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1. Introduction

1.1 Hodgkin Lymphoma

Hodgkin lymphoma (HL) is one of the most frequent lymphomas in the Western world, with an annual incidence of about 3 cases per 100,000 persons. This lymphoid malignancy involves peripheral lymph nodes and can also affect organs such as bone marrow, liver and lung.

HL is divided into two main types: nodular lymphocyte-predominant HL (NLPHL) and classical HL (cHL) with morphological, phenotypic, genotypic differences. This last includes the following subtypes (Cross RM, 1969):

- **Nodular sclerosis:** this is the most common type of Hodgkin disease in developed countries, accounting for about 60% to 80% of cases. It is most common in young adults, but it can occur in people of any age. It tends to start in lymph nodes in the neck or chest.

- **Mixed cellularity:** this is the second most common type (15% to 30%) and is seen mostly in older adults (although it can occur at any age). It can start in any lymph node but most often occurs in the upper half of the body.

- **Lymphocyte-rich:** this subtype accounts for about 5% of Hodgkin disease cases. It usually occurs in the upper half of the body and is rarely found in more than a few lymph nodes.

- **Lymphocyte-depleted:** this is the least common form of Hodgkin disease, making up less than 1% of cases. It is seen mainly in older people. The disease is more likely to be advanced when first found, in lymph nodes in the abdomen as well as in the spleen, liver, and bone marrow.

The diagnosis of cHL is based on the identification of mononucleated Hodgkin cells (HC) and bi- or multinucleated Reed-Sternberg cells (RSC). These cells measure 20- 60 μm in diameter and display a large rim of cytoplasm and at least two nuclei with acidophilic or amphophilic nucleoli (**Figure 1**) (Stein H, *et al*., 2008).

The neoplastic population also includes a variable number of mononuclear elements showing similar cytological features to HRSC (**Figure 1**). Although regarded as diagnostic, HRSC are not exclusive of cHL, since similar elements may be observed in reactive lesions (such as infectious mononucleosis), B- and T-cell lymphomas, carcinomas, melanomas or sarcomas. (Stein H, *et al*., 2008).

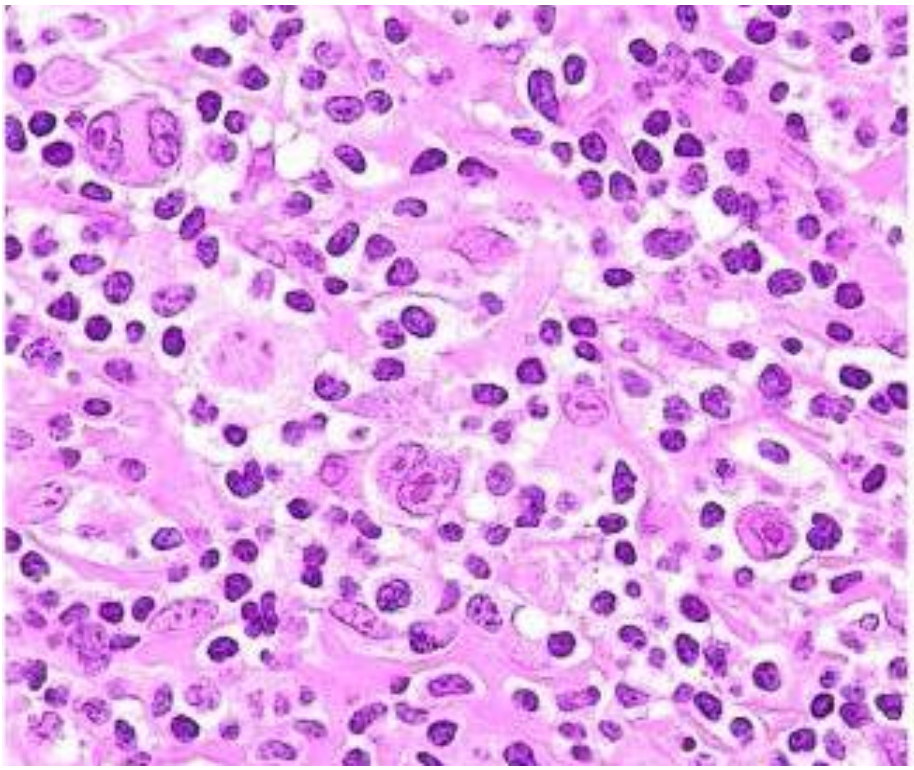

Figure 1. HRSC and HC in cHL (x400, Hematoxylin-Eosin).

1.1.1 Molecular biology

HRSC usually show multiple chromosomal abnormalities and are aneuploid. In addition, multiple subclonal aberrations are found, indicating chromosomal instability of the tumor (Weber-Matthiesen K, *et al*., 1995).

Some of them involve the oncogenes BCL1, BCL2, BCL3, BCL6, REL, and MYC, but for most cases the partner genes are unknown (Martin-Subero JI, *et al*., 2006; Szymanowska N, *et al*., 2008).

The detection of constitutive activity of the transcription factor NF-κB in HRSC prompted numerous studies to search for gene mutations that contribute to this activity (Bargou RC, *et al*., 1997). Genomic gains of REL, encoding an NF-κB factor, are present in about 30% of cases (Joos S, *et al*., 2002; Martin-Subero JI, *et al*., 2002). The positive regulator of the alternative NF-κB pathway, NIK, is also frequently affected by genomic gains in HRSC (Otto C, *et al*., 2012). Mutations in the genes of the NF-κB inhibitors Iκ-Bα and Iκ-Bε were found in about 10% – 20% of cases (Emmerich F, *et al*., 1999; Lake A, *et al*., 2009).

Other regulators of NF-κB, such as BCL3 and the tumor suppressor genes CYLD and TRAF3 are rarely mutated in HRSC (Martin-Subero JI, *et al.,* 2006). Another signaling pathway activated in HRSC for which genetic lesions have been found is the JAK/STAT pathway. JAK2 shows chromosomal gains in about 20% of HL, and in rare cases is translocated (Joos S, *et al*., 2000; Van Roosbroeck K, *et al.,* 2011). SOCS1, a main inhibitor of STAT activity, is affected by inactivating mutations in about 40% of classical HL cases (Weniger MA, *et al*., 2006).

The genomic region on chromosome 9p24, which shows gains in HRSC and in which the JAK2 gene is located, also encompasses the gene JMJD2C and the programmed death-ligand 1 (PD-L1) genes PD-L1 and PD-L2 (Green MR, *et al*., 2010). PD-L1s can inhibit PD-1–expressing T cells and thereby may contribute to an immunosuppressive microenvironment in HL (Green MR, *et al*., 2010). Several additional pathways are de-regulated in HRSC. These include the PI-3K/AKT, NOTCH1 and the MEK/ERK pathway (Dutton A, *et al.,* 2005; Zheng B, *et al*., 2003) (**Figure 2**).

