Histologically transformed follicular lymphoma exhibits protein profiles different from both non-transformed follicular and de novo diffuse large B-cell lymphoma

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The majority of untransformed follicular lymphoma (FL) follow an indolent clinical course and have a median overall survival that, in several series, exceeds one decade. Histological transformation (HT), usually to diffuse large B-cell lymphoma (DLBCL), occurs in 30% of all patients with grade I/II FL. HT is usually associated with a rapidly progressive clinical course, treatment resistance and poor survival. Although HT is a well-described clino-pathological event, the molecular mechanisms behind it are still largely unknown, particularly regarding changes in global protein expression. Moreover, no unequivocal prognostic tools have been identified to effectively predict the patients at risk of HT.

Therefore, we investigated the proteome in lymphoma samples reflecting distinct clinical settings to establish whether HT could be associated with specific protein profiles. First, we analyzed nodal FL tissue from two cohorts of patients as follows: those with a long (>10 years) indolent course and no verified transformation event (FL nonHT), and those characterized by the later occurrence of biopsy-verified HT (FLHT). Subsequently, we compared the protein expression profiles of pre- (FLHT) and post- (secondary DLBCL) transformation tissue samples. Finally, we compared the latter samples (secondary DLBCL) with a set of tissue biopsies from patients with DLBCL without known prior FL (de novo DLBCL; Figure 1a).

From each analysis, we identified differentially expressed protein spots (at least twofold and P < 0.05): 6 spots in the comparison FL nonHT vs FLHT (analysis A), 9 in the comparison FLHT vs secondary DLBCL (analysis B) and 28 in the comparison de novo vs secondary DLBCL (analysis C; Figure 1b) as listed in Table 1.

Several of the identified proteins showed complex expression patterns, for example, gelsolin, serotransferrin (TF), vimentin, hnRNP H, pyruvate kinase isozymes M1/M2 (PKM) and gyceraldehyde-3-phosphate dehydrogenase (GAPDH). Some of these were identified from more than one spot; some with approximately equal molecular masses but different isoelectric points and some from spots with lower molecular mass than expected for full-length protein. Furthermore, from some spots, various proteins were identified. The complex expression became further apparent by one-dimensional (1D) western blot (WB) analyses of these proteins.

Gelsolin showed high expression in the secondary DLBCL compared with de novo DLBCL (analysis C) identified from spot 4911, corresponding to the full-length protein. The gelsolin gene codes for two isoforms with similar molecular masses where only one of the isoforms was identified from the two-dimensional (2D) gels with a differential expression. Gelsolin expression assessed by 1D WB analysis showed a similar tendency of upregulation in the secondary DLBCL group as observed in the 2D gel analyses, although this was not significant (Supplementary Figure 1). Presumably, the expression of both the gelsolin isoforms is confined in the single major band observed in the WB and quantification with this method is thus restricted to an estimate of the total expression, as it was not possible to distinguish the two isoforms.

In the proteomic analyses, full-length TF (spot 7901) was downregulated in the secondary DLBCL group, both in comparison with FLHT (analysis B) and in comparison with de novo DLBCL (analysis C; Figure 1b). Downregulation in secondary DLBCL was confirmed by WB analysis at borderline significance. The quantification of TF expression was, as with gelsolin, based on several isoforms in that two distinct bands were observed, which migrated close together in the WB analysis (Supplementary Figure 1). Only one spot from the 2D gels containing TF was found to be differentially expressed, which indicated that only this isoform of TF was more than twofold downregulated in secondary DLBCL. Previous studies have identified TF from multiple spots in 2D gel analyses inferring the isoformic nature of TF in lymphoma tissue. Thus, it is unknown which band from the WB analysis corresponds to the identified differential spot on the 2D gels.

