Sortilin as a Novel Diagnostic and Therapeutic Biomarker in Chronic Lymphocytic Leukemia

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Abstract

Background: The overexpression of sortilin/neurotensin receptor has previously been reported in various human solid tumors but not in hematological malignancies. Here, we report the overexpression of sortilin in leukemic cells from patients with Chronic Lymphocytic Leukemia (CLL).

Methods: Flow cytometry was used to compare the expression of sortilin in CLL patients (n=52) and healthy individuals (n=26). Also, in vitro apoptosis induction was assessed in CLL Peripheral Blood Mononuclear Cell (PBMCs) following directly targeting of sortilin.

Results: The results showed a significant expression of sortilin on the surface of CLL PBMCs (range from 2.2 to 71.5%) in comparison to healthy individuals (range from 0.03 to 7.4%) (p ≤ 0.0001). The optimal cut-off value of sortilin expression was determined at 7.2% with high sensitivity and specificity. Treatment of leukemic cells with anti-sortilin antibody could induce apoptosis without any effect on normal cells.

Conclusion: Apoptosis induction in CLL cells together with a significant correlation between the expression of sortilin and CD23 represent a possible functional role of sortilin in leukemogenesis of CLL cells. Therefore, sortilin might be considered as a promising novel biomarker in diagnosis, monitoring, and therapy of patients with CLL.

Keywords: Apoptosis, Biomarker, Chronic Lymphocytic Leukemia, Monoclonal antibody, Sortilin

Introduction

B cell Chronic Lymphocytic Leukemia (B-CLL) is a clonal lymphoproliferative disorder characterized by accumulation of mature-appearing neoplastic lymphocytes with co-expressing CD5, CD19, and CD23. The majority of CLL patients live for a long time without any treatment. However, CLL has still to be considered as an incurable disease. Due to low-efficient therapy and risk of side effects, it is often advised waiting until the disease become progressive and the bothersome symptoms appear. Therefore, the discovery of molecular biomarkers enabling us to predict disease progression to start therapeutic interventions would be advantageous. Indeed, improvement in our knowledge about the biology of CLL will improve the therapeutic options.

Sortilin is a 95 kDa transmembrane glycoprotein with a deregulated expression in many human cancers and also neurological disorders. Human sortilin is encoded by SORT1 gene located on chromosome 1 and classified as a member of mammalian vacuolar protein sorting 10p domain (Vps10pD) family. As a multifunctional receptor, sortilin mediates transport of proteins such as neurotensin and neurotrophin to cell membrane or lysosomes, directing cell survival and tumorigenesis.

Several studies have reported that sortilin is deregulated in various human carcinomas including breast, colon, prostate, lung and melanoma. We have also previously reported the overexpression of sortilin in ovarian carcinoma using a developed specific mAb (clone 2D8). Here, we used this antibody to study the overexpression of sortilin in CLL patients in comparison with healthy individuals. The induction of apoptosis in CLL Peripheral Blood Mononuclear Cells (PBMCs) following 2D8 mAb treatment showed that sortilin may function as a survival factor in CLL. In this study, we attempted to evaluate sortilin/neurotensin re-
sorine were used simultaneously in other study 16. Af-

The controls including isotype control mAb, and stauro-

sporine (2 μM) (Sigma) was served as positive control.

Results

Sortilin expression in CLL and healthy PBMCs

The expression of sortilin on the cell surface of puri-

fied PBMCs from 52 CLL patients (median age 59

years, range 40-81) was compared to 26 healthy individu-

als (median age 46.5 years, range 25-70). The biologi-

cal characteristics and immunophenotyping of CLL pa-

tients are summarized in table 1.

