The significance of mTOR (mammalian target of rapamycin) activity in human lymphomas

Doctoral theses

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INTRODUCTION

1. The significance of the mTOR signaling pathway

The mTOR (mammalian target of rapamycin) serine-threonine kinase is a 289 kDa protein, which integrates numerous positive and negative signals from different pathways. Therefore, mTOR signaling has an important role in the regulation of essential cell functions such as cell growth, proliferation, survival and motility.

The active form of mTOR kinase exists in two complexes termed mTOR complex 1 (mTORC1) and 2 (mTORC2), which possess distinct functional roles and positions in the signaling network. The elements of mTORC1 are mTOR, Raptor, mLST8, PRAS40 and Deptor, whereas those of mTORC2 are mTOR, Rictor, mLST8, mSin1, Protor and Deptor. Protein synthesis is the best characterized process controlled by mTORC1. S6 kinase 1 (S6K1) and eukaryotic translation initiation factor 4E (eIF4E)-binding protein 1 (4EBP1) can be directly phosphorylated by mTORC1. Phosphorylation of 4EBP1 results in the release of eIF4E, allowing cap-dependent translation of proliferation and survival promoting proteins (C-MYC, BCL-2, FGF-2, cyclin-D1, survivin). The activation of S6K1 promotes ribosome biogenesis.

mTORC2 controls several members of the AGC subfamily of kinases (AKT, serum- and glucocorticoid-induced protein kinase 1 [SGK1], and protein kinase C-α [PKC-α]). mTORC2 directly activates Akt by phosphorylating its hydrophobic motif (Ser473), a site required for its maximal activation. Therefore, mTORC2 influences survival by promoting antiapoptotic mechanisms, and regulates cell motility.

Genetic mutations have been identified in certain hematological malignancies (eg. leukemias, mantle cell lymphoma, multiple myeloma), which lead to the deregulation of the PI3K/AKT/mTOR pathway. Nevertheless, there is little information about mTOR activity in most lymphoma types.

2. Targeted therapy

Small molecules and monoclonal antibodies used in targeted therapy block the growth of cancer cells by interfering with specific molecules needed for carcinogenesis and tumor growth – mainly growth factor receptors, tyrosine kinase receptors and different proteins in survival pathways. Classical mTOR inhibitors – rapamycin and its analogs (rapalogs) – and

new generation mTOR inhibitors – dual mTORC1/mTORC2 inhibitors – are in the focus of pharmacological development, and preclinical as well as clinical trials. The Food and Drug Administration (FDA) approved the rapalog Temsirolimus for the treatment of advanced-stage renal cell carcinoma in 2007, making it the first mTOR inhibitor approved for cancer therapy.

3. Characteristics of diffuse large B cell lymphomas (DLBCL) and Hodgkin lymphomas (HL)

DLBCL is a heterogeneous group of lymphomas, accounting for 30-40% of non-Hodgkin lymphomas. Lymph nodes in DLBCL demonstrate a diffuse proliferation of large lymphoid cells that totally or (less frequently) partially efface the architecture. Currently used chemotherapy can achieve complete remission in 60-80% of patients, and 5-10 year survival is 50-70%. Two molecular subtypes have been distinguished, based on immunophenotypic and molecular genetic studies: germinal center (GC) and non-GC – also termed as activated B cell (ABC) – DLBCL, the latter of which carries a poorer prognosis.

HL is a monoclonal B cell derived lymphoid neoplasm often occurring in young ages. The neoplastic tissue usually contains a small number of scattered large mononucleated and multinucleated tumour cells (designated Hodgkin and Reed-Sternberg cells, respectively, collectively referred to as HRS cells) residing in an abundant heterogeneous admixture of non-neoplastic inflammatory and accessory cells. Hodgkin lymphomas are comprised of two disease entities: nodular lymphocyte predominant HL (NLPHL) and classical HL. Classical HL has been further subclassified into nodular sclerosis, lymphocyte rich, lymphocyte-depleted and mixed cellularity HL. Currently used chemotherapy can achieve complete remission in 70-80% of patients, and 5 year survival is 85-88%. The main challenge in the near future will be the development of strategies that decrease late morbidity (eg. due to secondary tumors such as lung cancer, melanoma, breast cancer and lymphoma) and mortality, but retain or increase the efficacy of current regimens.