Vimentin was identified from two spots, that is, one spot together with tubulin (spot 1710) that migrated corresponding to full-length vimentin/full-length tubulin with high expression in secondary DLBCL compared with de novo DLBCL (analysis C) and from spot 0501 with high expression in FL nonHT compared with FLHT (analysis A) migrating with a lower molecular mass than expected for the full-length protein (Figure 1b, Table 1). Vimentin is also known to have several isoforms that are distinguishable by 2D gel analyses. These isoforms migrate with equal molecular masses and a 1D WB method is insufficient to discriminate the distinctive isoforms, as shown in the WB analysis (Supplementary Figure 1). As two proteins were identified from the single differentially expressed spot 1710, it is not possible to determine which protein is responsible for the expression change seen. Another aspect of the putative differential expression of vimentin was found in the spot corresponding to a fragment (spot 0501). The antibody against vimentin recognized both full-length vimentin and some fragments with lower molecular masses in WB analysis (Supplementary Figure 1). The signal from the band just below the full-length protein, ~40 kDa, presumably corresponding to spot 0501, was observed with a too low signal for quantification. Even longer exposure time, higher antibody concentration and higher amount of total protein were not able to generate a quantifiable signal from this band owing to the relatively higher amount of the full-length protein. The same was found for hnRNP H (spot 6202), which was identified with high expression in de novo DLBCL compared with secondary DLBCL (analysis C). This spot exhibited a lower molecular mass than expected for the full-length protein, and WB analysis was only able to show the fragment levels that were too low to be quantified (Figure 1b, Supplementary Figure 1). Full-length hnRNP H showed no significant differential expression in the WB analysis in agreement with no identified differentially expressed spots in the proteomic analysis with a molecular mass corresponding to full-length hnRNP H.

PKM was identified from two spots (8803 and 8805) with equal molecular masses corresponding to the full-length protein but with different isoelectric point (pI; Figure 1b). Spots 8803 and 8805 showed higher expression in secondary DLBCL in comparison with de novo DLBCL (analysis C) and for spot 8803 also in comparison...
Figure 1. (a) Schematic outline of the study. Four patient groups were analyzed. FLnonHT: patients diagnosed with FL and followed at least 11 years with no transformation to DLBCL ($n = 5$); FLHT: patients diagnosed with FL with subsequent transformation to DLBCL at a time point from 1 to 10 years after first diagnosis with FL ($n = 7$); secondary DLBCL: patients diagnosed with DLBCL following a previously known FL diagnosis ($n = 6$); and de novo DLBCL: patients diagnosed with DLBCL with no evidence of previous FL ($n = 9$). Protein expression profiles were compared between FLnonHT and FLHT (analysis A), between FLHT and secondary DLBCL (analysis B), and between secondary and de novo DLBCL (analysis C). (b) 2D PAGE analyses. Representative gels are shown from each group. Spots identified as differentially expressed are shown on the representative gels in the specified analyses. Analysis A: protein profiles compared between FLnonHT and FLHT (6 spots); analysis B: between FLHT and secondary DLBCL (9 spots); and analysis C: a comparison between secondary and de novo DLBCL (28 spots).
| Fold change (ratio) | Identified protein | SwissProt database code | Number of identified peptides | Mascot score | Molecular mass (Da) | Spot no. on 2D gels |
|---------------------|--------------------|-------------------------|------------------------------|--------------|------------------|------------------|
| Analysis A (FL<sup>HT</sup>/FL<sup>nonHT</sup>) |
| 0.20 | Glyceraldehyde-3-phosphate dehydrogenase | G3P_HUMAN | 8 | 612 | 36201 | 8301 |
| 0.25 | Fructose-bisphosphate aldolase A | ALDOA_HUMAN | 1 | 74 | 39851 | |
| 0.26 | Serum albumin fragment | ALBU_HUMAN | 1 | 65 | 71317 | 3604 |
| 0.32 | Vimentin fragment | VIME_HUMAN | 9 | 480 | 53676 | 0501 |
| 0.41 | ATP synthase subunit delta, mitochondrial | ATPD_HUMAN | 2 | 133 | 17479 | 0006 |
| 3.23 | Serum albumin fragment | ALBU_HUMAN | 3 | 147 | 71317 | 3602 |