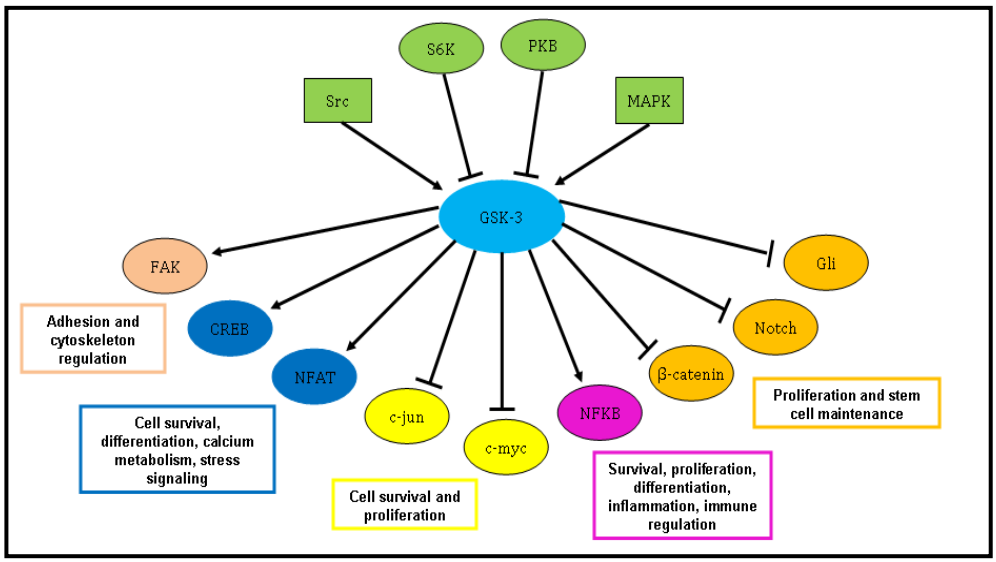

Figure 2. The scheme illustrates the GSK-3β involvement in several signalling pathways.

1.1.2 The role of the microenviroment

The microenvironment that surrounds the malignant cells of HL is a critical determinant of its initiation and progression. HRSC interact with CD4+ and CD8+ T cells, B cells, plasma cells, macrophages, mast cells, dendritic cells, neutrophils, eosinophils and fibroblasts and indeed actively attract them via the secretion of cytokines and chemokines (**Figure 3**). This interaction is presumably a very important factor for the survival and proliferation of HRSC. Numerous interactions can be envisioned. For example, CD40 stimulation leads to NF-κB activation and signaling through CD80 is an important costimulatory signal in B cell–T cell interaction. Other factors and interactions help to rescue HRSC from an immunological attack, including inhibition of cytotoxic T cells by Tregs (Marshall NA, *et al*., 2004). Cytotoxic T cells are also inhibited through expression of the PD1 and CD95 ligands and secretion of IL-10, TGF-β, and galectin-1 by the HRSC (Newcom SR, *et al*., 1995).

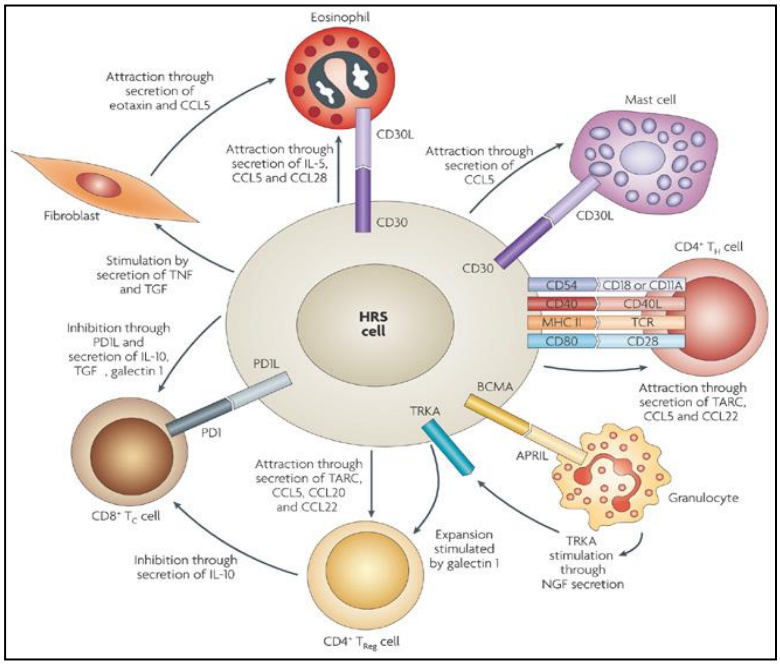

Figure 3. The microenvironment formation. The figure shows the molecules involved directly or indirectly in the recruitment and/or proliferation of cells constituting the cHL microenviroment.

1.2 Glicogen syntase kinase-3

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase (Embi N, *et al*., 1980; Rylatt DB, *et al*., 1980) involved in a number of cellular processes, including the division, proliferation, differentiation, and adhesion of cells (Kockeritz L, *et al*., 2006). Dysfunction of GSK-3 is implicated in several human diseases, including Alzheimer's Disease (AD), Parkinson's Disease (PD), type-2 diabetes, bipolar disorder (BPD) and cancer (Kockeritz L, *et al*., 2006; Frame S, *et al*., 2001). Two isoforms of GSK-3 have been identified, namely, GSK-3*α* (51kDa) and GSK-3β (47kDa), which although encoded by different genes are similarly regulated (Woodgett JR, *et al*., 1990). GSK-3β is the most studied form of GSK-3; its gene including 12 exons is located on the chromosome 3 (q13.3) and produces a 7134 bp mRNA (**Figure 4**).

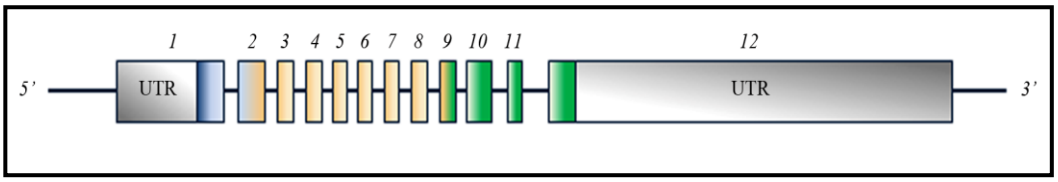

Figure 4. The GSK-3β gene structure. GSK-3β gene with its 12 exons; the grey boxes are untranslated regions (UTR).

Human GSK-3β is a 47 kDa protein with a small N-terminal domain, a kinase domain, presenting an ATP binding site and a protein active site and, finally, a C-terminal domain (**Figure 5**)

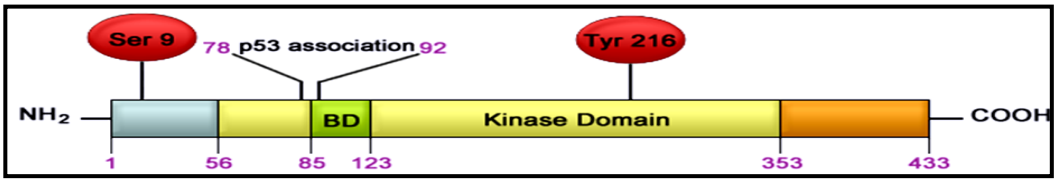

Figure 5. The GSK-3β gene and protein structure. Protein structure from the amino to the carboxy-terminal region.