4. Microenvironment and galectin-1 expression in Hodgkin lymphomas

In HL, malignant cells are typically outnumbered by reactive cells in the microenvironment, which have an important role in tumor progression. Complex crosstalk between tumor cells

and microenvironmental cells warrants cell proliferation and survival. The most abundant cell types are T helper 2 (T_H-2) and regulatory T cells. Hodgkin/Sternberg-Reed cells (HRS) recruit these cells through the expression of specific chemokines and cytokines (CCL5, CCL17, CCL22). Galectin-1 expressed by HRS cells has a role in T cell selection, and it can induce apoptosis in reactive T cells, thereby hindering inflammation and autoreactivity. Galectin-1 can help regulatory T cell proliferation by promoting IL-10 expression.

AIMS

1. The mTOR pathway plays a key role in the survival of tumor cells in several neoplasms. Little information is available about mTOR signaling activity in human lymphomas. Thus, we wished to investigate mTOR activity in different lymphomas.

    1.1. We wished to characterize the connection between prognostic data and mTOR activity in diffuse large B cell lymphomas and Hodgkin lymphomas in an extended number of cases.
    1.2. We wished to investigate the functional role of high mTOR activity in HL.
    1.3. We wished to examine the *in vitro* and *in vivo* effects of mTOR inhibition on lymphoma cell growth, survival and protein expression.

2. We wished to characterize the microenviroment of HL, in particular, the presence of regulatory T cells and galectin-1 expression.

METHODS

1. Tissue microarray (TMA)

Different lymphoma types (6 Burkitt-lymphomas [BL], 23 HL, 11 MCL, 9 anaplastic large-cell lymphomas [ALCL], 9 DLBCL, 12 marginal zone lymphomas [MZL], 13 chronic lymphoid leukemias/small lymphocytic lymphomas [CLL], 10 follicular lymphomas and 12 peripheral T-cell lymphomas) were included in the first TMA study.

The study was extended to include a total number of 83 HL patients (40 females, 43 males; age: 8-82 years, mean age: 29.8 years) and 68 DLBCL patients (34 females, 34 males; age: 13-87 years, mean age: 59 years).

2. Cell cultures and treatments

Hodgkin-lymphoma (KM-H2, L428, L1236, HDLM2, DEV), non-Hodgkin lymphoma (BHD1, HT58, BL41, BL41/95, Ramos, Raji, U937, SC1), multiple myeloma (U266), T-ALL (CEM, Jurkat), B-ALL (MN60, Nalm6), CML (K562), AML (HL60) and adherent cell lines (HeLa – cervix carcinoma, MDA-MB-231 – breast carcinoma) were treated with rapamycin, dual inhibitors (NVP-BEZ-235, PP-242), nocodazole, staurosporine, vincristine, etoposide and doxorubicin. Cell morphology was evaluated on methanol fixed and hematoxylin-eosin (HE) stained cytospin preparates.

3. Flow cytometry

The DNA content of cells was acquired using a FACScan flow cytometer (BD Biosciences). Data was analyzed with WinList software (Verity Software House).

4. Proliferation assay

Cells were incubated with resazurine solution (Alamar blue assay) and the resulting fluorescence was read on a spectrophotometer (absorbance: 570-590 nm) to assess viability and proliferation activity.

5. ELISA

Sandwich ELISA Kit (p4EBP1 – Thr37/Thr46, Cell Signaling) was used for the detection of phospho-4EBP1 according to the manufacturer's instructions. Optical density (OD) was measured at 450 nm wavelength.

6. Immunocytochemistry and immunohistochemistry

P-S6, mTOR, p-mTOR, Rictor, Raptor, Galectin-1 and p-Histone-H3 expression was detected in lymphoma cell lines on cytospin preparates.

Human and xenograft samples were investigated by IHC using p-S6, p-mTOR, p-4EBP1, p-p70S6K, p-Histone-H3, cleaved/activated caspase3, Rictor, Raptor, CD15, CD30, MUM-1, BCL-xL, BCL-2, NF-kappaB-p50 and Survivin antibodies. Vectastain or Novolink system and DAB chromogen was used for detection.

7. Duolink

Duolink immunocytochemistry was used for the in situ detection of the active form of ribosomal S6, and colocalised Rictor and mTOR kinase present in mTORC2.

8. Western-blotting

Protein extracts were transferred to PVDF membranes after SDS-PAGE. Membranes were incubated with p-mTOR, mTOR, p-p70S6K, p-S6, Rictor and galectin-1 antibodies, followed by HRP-conjugated secondary antibodies. Reactions were developed with ECL. β-actin was used to confirm equal protein loading.

9. Real-time PCR

RNA was isolated (Micro-to-Midi RNA isolation kit, Invitrogen) and reverse transcribed. Real-time PCR (Hs00899709_m1, TaqMan® Gene Expression Assay, Life Technologies)

was used to characterize the gene expression of galectin-1 in lymphoma cell lines. Gene expression was normalized to GAPDH.