| Analysis B (secondary DLBCL/ FL<sup>HT</sup>) |
| 0.28 | NI | | | | | |
| 0.31 | Actin<sup>a</sup> fragment | ACTB_HUMAN | 5 | 456 | 42052 | 6003 |
| 0.40 | Serine/arginine-rich splicing factor 2 | SRSF2_HUMAN | 1 | 100 | 25461 | 1305<sup>c</sup> |
| 0.45 | HLA class I histocompatibility antigen, B-15 alpha chain | HLA-B15_HUMAN | 4 | 220 | 40648 | 3504 |
| 0.47 | Serotransferrin | TFRE_HUMAN | 1 | 68 | 79294 | 7901<sup>d</sup> |
| 0.49 | NI | | | | | |
| 3.67 | Serine/arginine-rich splicing factor 1 | SRSF1_HUMAN | 2 | 74 | 27842 | 2309 |
| 2.37 | Peroxiredoxin-1 | PROX1_HUMAN | 2 | 79 | 22324 | 7104 |
| 2.21 | Pyruvate kinase isozymes M1/M2 fragment | KPYM_HUMAN | 21 | 1465 | 58470 | 8803<sup>da</sup> |
| 0.47 | Catalase | CAT_A_HUMAN | 2 | 126 | 59947 | |

| Analysis C (secondary DLBCL/de novo DLBCL) |
| 0.26 | Macrophage-capping protein fragment | CAPG_HUMAN | 1 | 65 | 38760 | 3207<sup>ed</sup> |
| 0.26 | Coromin-1A | COR1A_HUMAN | 6 | 388 | 51678 | 5802 |
| 0.31 | Tubulin<sup>b</sup> fragment | TBA1_HUMAN | 4 | 306 | 50820 | 5401 |
| 0.33 | Actin<sup>a</sup> | ACTA_HUMAN | 1 | 77 | 42367 | 2409<sup>ca</sup> |
| 0.33 | Tubulin fragment | TBA1B_HUMAN | 3 | 239 | 50804 | 2403<sup>a</sup> |
| 0.36 | Coflin-1 fragment | COF1_HUMAN | 1 | 75 | 18719 | 3003 |
| 0.37 | Serum albumin fragment | ALBU_HUMAN | 2 | 93 | 71317 | 5202 |
| 0.42 | Serum albumin fragment | ALBU_HUMAN | 5 | 316 | 71317 | 3603<sup>ea</sup> |
| 0.45 | Heterogeneous nuclear ribonucleoprotein H fragment | HNRH1_HUMAN | 4 | 276 | 49484 | 6202 |
| 0.45 | HLA class I histocompatibility antigen<sup>b</sup> | HLA-A102_HUMAN | 3 | 114 | 41181 | 5507 |
| 0.45 | Pyruvate kinase isozymes M1/M2 fragment | KPYM_HUMAN | 6 | 251 | 58522 | 6408 |
| 0.45 | Serotransferrin | TFRE_HUMAN | 1 | 68 | 79294 | 7901<sup>d</sup> |
| 0.46 | NI | | | | | |
| 0.46 | NI | | | | | 4002 |
| 0.46 | Glyceraldehyde-3-phosphate dehydrogenase | G3P_HUMAN | 4 | 292 | 36201 | 7305 |
| 0.47 | Tubulin<sup>b</sup> fragment | TBB5_HUMAN | 6 | 324 | 50095 | 4403 |
| 0.48 | Actin<sup>a</sup> | ACTB_HUMAN | 6 | 523 | 42052 | 3405 |
| 3.45 | Pyruvate dehydrogenase E1 component subunit beta, mitochondrial | ODPB_HUMAN | 2 | 140 | 39550 | |
| 3.33 | Gelsolin | GELS_HUMAN | 2 | 72 | 86043 | 4911 |
| 3.13 | Brain acid soluble protein 1 | BASP1_HUMAN | 4 | 163 | 22680 | 0610 |
| 2.70 | Glyceraldehyde-3-phosphate dehydrogenase | G3P_HUMAN | 7 | 584 | 36201 | 8403 |
| 2.70 | Glyceraldehyde-3-phosphate dehydrogenase | G3P_NEUCR | 1 | 52 | 36384 | |
| 2.50 | Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta | GB1_HUMAN | 3 | 182 | 38151 | 3407<sup>ae</sup> |
| 2.44 | Pyruvate kinase isozymes M1/M2 | KPYM_HUMAN | 20 | 1374 | 58470 | 8805<sup>ae</sup> |
| 2.38 | Vimentin | VIIM_HUMAN | 9 | 446 | 53676 | 1710 |
| 2.33 | Serum albumin | ALBU_HUMAN | 16 | 1030 | 71317 | 4906 |
| 2.33 | Pyruvate kinase isozymes M1/M2 | KPYM_HUMAN | 21 | 1465 | 58470 | 8803<sup>da</sup> |
| 2.17 | Perilipin-3 | PLIN3_HUMAN | 2 | 240 | 47046 | 1606 |
| 2.04 | Proteasome subunit beta type-9 | PSB9_HUMAN | 4 | 260 | 23364 | 0103 |