1.2.1 Phosphorylation

GSK-3β is inactivated by diverse stimuli and signaling pathways. In particular, phosphorylation at the N-terminal Serine 9 (S9) residue is the most frequently examined mechanism that negatively regulates the activity of the kinase. This modification induces the interaction between the S9 and the substrate docking motif in the binding domain, generating a pseudosubstrate that inhibits the substrate access to the catalytic groove of the kinase (Doble BW, *et al.*, 2003). Several kinases can phosphorylate this serine, including Akt, protein kinase A (PKA), protein kinase C (PKC), p70 S6 kinase (S6K) and p90 ribosomal S6 kinase (Patel S, *et al*., 2008; Sutherland C, 2011; Kockeritz L, *et al*., 2006) (**Figure 6**).

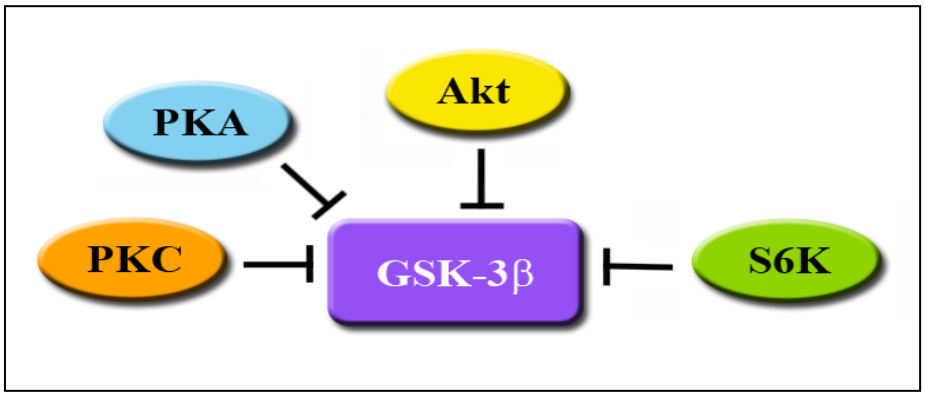

Figure 6. Kinases primarily involved in GSK-3β inhibition.

Thus, many signaling pathways that activate these kinases can inhibit GSK-3β by phosphorylation at S9 residue. A consequence of the GSK-3β inhibition is that concentration of primed substrates increases sufficiently to compete with the pseudo-substrate. However, it should always be borne in mind that the pS9 inhibitory mechanism does not necessarily regulate the phosphorylation of non-primed substrates by GSK-3β. Therefore, if a non-primed substrate is under investigation, examining changes in the pS9 of the kinase should be interpreted cautiously. In opposition to this inhibitory regulation, GSK-3β is activated by phosphorylation at Tyrosine 216 (Y216) residue, that is located in the "activation loop" of the enzyme (**Figure 7**).

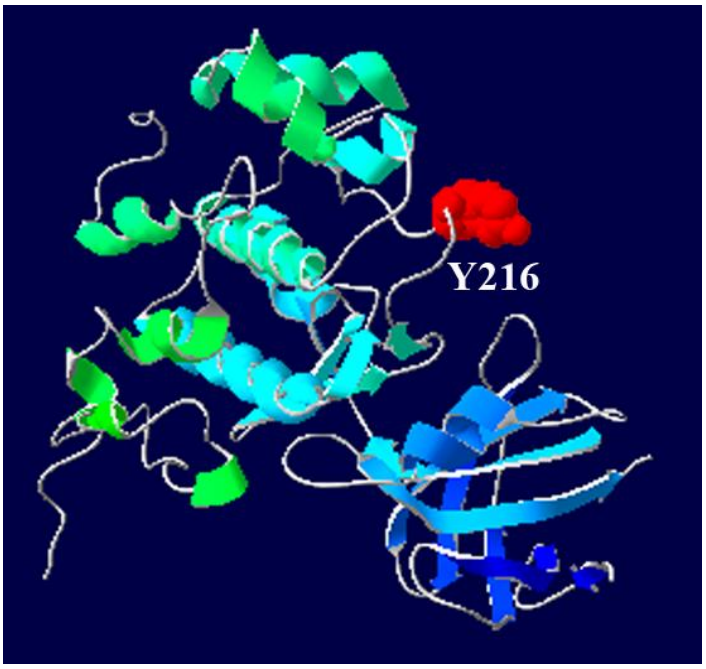

Figure 7. GSK-3β activation by Y216 phosphorylation. Y216 (red residue) is located in the "activation loop" of the enzyme, between the kinase domain and the binding domain.

Y216 might act as an autophosphorylation site or as a substrate for other protein tyrosine kinases, such as Pyk2, MEK1 and SRC-family tyrosine kinases (Cole A, *et al*., 2004). Several proapoptotic stimuli were also demonstrated to increase the activity and Y216 phosphorylation of GSK-3β (Bhat RV, *et al*., 2000), but the kinases mediating this modification remain unclear.

1.2.2 Cellular localization and complex formation

In addition to phosphorylation, mechanisms that regulate the intracellular localization of GSK-3β control its access to substrates. Although GSK-3β is traditionally considered a cytosolic protein, it is also located in nuclei and mitochondria, where it is highly activated compared with its cytosolic counterpart (Bijur GN, *et al.*, 2001; Bijur GN, *et al*., 2003). Nuclear GSK-3β is particularly interesting because it regulates many important transcription factors, such as cAMP response element-binding protein (CREB), GATA binding protein 4 (GATA4), hypoxia-inducible factor 1 (HIF-1), nuclear factor of activated T-cells (NFAT), NF-κB, Notch and p53. Meares GP, *et al.* reported the existence of a bipartite nuclear localization sequence in GSK-3β, consisting of residues 85–103, that was identified by assessing the subcellular localization of mutants created by site-directed mutagenesis (Meares GP, *et al*., 2007). Further studies will be needed to better understand the regulation of the nuclear and mitochondrial localization of GSK-3β.

1.2.3 GSK-3β regulation of the Wnt/β-catenin signaling

The Wnts are a family of secreted, cysteine-rich, glycosylated, protein ligands that influence cell growth, differentiation, migration (Miller JR, 2002). One of the pathways regulated by Wnt molecules is the canonical Wnt/β-catenin pathway (Hualsken J, *et al.*, 2002).

β-catenin can play multiple roles in cell physiology and it may be present in several cellular compartments, such as the cytoplasm and the nucleus where it forms an active complex containing T-cell factor/lymphoid enhancer factor transcription factors (TCF/LEF) (Graham TA, *et al*, 2000).

It has a central region which presents armadillo domain repeats important for the binding of partners, such as Axin-1 and adenomatous polyposis coli protein (*APC*) as well as transcription factors (Graham TA, *et al*., 2000) (**Figure 8**).