10. Xenograft model

HL, BL and DLBCL xenograft tumors were established in SCID mice, and mTOR inhibitory treatment was performed for 3-8 weeks. Rapamycin (Sirolimus, Rapamune) was administered orally; DLBCL xenografts were also treated with a subcutaneous rapalog (Temsirolimus). Body weight and tumor diameter was measured 1-2 times per week. Tumor weight was measured in euthanized animals at the end of the experiments. Tumor tissues were formalin-fixed, paraffin-embedded and immunostained with human CD15, human CD30, p-mTOR, p-S6, cleaved/activated caspase3 (apoptotic marker), p-HH3 (proliferation marker) and galectin-1 antibodies.

11. Statistics

Statistics was calculated with paired Student's t-test, Chi square test and Fisher'exact test using SPSS (SPSS Inc., Chicago, IL, USA) and PAST softwares (PAST free software downloaded from http://folk.uio.no), and log-rank test using GraphPad software (GraphPad, San Diego, California, USA).

RESULTS

1. mTOR activity in lymphoma cell lines

The expression of mTOR kinase was similar in normal and malignant lymphoid cells. mTOR activity was confirmed and quantitated by investigating the active form of mTOR (p-mTOR) and its phosphorylated target molecules (p-4EBP1, p-p70S6K, p-S6) using ELISA, immunocytochemistry and Western-blotting. Lymphoma and leukemia cell lines expressed significantly higher p-4EBP1 levels than normal B and T cells, indicating increased mTOR activity. The expression of p-mTOR and its indirect target p-S6 was increased in these cell lines.

2. mTOR activity in mitotic lymphoid cells

We observed strong phospho-S6 immunopositivity in the dividing lymphoid cells of reactive lymph nodes, in different lymphoma biopsies and in lymphoma cells cultured *in vitro*.

3. Investigation of mTOR activity in human lymphoma biopsy samples

Immunostainings of several lymphoma types revealed high mTOR activity in mantle cell lymphoma, Burkitt lymphoma, diffuse large B cell lymphoma, anaplastic large cell lymphoma and Hodgkin lymphoma cases.

Regarding other lymphoma types, no or only low (0/+) mTOR activity was detected in marginal zone lymphomas, chronic lymphoid leukemias/small lymphocytic lymphomas and peripheral T cell lymphomas.

4. DLBCL and HL studies with an extended number of cases

High mTOR activity was found in 62% of the analysed DLBCL cases. mTOR-related phosphoprotein positivity was observed in 80% of non-germinal center-derived diffuse large B cell lymphoma cases, indicating mTOR activity. Moreover, Rictor (a characteristic protein of the mTOR complex2) was overexpressed in 43% of all diffuse large B cell lymphomas and in 63% of mTOR-active non-germinal center diffuse large B cell lymphoma samples. Rictor

overexpression with mTOR activity indicated significantly worse survival for patients than mTOR inactivity, or mTOR activity with low Rictor expression.

High mTOR activity was confirmed as a characteristic feature of HL, independently of the subtypes. All cases with low mTOR activity were in complete remission with at least 5-year disease-free survival. Overall survival in cases with high mTOR activity was 85%. Rictor overexpression (indicating potential mTORC2 dominant expression) was detected only in one out of 83 HL cases.

5. Anti-apoptotic protein expression and a possible connection to mTOR activity

We searched for a potential correlation between anti-apoptotic proteins (BCL-2, BCL-xL, Survivin and NFκB-p50) known to be overexpressed in HLs and the role of mTOR activity behind their expression. We found that BCL-xL and NFκB-p50 expression may correlate with mTOR activity in HLs, but this correlation was not significant (Fisher's exact test; $p=0.07$ and $p=0.86$, respectively).

6. Examination of HL microenvironment

FOXP3 positive regulatory T cells (Treg) were immunostained in HL samples. The numbers of Treg cells in tumor cell rich and tumor cell free areas were compered. The number of Treg cells was significantly higher in the microenvironment of HRS cells. Prognosis correlated positively with the number of Treg cells: more than 17% Treg cells in the microenvironment were indicators of good prognosis (100% disease free survival at 5 years).

7. Galectin-1 expression in HL microenvironment and in HRS cells

Strong galectin-1 expression was confirmed in tumor cells and in the extracellular matrix (ECM) in the majority of HL samples (63/73, 86%). Low mTOR activity and low galectin-1 expression was observed in parallel in two cases. Galectin-1 expression pattern did not show correlation to HL subtypes. Strong expression of galectin-1 in the ECM along with a high Treg cell number was characteristic in HL samples.

