; CI: 2.0-10.1; $p<0.001$ for DAP-kinase hypermethylation and bcl2/IgH, respectively). Restricting the analysis to the 128 BM samples obtained during follow-up, DAP-kinase hypermethylation and bcl2/IgH continued to be independent predictors of disease activity (OR: 2.6; CI: 1.1-5.9; $p=0.026$ and OR: 3.9; CI: 1.6-9.8; $p=0.004$). Figure 3A shows DAP-kinase methylation status in seven patients during follow-up.

DAP-kinase hypermethylation is a frequent event in FL, but there are no data on the prognostic impact of this event in FL or on its role as a marker of residual disease, an alternative to the bcl2/IgH rearrangement. FL is a chemosensitive and radiosensitive disease and is also susceptible to immunotherapy. The spectrum of therapeutic possibilities vary from watch and wait strategies to high-dose therapies and it is unclear which patients will benefit most from a particular treatment. However, the great majority of patients with FL will eventually relapse. Clinical and laboratory characteristics have been used to predict prognosis, and a Follicular Lymphoma International Prognostic Index (FLIPI) has recently been developed. The molecular characterization of FL may help to define the prognosis better and to identify new therapeutic targets. Methylation of DAP-kinase has recently been shown to be a frequent alteration in B-cell lymphomas, in particular in FL. Here, we confirm this finding: 71% of lymph node biopsies from patients with FL showed DAP-kinase hypermethylation. We found frequent DAP-kinase hypermethylation in DNA from BM aspirates at diagnosis (71%, 32/45); this finding did not correlate with the presence of the bcl2/IgH rearrangement, indicating that these two events targeting the apoptotic pathway are frequent, but independent, in the pathogenesis of FL.
Although treatment in our series was heterogeneous, we confirmed the data of the whole patient group and other solid tumors, but its prognostic significance in B-cell malignancies has not been reported so far.

We further evaluated DAP-kinase methylation as a marker of disease in the BM during the follow-up of patients with FL. No alternative markers have been described for patients with FL who are negative for DAP-kinase hypermethylation and the presence of bcl2/IgH. We therefore conclude that the DAP-kinase hypermethylation signal in BM samples of FL patients is most probably due to the presence of FL cells. This situation appears to have analogy with the finding of the bcl2/IgH rearrangement in highly enriched B-cell populations of normal individuals, while the bcl2/IgH PCR signal in FL patients during follow-up indicates the presence of FL cells.

DAP-kinase is frequently hypermethylated in myelodysplastic syndromes, which have been shown to occur in up to 10% of aggressively treated FL. Although we cannot exclude that DAP-kinase methylation was due to the presence of a dysplastic myeloid clone after therapy, associations with bcl2/IgH and disease status suggest a lymphomatous origin of the methylation PCR signal. Studies on selected cell populations could help to clarify this point.

In conclusion, this is the first report on DAP-kinase hypermethylation identifying a subset of FL patients with a negative prognostic profile. DAP-kinase hypermethylation may be used as a marker of residual disease, especially in those cases in which the bcl2/IgH rearrangement cannot be amplified or is not present. Prospective clinical studies will be needed to confirm the clinical value of DAP-kinase hypermethylation for molecular monitoring.

MTV: conception of the study, analysis of data, wrote the manuscript; DG: performed experiments, analysis of data; FD: analysis of data, wrote the manuscript; FG: performed experiments, analysis of data; GD: analysis of data, wrote the manuscript; MM: patients’ data collection, analysis of data; LML: collection and analysis of lymph node specimens, revision of manuscript; SH: conception of the study, analysis of data, wrote the manuscript; GL: revision of manuscript. This work was supported by a grant from Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.).

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