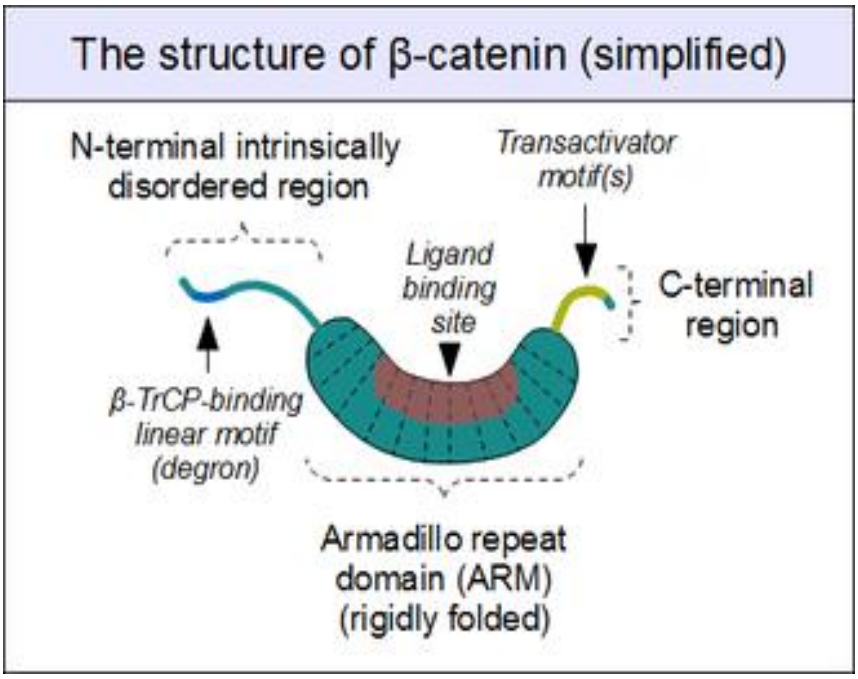

Figure 8. The simplified structure of β-catenin.

GSK-3β play an inhibitory role in this pathway. In a stimulated cells (**Figure 9**, **left**), after binding of Wnt to the receptor complex, the signal is transduced to cytoplasmic phosphoprotein Dishevelled (Dsh/Dvl).

Dsh inhibits the activity of the GSK-3β enzyme, and activates a complex series of events that lead to the prevention of β-catenin degradation and its consequent stabilization and accumulation in the cytoplasm (Hatsell S, *et al*., 2003). Stabilized β-catenin can translocate into the nucleus by a mechanism that remains poorly understood.

In unstimulated cells (**Figure 9, right**), cytoplasmic β-catenin is targeted for degradation by a destruction complex composed of proteins named axin-1, adenomatosis polyposis coli (*APC)*, GSK-3 and the casein kinase 1 (*CK1*) (MacDonald BT, *et al*., 2009) (**Figure 10**).

Axin-1 and APC act together as scaffolding proteins through binding of β-catenin, and enhance its N-terminal phosphorylation by GSK-3 and CK1. The first phosphorylation event is generated by *CK1* at S45 which allows the GSK-3 mediated sequential phosphorylation of Thr41, S37, and S33 followed by proteasomal degradation (Liu C, *et al*., 1999; Liu C, *et al*., 2002).

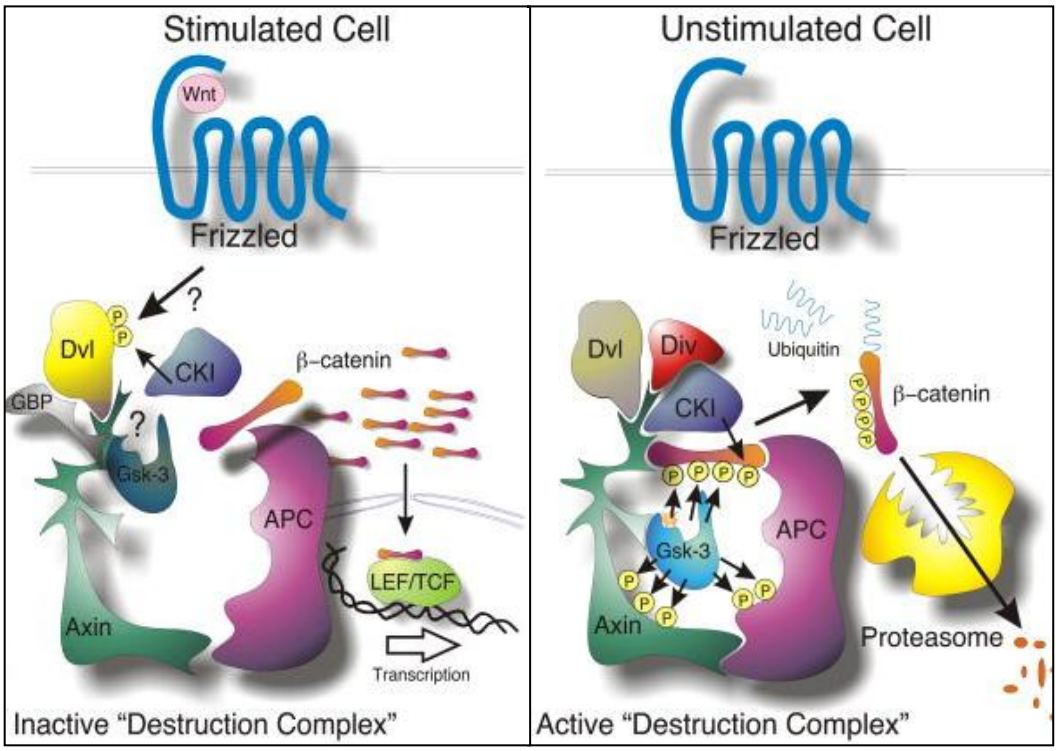

Figure 9. Central role of GSK-3 in the Wnt/β-catenin pathway. Wnt stimulation activates the receptor Frizzled which then signals through Dishevelled (*Dvl*) to inactivate β-catenin phosphorylation. Unphosphorylated β-catenin translocates to the nucleus where it binds the LEF/TCF transcription factors. In unstimulated cells, the destruction complex composed by *CK1*, axin-1, *APC* and GSK-3 binds the β-catenin, promoting its ubiquitination and proteasomal degradation.