High galectin-1 expression was observed in HL cell lines. The alteration in galectin-1 expression after rapamycin treatment was investigated by real-time PCR and Western-

blotting. The translation of galectin-1 was decreased after mTOR inhibition. We confirmed the reduction of galectin-1 protein expression as a consequence of mTOR inhibition in HL xenograft tissues as well.

8. The effect of mTOR inhibitors in lymphomas *in vitro*

Lymphoma/leukemia cell lines were treated with classical and new generation mTOR inhibitors (rapamycin and dual inhibitors, respectively). Apoptosis induction and proliferation inhibiton was examined after 24-72 hours. mTOR inhibition lead to G1 cell cycle block in all cell lines without apoptosis induction after 72 h. Dual inhibitors had a stronger effect on proliferation than rapamycin. The effect of long term mTOR inhibitory treatment (96-120 h) was established in KMH2 and BHD1 cells (HL and DLBCL lines, respectively). Rapamycin was able to induce apoptosis in HL cell lines after 96 h, while dual inhibitor treatment was required to achieve an apoptotic effect in DLBCL cell lines. Rapamycin augmented the effect of chemotherapeutic agents and TGFβ.

9. The effect of mTOR inhibitors in lymphomas *in vivo*

Hodgkin, Burkitt and diffuse large B cell lymphoma xenografts were created and used to examine the effect of mTOR inhibition. Rapamycin treatment significantly reduced tumor volume and tumor weight in the treated animals. The anti-proliferative and apoptotic effect of *in vivo* treatment was also confirmed in xenograft biopsies.

10. Detection of mTOR activity in situ

A new method (Duolink) was established to quantitatively detect the amount of p-S6 and mTORC2 complex in cell lines. Using rapamycin treatment, we demonstrated that this method is able to quantitatively determine alterations in protein expression.

CONCLUSION

I. We have characterized mTOR activity in different lymphomas

a. We showed that p-S6 expression is increased in mitotic lymphoid cells compared to interphasic cells. This observation supports increased mTOR activity.

b. We showed that mTOR activity is a characteristic feature of MCL, BL, DLBCL, ALCL and HL.

c. High mTOR activity of tumor cells in HL and DLBCL cases was further elucidated. We confirmed that high mTOR activity is associated with poor prognosis in the ABC/non-GC subtype of DLBCL.

d. Rictor overexpression occured in 63% of mTOR active cases in DLBCLs. Rictor overexpression along with high mTOR activity indicated significantly worse survival for patients.

e. Increased mTOR activity was confirmed in more than 90% of HLs. Low mTOR activity had a good prognostic value with more than 5 year disease free survival in patients, with complete remission. In contrast to DLBCLs, Rictor overexpression (mTORC2 activity) was not characteristic in HLs.

II. We examined the expression of several extra- and intracellular proteins, which may play a role in the survival mechanisms of neoplastic cells in HL.

a. We observed the overexpression of the antiapoptotic protein Bcl-xL and NFκB-p50 in the majority of mTOR active HLs.
b. We showed increased galectin-1 expression in HL cells and in the extracellular matrix, which was independent of HL subtypes.
c. A high regulatory T cell number was characteristic in HLs, compared to reactive lymph nodes. Higher numbers of regulatory T cells had a good prognostic value in HL cases.
d. We confirmed the inhibitory effect of rapamycin treatment on galectin-1 translation *in vitro* and *in vivo*.

III. We characterized the effect of mTOR inhibition on tumor growth.

a. Long-term rapamycin treatment had an antiproliferative effect and induced apoptosis in Hodgkin lymphoma cell lines *in vitro*, and in xenografts *in vivo*. However, dual inhibitor treatment was necessary for apoptosis induction in the BHD1 DLBCL cell line.

b. We investigated the expression of Raptor and Rictor, characteristic for different mTOR complexes. Based on our *in vitro* experiments, the variable sensitivity of cell lines to mTOR inhibitors can be explained by the presence of different mTOR complexes (mTORC1 vs. mTORC2).
c. Rapamycin increased the apoptotic effect of chemotherapeutic agents and negative regulators (such as TGFβ) in lymphoma cells.

Taken together, mTOR activity may be a potential therapeutic target in different lymphoma types. However, patient and inhibitor selection criteria must be carefully considered. The combination of mTOR inhibitors with other agents will probably offer the highest efficiency for achieving the best clinical response, and may also allow dose reduction in order to decrease late treatment toxicity in these cases.

PUBLICATIONS

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