Flow cytometry results showed that 2D8 mAb spe-

cifically detected sortilin on the surface of CLL cells, but not healthy PBMCs (Figure 1A). Results demon-

strated that the median expression of sortilin in CLL pa-

tients was 24.6% (range from 2.2% to 71.5%) in com-

parison with healthy individuals which was 4.2% (range

from 0.03% to 7.4%) (p ≤ 0.0001) (Figure 1B). However,

no significant difference in sortilin expression was ob-

served between progressive and non-progressive cases

Table 1. Clinical characteristics of CLL patients

<table>
<thead>
<tr>
<th>Gender</th>
<th>N=52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male/Female</td>
<td>30/22</td>
</tr>
<tr>
<td>Age</td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>62</td>
</tr>
<tr>
<td>Range</td>
<td>42.85</td>
</tr>
<tr>
<td>Treatment history</td>
<td>(N)</td>
</tr>
<tr>
<td>Prior treatment</td>
<td>0</td>
</tr>
<tr>
<td>Rai stage</td>
<td>(N)</td>
</tr>
<tr>
<td>0 (n=38), I (n=7), II (n=5), III and IV (n=0)</td>
<td>50</td>
</tr>
<tr>
<td>Lymphocyte count</td>
<td>(10^9/l)</td>
</tr>
<tr>
<td>Median (range)</td>
<td>30 (11-150)</td>
</tr>
<tr>
<td>CD23 (%)</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>58±3</td>
</tr>
<tr>
<td>Median (range)</td>
<td>63 (2-98)</td>
</tr>
<tr>
<td>CD5^+/19^+ (%)</td>
<td></td>
</tr>
<tr>
<td>Mean±SEM</td>
<td>77±1.8</td>
</tr>
<tr>
<td>Median (range)</td>
<td>78 (47-97)</td>
</tr>
<tr>
<td>ZAP-70 (N)</td>
<td></td>
</tr>
<tr>
<td>Greater than 20%</td>
<td>1</td>
</tr>
<tr>
<td>Lower than 20%</td>
<td>51</td>
</tr>
<tr>
<td>CD38 (N)</td>
<td></td>
</tr>
<tr>
<td>Greater than 30%</td>
<td>16</td>
</tr>
<tr>
<td>Lower than 30%</td>
<td>36</td>
</tr>
</tbody>
</table>
or even between different stages (0, I and II) of the malignancy. The commercial polyclonal antibody did not detect any cell surface expression of sortilin neither in healthy nor in leukemic cells.

**Determining cut-off value**

The cut-off value of sortilin expression in CLL patients was determined at 7.2% (AUC: 0.98, sensitivity: 96.1%, specificity: 94.2%) (p ≤ 0.0001) (Figure 2). Considering cut-off value of sortilin expression (7.2%), 3 out of 52 patients (5.7%) were identified as negative, and only one out of 26 healthy individuals (3.8%) was presented as false positive.

**Immunofluorescent staining**

The expression of sortilin in CLL patients was also investigated by immunofluorescent staining. Figure 3 shows that sortilin is expressed in leukemic cells but not in healthy PBMCs. MAb 2D8 also detected sortilin in CLL cell lines including 232-B4, WA-C3CD5+ and I83-E95 (Figure 3). Caov-4 ovarian cancer cell line was used as positive control. Nuclei were counterstained with DAPI (blue color).

**Western blot**

Cells were lysed in a lysis buffer (1% Triton X-100, 50 mM Tris, pH=7.4, 150 mM NaCl, 5 mM EDTA, 1 mM NaF, 20 mM Na4P2O7, 1% glycerol, 0.1% sodium dodecyl sulfate) containing 10% phosphatase inhibitor and used as positive control. However, sortilin expression was not detected in acute T cell leukemia (Jurkat) cell line as negative control.