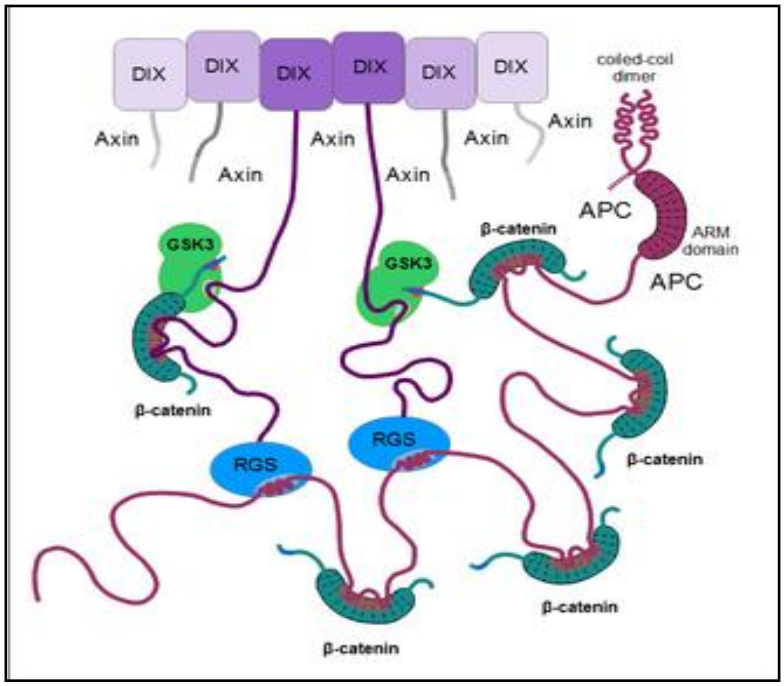

Figure 10. The simplified structure of β-catenin destruction complex.

1.2.4 GSK-3β and cancer

The role of GSK-3β in cancer remains complex and controversial. GSK-3β may play positive roles in cell proliferation and its aberrant expression as a tumor promoter. Several studies have reported an overexpression of GSK-3β in various tumor types including colon, liver, ovarian and pancreatic tumors (Luo J, 2009; Shakoori A, *et al*., 2005; Ougolkov AV, *et al*., 2005).

Farago *and collegues* showed that antagonizing the endogenous activity of GSK-3β by tissue-specific expression of a kinase-inactive GSK-3β in mouse mammary glands promoted mammary tumorigenesis (Farago M, *et al*., 2005). The promotion of mammary tumorigenesis by this kinase-inactive GSK-3β is accompanied by the accumulation of β-catenin and cyclin D1, suggesting that the promotion was mediated by the dysregulation of the Wnt/β-catenin pathway.

Moreover, emerging evidence earmark GSK-3β as a therapeutic target in acute human leukemias (MLL-associated), multiple myeloma and chronic lymphocytic leukemia indicating, a multifaceted role of this kinase in normal and malignant hematopoiesis (Wang Z, *et al*., 2008; Piazza F, *et al*., 2010; Ougolkov AV, *et al*., 2007).

Furthermore, GSK-3β has been implicated in cancer stem cell biology (McCubrey JA, *et al*., 2014) and, recently, it has been observed that targeting GSK-3β promotes imatinib-mediated apoptosis of chronic myeloid leukaemia stem cells (Reddiconto G, *et al*., 2012).

In a recent work, it has been documented an abnormal high expression of β-catenin and pGSK-3β (S9) in mantle cell lymphoma patients and the positive pGSK-3β expression significantly correlated with positive nuclear expression of β-catenin. Of the clinical parameters, negative pGSK-3β significantly correlated with a longer overall survival (Chung R, *et al.*, 2010).

GSK-3β also functions as a tumor suppressor. The dysregulation of GSK-3β phosphorylation and inhibition of GSK-3β activity has been shown to be involved in hepatocarcinogenesis (Desbois-Mouthon C, *et al*., 2002). In a recent study, it has been observed that the expression of GSK-3β in HCC tissues was significantly lower than that in normal liver tissues and pericancerous tissues (Huang KT, *et al*., 2012). In addition, low expression of the kinase was correlated with advanced clinicopathological characteristics and poor prognosis of HCC patients.

1.2.5 GSK-3β, inflammation and cancer

Cytokines generated by activated immune cells are considered important components in orchestrating the relationship between inflammation and cancer. Studies conducted over the last several years have elucidated the molecular mechanisms of intracellular signaling pathways of inflammatory cytokines for tumor development (Kundu JK, *et al*., 2008; Masuhara M, *et al*., 1997). GSK-3β has been identified by recent findings as vital factor in the inflammation process (Martin M, et al., 2005).

GSK-3β is mostly known as a pro-inflammatory agent and drugs that inhibit its activity are being developed for diseases such as Alzheimer's, cancer, diabetes and immune disorders. Indeed, Balamurugan *et al*. demonstrated that GSK-3β can act in cooperation with the protein FBXW7 as inhibitor of the inflammatory response (Balamurugan K, *et al*., 2013). In this work, authors showed a dual role of the kinase that might complicate clinical applications of drugs targeted at inhibiting GSK-3β.

Another study has pointed out how the overexpression of the C-C chemokine receptor type 7 (CCR7), involved in the development and progression of chronic inflammatory diseases and cancer, was partly mediated by the Akt/GSK-3β signaling pathway in colon cancer (Yu S, *et al*., 2015). The inhibition of the Akt/GSK-3β cascade may emerge as potential therapeutic strategy to reduce CCR7 expression in this neoplasm.

Moreover, the ability of GSK-3β inhibition to differentially regulate pro- and anti-inflammatory cytokine production and its functional role in adaptive immune responses might play an important role in the progression of esophageal cancer (Gao S, *et al*., 2014).

It has also been demonstrated that some inflammatory mediators in tumor microenvironment, including TGF-β and IL-6, contributed to cancer invasion and metastasis (Wu Y, *et al*., 2009). In particular, Salim *et al*. suggested a direct effect of the pro-inflammatory mediator leukotriene D4, a component of the tumor microenvironment, in regulating the proliferation and migration of colon cancer cells, most likely via a GSK-3β/β-catenin signaling pathway (Salim T, *et al*., 2014).

In a recent study, authors investigated the effect of flavonoid apigenin treatment on the expression of genes involved in inflammation and cancer in human pancreatic cancer cells (Johnson JL, *et al*., 2013). The results showed that apigenin inhibited the GSK-3β/NF-κB signaling pathway, leading to an induction of the mitochondrial pathway of apoptosis in cell lines. Moreover, gene expression analysis revealed apigenin treatment upregulated 59 genes and downregulated 63 genes related to inflammation and cancer.

It is currently recognized that chronically elevated TNFα, a major pro-inflammatory cytokine, in tissues may promote tumor growth, invasion and metastasis (Szlosarek P, et al., 2006). Michalaki *et al*. demonstrated that TNFα expression is significantly increased in the serum of prostate cancer patients and associated with tumor metastasis (Michalaki V, *et al*., 2004).

Furthermore, Vadrot *et al*. reported that GSK-3β was involved in TNFα-induced mitochondrial DNA (mtDNA) depletion and that p53 was necessary for the recovery of mtDNA content (Vadrot N, *et al*., 2012). They suggested that p53 binding to GSK-3β, TFAM and mtDNA regulatory region D-loop could participate in this recovery by stimulating mtDNA repair.

1.2.6 GSK-3β inhibitors: application in cancer

Given the central role of GSK-3β in all the aforementioned pathways, the therapeutic potential of its inhibitors has become an important area of investigation (**Table 1**).