**Abbreviations:** DLBC, diffuse large B-cell lymphoma; 2D, two-dimensional; FL, follicular lymphoma; HT, histological transformation; no., number; NI, Not identified. *Mascot score: Ion score is \(-10\log(P)\), where P is the probability that the observed match is a random event. **A number of variants was found. Top hit listed. *Contaminated with tubulin from previous samples. *Identified from analysis B as well as analysis C. *Few additional significant peptides were found with scores just above significance level.
with FLHT (Figure 1b). In addition to PKM, catalase was also identified in both spots (Table 1). The expression of PKM was assessed by 1D WB analysis in which the isoforms are merged and seen as one band (Supplementary Figure 1). This combined PKM band revealed no significant difference, indicating that additional isoforms of PKM may be present. PKM was identified from spot 6408 as well. This spot migrated with a lower molecular mass than expected for full-length PKM. No apparent bands with lower molecular masses than full-length PKM were observed in the WB analysis, either because of low expression at a level below the detection limit or because the fragment was not recognized with the chosen antibody (Supplementary Figure 1).

GAPDH was identified from three spots with a complex expression, that is, spot 8301: GAPDH and fructose-bisphosphate aldolase A; spot 8403: GAPDH, mitochondrial malate dehydrogenase, and annexin A2; and spot 7305: GAPDH. Low expression of spot 8301 was observed in FLHT compared with FLnonHT (analysis A). In the comparison of the DLBCL presentation (analysis C), spot 8403 showed high expression in secondary DLBCL and spot 7305 showed high expression in de novo DLBCL. Spots 7305 and 8301 migrated almost entirely together and may represent two isoforms of GAPDH with different pl and equal molecular masses, whereas spot 8403 showed a slightly higher molecular mass as well as altered pl (Figure 1b). GAPDH has previously been shown to be post-translationally modified as reviewed in Sirover et al. and Butterfield et al. Interestingly, in the comparisons of the two DLBCL presentations (analysis C), the GAPDH-positive spots showed high expression (spot 8403) or low (spot 7305) expression in secondary DLBCL. Thus, there were opposite expression patterns for the GAPDH isoforms in the two DLBCL presentations. The presence of several isoforms from the 2D gels was also apparent by 1D WB analysis in which the expression of GAPDH was observed as a band corresponding to the molecular mass of full-length GAPDH together with a number of lower molecular mass bands (Supplementary Figure 1).

There is a clinical need for biomarkers that may allow risk stratification of FL patients based on their risk of HT. In DLBCL, only prior evidence of previous FL can distinguish secondary transformation from de novo DLBCL. Therefore, a characterization of biological features that allows a distinction between these patient subsets may be useful to identify novel biomarkers of potential therapeutic relevance. In this study, we identified several protein spots showing novel differential expression with regard to possible risk of HT. The majority of differentially expressed spots were identified from a comparison of two DLBCL presentations, namely secondary and de novo DLBCL, implying that these morphologically similar entities differ in disease biology. We will further investigate these putative biomarkers in a larger, previously published cohort together with functional studies of the complex protein expression observed. Such supplementary studies, both in our own material and in unrelated cohorts, are warranted to establish the clinical relevance of these putative markers with regard to HT.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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