**Figure 2. ROC curve shows the trade-off between sensitivity and specificity for cut-off value of sortilin expression in CLL patients. The optimal cut-off value was determined at 7.2% with the sensitivity and specificity of 96.1 and 94.2%, respectively. The Area Under the Curve (AUC) was 0.98.**

**Figure 3. Detection of sortilin expression using immunofluorescent staining. MAb 2D8 detected sortilin (green color) in CLL but not in healthy PBMCs. Sortilin was also detected in CLL cell lines (232-B4, WA-C3CD5+ and I83-E95). Caov-4 ovarian cancer cell line was used as positive control. Nuclei were counterstained with DAPI (blue color).**
sequent electro-transferring to PVDF membrane. The yacrylamide gel electrophoresis (SDS-PAGE) with sub-
loaded to each well of 10% Sodium dodecyl sulfate pol-
membranes were probed with primary antibodies in-

Apoptosis assay

Correlation between sortilin and CD23

A significant correlation was observed between sor-
tilin and CD23 expression in leukemic cells (r=0.27, p=0.045) (Figure 4). However, no correlation between sortilin and other CLL immunophenotypic markers includ-
ing CD38 and ZAP70 was identified (data not shown).

Expression of sortilin was studied in different stages
(0, I and II) of CLL; however, no statistically difference
was found. Perhaps more comprehensive studies could
assist in defining the sortilin as an adequate staging
marker in CLL patients. We found a significant relation-
ship between expression of sortilin and CD23 as an im-
munophenotypic marker of CLL. Since CD23 contrib-
utes to accumulation of long-lived B CLL cells, it may
conclude that sortilin can play a role in B cell survival
akin to CD23. CD23 is a cell surface protein considered
to be important in differentiation of CLL from Mantle
Cell Lymphoma (MCL) and Marginal Zone Lymphoma
(MZL) 21. Therefore, it is wise to investigate the lack/
presence of sortilin in patients with MCL and MZL;
which may add another tumor marker to the current di-
agnostic panel for differentiating CLL from other Non-
Hodgkin’s lymphomas.

Although it is not obvious why CLL cells harbor cell
surface sortilin, several studies suggest that sortilin may
also undertake vital tasks in the pathophysiology of
other cancers. Recent finding suggests a role of sortilin
in angiogenesis through releasing of exosomes to the
extracellular media 22. A complex composed of sortilin and
results shows an ectopic expression of sortilin by local-
izing the protein on the surface of malignant B cells
which differs from its subcellular localization either in-
tracellular or secreted forms in normal B cells 18. The
Western blot data using 2D8 mAb (Figure 6) clearly
shows expression of a 95 kDa band in CLL lysates but
not in normal healthy B cells (left panel) confirming our
immunocytochemistry data in normal B cells (Figure 3).
Additionally, our antibody detects a strong band of 40
kDa in CLL cells with a faint band in normal B cells.

Furthermore, using a commercially available anti-
sortilin antibody in Western blot experiments a 60 kDa
band of sortilin variant (in addition to the 95 kDa band)
was observed in CLL cells but not in normal healthy in-
dividuals (Figure 6, right panel). This may suggest that
the structure of sortilin protein expressed in malignant B
cells differs from its normal counterpart. Such difference
has been reported for ectopic expression of a 38
kDa proline/arginine rich end leucine-rich repeat protein
(PRELP) in leukemic B cells from CLL patients 19. It
seems that variation in the structure of ectopically ex-
pressed proteins may affect the subcellular localization,
representing a hallmark of cancer cells. This notion
should be considered in discovery of new markers tar-
geting cancer.

Here we report overexpression of sortilin on the
surface of leukemic B cells in comparison with healthy
PBMCs. Therefore, sortilin might be categorized as a di-
agnostic biomarker in CLL patients. As there was no
significant difference in sortilin expression between two
groups of progressive and non-progressive cases, it may
not be classified as a prognostic indicator of CLL. This
might imply that sortilin expression is a genuine phe-
nomenon of malignant B cells initiated since the for-
mation of leukemic cells.