Table 1. Clinical trials with GSK-3β inhibitors (Clinical Trials.gov)

Inhibitor Name	Therapeutic application	Ref/Clinical Trials
Tideglusib	Alzheimer's Dementia Cerebrovascular Diseases	NCT00948259 NCT01049399
LY-2090314	Advanced/metastatic cancer Leukemia	NCT01632306 NCT01287520 NCT01214603
Valproic acid sodium salt	Epilepsy, Mania, Bipolar disorder Alzheimer's Dementia	NCT00088387
Indirubin	Myeloid Leukemia	Damiens E, *et al*., 2000.

Identifier

NCT00948259: Phase II of Tideglusib used for treating patients with Alzheimer's disease.

NCT01049399: Phase II of Tideglusib for progressive supranuclear palsy.

NCT01632306: Phase I/II of LY-2090314 used in combination with different chemotherapies in treating advanced/metastatic pancreatic cancer.

NCT01287520: Phase I of LY-2090314 in combination with pemetrexed and carboplatin in patients with advanced/metastatic cancer.

NCT01214603: Phase II study of intravenous LY-2090314 in acute leukemia patients.

NCT00088387: Valproic acid sodium salt is used in clinical trial in combination with lithium for treatment of patients suffering from Alzheimer's disease.

Many tumors presents defects in elements of the Wnt pathway that result in the abnormal activation of Wnt signaling and accumulation of β-catenin (Lustig, B and Behrens J, 2003). Furthermore, the inhibition of GSK-3β stabilizes three cell-cycle regulators, cyclin D1, cyclin E and c-Myc, the overexpression of which is linked with tumorigenesis. Because GSK-3β inhibition is expected to mimic Wnt signaling and stabilize oncogenic proteins, there is concern that its inhibitors might be potent inducers of cancer. Several data have showed that long-term use of lithium at concentrations that inhibit the enzyme was not associated with increased cancer morbidity in patients with bipolar disorder (Cohen, Y, *et al.*, 1998). Under some circumstances, GSK-3β inhibitors should be examined for potential use in cancer therapy. An alternative way to circumvent the potential Wnt-mimetic activity of GSK-3β inhibitors is to design inhibitors that either target the phosphorylation of non-Wnt pathway substrates or inhibit GSK-3β activity only in a specific cell compartment.

2. Aim

GSK-3β is a serine/threonine kinase involved in cell cycle progression, differentiation, embryogenesis, migration, metabolism, survival and cellular senescence (Embi N, *et al*., 1980; Rylatt DB, *et al*., 1980).

The aberrant expression of GSK-3β has been implicated in the pathogenesis of many disorders such as diabetes, atherosclerosis, neurodegenerative diseases and cancer (Kockeritz L, *et al*., 2006; Frame S, *et al*., 2001).

Emerging evidence showed GSK-3β as a therapeutic target in acute human leukemias (MLL-associated), multiple myeloma and chronic lymphocytic leukemia indicating a multifaceted role of this kinase in normal and malignant hematopoiesis (Wang Z, *et al*., 2008; Piazza F, *et al*., 2008; Ougolkov AV, *et al*., 2007).

A recent study has demonstrated the biological and clinical relevance of the GSK-3β inactivation in mantle cell lymphoma (Chung R, *et al*., 2010).

The aim of the study is to clarify the biological significance of the kinase in untreated cHL patients, focusing our attention on its involvement in the β-catenin regulation in the canonical Wnt pathway.

3. Materials and Methods

3.1 Cell cultures

CHL cell lines were purchased from German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). L-1236 and L-428 cell lines were cultured in in RPMI 1640 medium (GE Healthcare, Piscataway, NJ) supplemented with 10% heat inactivated fetal bovine serum (EuroClone, Pero, Milan, Italy), 2 mM L-glutamine (EuroClone), penicillin (100 U/ml) and streptomycin (100 μg/ml) (GE Healthcare) and incubated at 37°C in a 5% CO2. Testing for Mycoplasma infection was carried at a monthly basis. Mononuclear cells (PBMCs) from healthy donors were separated by density gradient centrifugation using Lymphosep (Biowest, Nuaillé, France) and B lymphocytes were purified using CD19-coated magnetic MicroBeads according to the manufacturer's protocol (Miltenyi Biotech).

3.2 Blood samples, PBMCs collection and CD19+ cell isolation

Buffy coat from 10 healthy volunteers (HV) were supplied by the Officina Trasfusionale, Pievesestina. Donors were anonymous to us. The need of donor consent was waived by the Ethics Committee. The use of buffy coat was acknowledged by the Ethics Committee of Area Vasta Romagna. Mononuclear cells (PBMCs) from healthy donors were separated by density gradient centrifugation using Lymphosep (Biowest, Nuaillé, France) and B lymphocytes were purified using CD19-coated magnetic MicroBeads according to the manufacturer's protocol (Miltenyi Biotech).

3.3 Tissue samples

Cases were retrospectively collected from the Haematopathology Unit archives, Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna. The study was conducted according to the principles of the Declaration of Helsinki after approval of the Internal Review Board. All the cases were diagnosed according to WHO classification:

- 8 were sub classified as nodular sclerosis type 1 (sec. BNLI).
- 30 as nodular sclerosis type 2 (sec. BNLI).

- 12 as mixed cellularity.

3.4 Western Blot

Total protein extracts were prepared in ice-cold lysis buffer (0.5% Nonidet P-40 250 mM sodium chloride, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid [Hepes], 5 mM ethylenediaminetetraacetic acid [EDTA], and 0.5 mM ethyleneglycol bis (beta-aminoethylether)-N,N,N',N tetraacetic acid [EGTA]) containing phosphatase inhibitor cocktail 2 (Sigma-Aldrich), protease inhibitor (Clontech, Mountain View, CA), and dithiothreitol (Invitrogen, Carlsbad, CA). BCA protein assay kit (Pierce, Rockford, IL) was used to measure protein concentration. Proteins were separated by polyacrylamide gel (Bio-Rad) electrophoresis and were transferred to 0.2 μm nitrocellulose membranes (Bio-Rad). The following antibodies were used:

- Anti-GSK-3β (clone D5C5Z, Cell Signaling, 1:1000).
- Anti-pGSK-3β S9 (clone D85E12, Cell Signaling, 1:1000).
- Anti-pGSK-3β Y216 (Abcam, 1:1000).
- Anti-β-catenin (clone E247, Abcam, 1:10000).
- Anti-β-actin (clone AC-15, Abcam, 1:50000).

Proteins were detected by chemiluminescence. Quantitative analysis was carried out with Quantity One software.

3.5 Immunohistochemistry

Three tissue microarrays (TMAs) for immunohistochemical studies were obtained from formalin-fixed paraffin-embedded samples collected at diagnosis from 100 cHL patients. TMA sections were investigated by the following antibodies:

- Anti-GSK-3β (clone 3D10, Novus Biological, 1:1000).
- Anti-pGSK-3β S9 (clone D85E12, Cell Signaling, 1:50).
- Anti-pGSK-3β Y216 (Abcam, 1:800).
- Anti-β-catenin (Abcam, 1:200)

The marker expression in HC and RSC was recorded as the percentage of positive cells in relation to the overall neoplastic population. Three samples of hyperplastic lymph nodes were added to investigate the expression of the same markers in the reactive lymphoid tissue. Immunohistochemical preparations were visualized and images were captured using Olympus Dot-slide microscope digital system equipped with the VS110 image analysis software.

The Fisher exact test on a 2x2 contingency table was then used to find significant correlation between expression of pY216 GSK-3β, S9 GSK-3β and β-catenin proteins in cHL neoplastic cells.

4. Results

4.1 Detection of GSK-3β and its phosphorylated forms in cHL cell lines

WB analysis of GSK-3β and its phosphorylated forms was performed in cHL cell lines and peripheral CD19-positive B-cells from healthy donors (**Figure 11 A**).

No difference was observed in terms of basal levels of GSK-3β expression between cHL cell lines and normal B-cells compared to pGSK-3β S9 which is the inhibitory form and pGSK-3β Y216 which is the stimulatory form. Indeed in both cell lines, the expression of inhibitory pGSK-3β was higher compared to stimulatory pGSK-3β. Conversely, the both phosphorylated forms of the kinase were equally expressed in B-cells. We also evaluated the expression of β-catenin in the nuclear fraction where it was present in the both cell lines (**Figure 11 B**), suggesting an activation of β-catenin signalling.

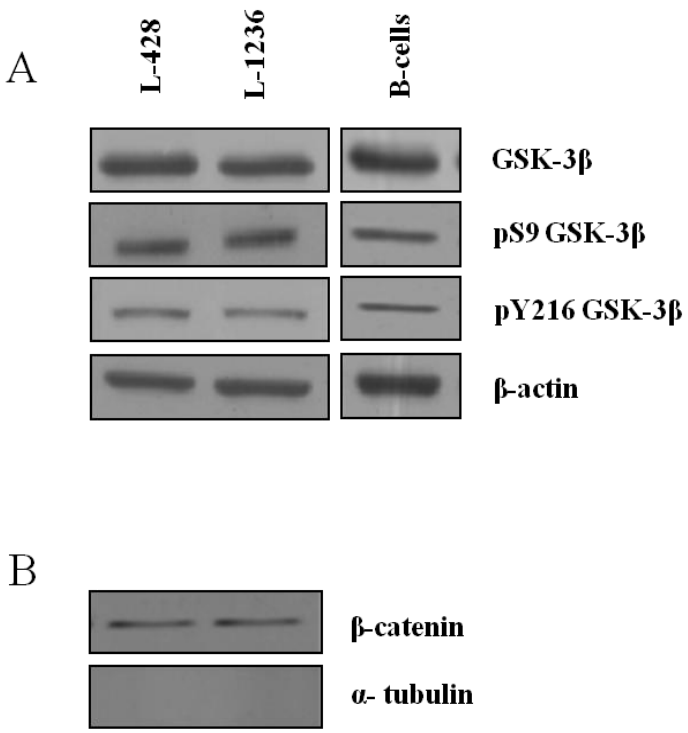

Figure 11. A) Representative immunoblots for GSK3β, pGSK3β S9, pGSK3β Y216 on 20 μg of total proteins from L-428 and L-1236 cells and from normal human B-cells. β-actin was used as loading control. B) Nuclear levels of β-catenin in both cell lines. α-tubulin was used for the purity of the nuclear fraction.

4.2 GSK-3β expression in normal B cell compartment and cHL samples

We used immunohistochemistry to evaluate the expression and cellular localization of total GSK-3β in normal B cells derived from reactive lymph nodes (**Figure 12**) and cHL samples (**Figure 13**). The germinal centres (GC) of the follicles showed diffuse cytoplasmic positivity in both CB and CC. Only scattered weak positive cells were observed in the mantle zones (MZ) **(Figure 12)**. In cHL samples, instead, the kinase was, predominantly, relocated in the cytoplasm of the HC and HRSC identified with the expression of CD30 (**Figure 13 A, B**). Among the 100 samples, we observed that GSK-3β was present in 100% (100/100) of cHL cases with a range of positivity from 10% to 100% and a mean expression of 65% of positive neoplastic cells (**Table 2**).

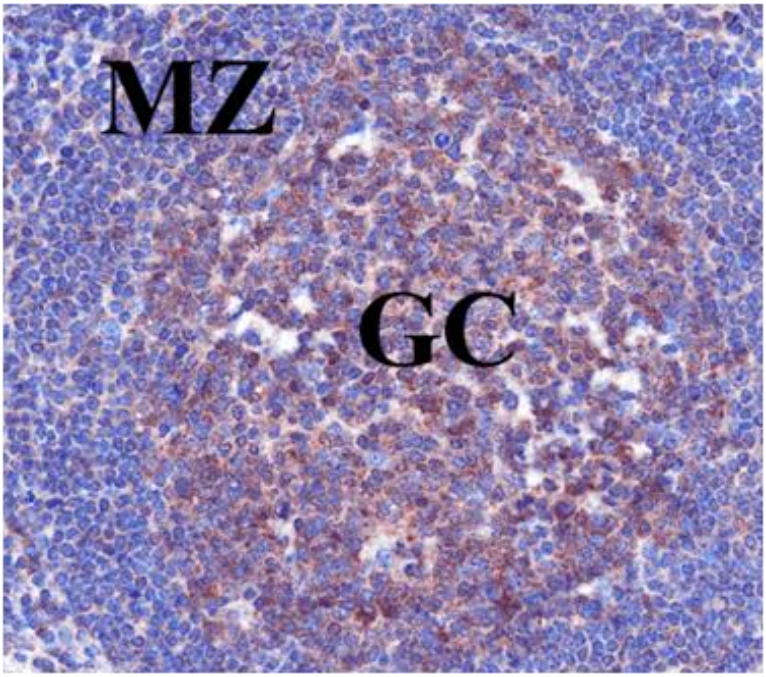

Figure 12. Immunohistochemical analysis shows the GSK-3β expression in GC of a reactive lymph node, being MZ negative (x200).