Avicenna Journal of Medical Biotechnology, Vol. 11, No. 4, October-December 2019
Expression of Sortilin in CLL

two tyrosine kinase receptors containing TrkB and EGFR in exosome structure mediated communication and signaling events between the lung cancer cells and its target, the endothelial cells. Immunohistochemistry experiments performed in a cohort of breast cancers patients showed an increase in the expression of sortilin which was associated with the breast cancer aggressiveness and invasion. In other study, it was shown that activation of matrix metalloproteinases in HT29 colorectal cancer cell line led to cleavage of the luminal part of sortilin resulting in release of its soluble form (sSortilin). Subsequently, activation of Focal Adhesion Kinase pathway by sSortilin regulates numerous downstream intracellular pathways mediating either cell migration or metastasis. Sortilin as a neurotrophin transporter is implicated in cell proliferation and anti-apoptotic effect of Brain-Derived Neurotrophic Factor (BDNF) in colorectal cancer cell. The phenomenon is accelerated by high affinity binding of sortilin to pro-BDNF as an apoptosis inducer in this type of malignancy. Knocking down of sortilin using siRNA treatment showed that sortilin plays a survival role in Caov-4 ovarian carcinoma cell line. In the same way, sortilin silencing resulted inhibition of cell survival and migration through decreased activation of extracellular signal-regulated kinases (ERK) in CAL-62 thyroid cancer cell line.

To investigate the potency of sortilin as a therapeutic biomarker in CLL, we explored the apoptosis induction by targeting sortilin using 2D8 mAb. As shown in figure 5A, treatment with 2D8 mAb considerably led to apoptosis in CLL cells but not in healthy PBMCs (p≤0.01). It is not exactly clear how 2D8 mAb induces cell de-
struction; however, it is obvious that this antibody directly induces apoptosis in CLL PBMCs. Mechanisms like antigen crosslinking, blockade of ligand-receptor growth or survival pathways, or activation of death receptors potentially may elicit the induction of direct apoptosis in targeted cells 25. Previously, it was shown that sortilin forms a functional complex by heterodimerization with Neurotensin Receptor 1 (NT-R1), mediating internalization of neurotensin growth signaling pathways 26,27. Therefore, it is also possible that 2D8 mAb may induce direct apoptosis in CLL cells by blockade of this type of heterodimerization. Therefore, it is necessary to elucidate the expression of NTR1, its heterodimerization with NTR3, and also endogenous expression of neurotensin in CLL cells to firmly state this hypothesis. In overall, beyond the mechanism of action, mAbs can induce targeted cell killing alone or can enhance target cell susceptibility to chemo- or radio-therapeutics by affecting the modulation of anti-apoptotic pathways 28.

Here we suggest a combination of chemo- and immunotherapy regimens containing specific mAbs targeting sortilin which might increase the efficacy of responses in CLL. Akin to the improved response achieved from combination of anti-CD20 antibody (of atumumab) plus fludarabine and cyclophosphamide in patients with relapsed CLL 29. Since CLL is a complex malignancy with a very heterogeneous pattern, and many signaling pathways may act in parallel in cancer cells; a combination of multi-targeting antibody-based therapy is necessary 29.

Based on a report on expression profile of sortilin in normal resting B cells and few malignant B cell lines, no surface expression of sortilin was detected either in normal or malignant B cells 18. Although sortilin is expressed in normal B cells but its localization differs in normal and malignant conditions. Our results clearly show that our anti-sortilin antibody detects sortilin on the surface of malignant B cells. Such discrepancy in findings might be due to the nature of immunogen used for antibody generation, as our antibody generated against the very N-terminal part of sortilin encompassing signal peptide 12.

Conclusion

In conclusion, our results from apoptosis induction in CLL cells by targeting sortilin might be attributed to the significant role of sortilin in CLL survival. Therefore, we believe that this is an important finding, which may potentially contribute to diagnosis, monitoring, and therapy of patients with CLL.

Acknowledgement

We are very grateful to all blood donors participated in this study. This research was supported by a grant from Avicenna Research Institute.

Conflict of Interest

The authors declare no conflict of interest.

References