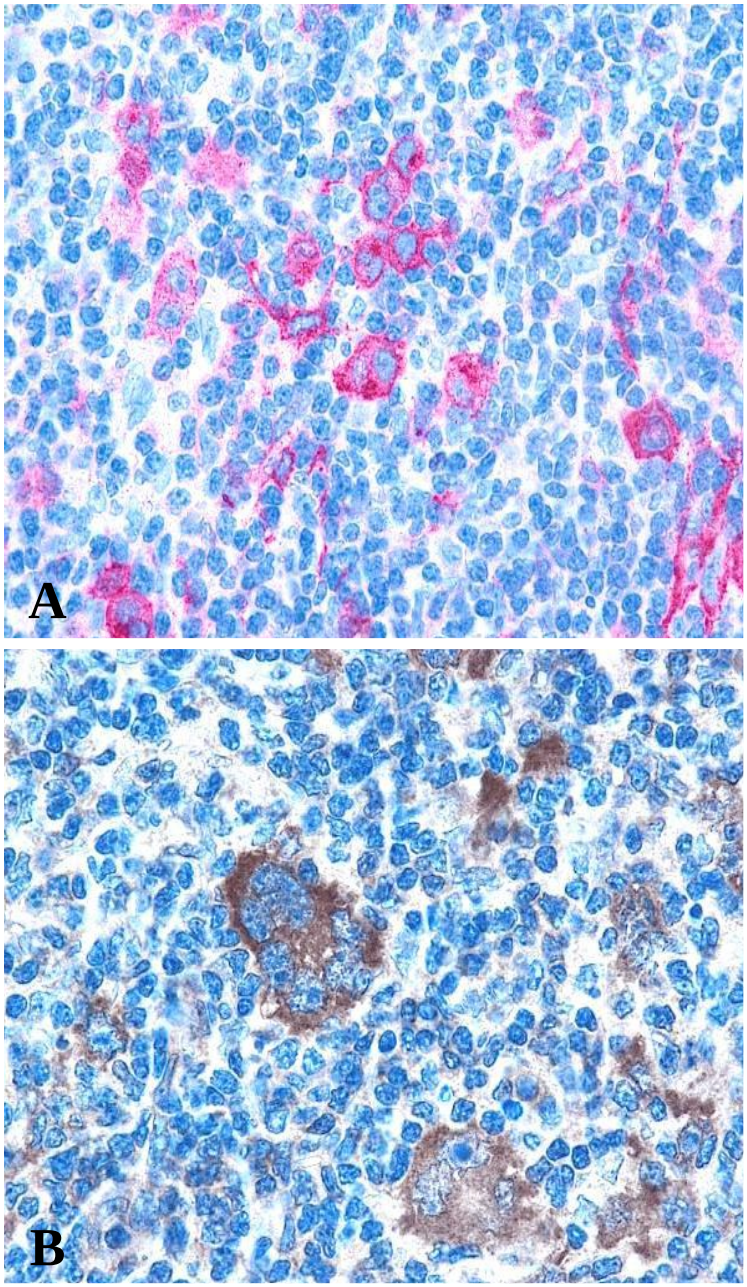

Figure 13. Immunohistochemical analysis shows: A) CD30 expression in HC and HRSC (x400); B) the GSK-3β expression in the cytoplasm of HC and HRSC (x400).

4.3 Constitutive GSK-3β activation prevents β-catenin accumulation in cHL samples

We also employed immunohistochemistry to evaluate the expression of stimulatory pY216 GSK-3β in cHL samples (**Figure 14**). The kinase was expressed in 100/100 (100%) of the cases, with a range of positivity in the neoplastic population, varying from 8% to 100% and a mean expression of 56% of malignant cells (Table 2). In line with the observed expression of the stimulatory GSK-3β in HC and HRSC, β-catenin was detected in the nucleus of only 12/100 (12%) of the cases, with a range of positivity in HC and HRSC, varying from 2% to 36% and a mean expression of 6% of malignant cells (Table 2).

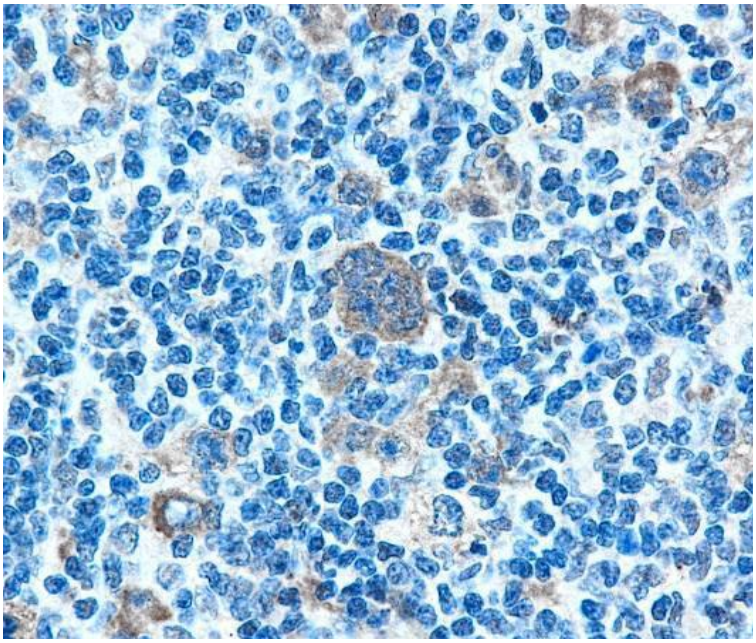

Figure 14. Immunohistochemical analysis shows a strong positivity of Y216 pGSK-3β in HC and HRSC (x400).

4.4 The stimulatory pY216 GSK-3β is aberrantly localized in nuclei of HC and HRSC

We evaluated the cellular localization of the stimulatory GSK-3β in normal B counterpart and cHL samples. In normal B compartment pY216 GSK-3β antibody showed a restricted cytoplasmic positivity in centroblasts and centrocytes (**Figure 15 A)**. Conversely, in 78/100 (78%) of the cHL cases, the pY216 GSK-3β protein was localized in both cytoplasm and nuclei of HC and HRSC (**Figure 15 B**).

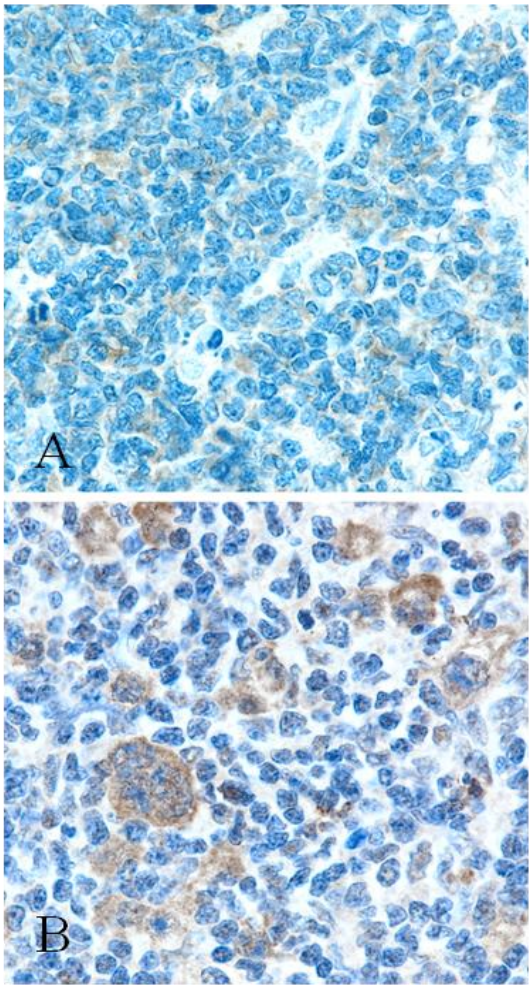

Figure 15. Immunohistochemical analysis shows: A) a restricted cytoplasmic localization of Y216 pGSK-3β in normal B cells (x400); B) a cytoplasmic and nuclear localization of the kinase in HC and HRSC (x400).

4.5 The inhibitory pGSK-3β significantly correlates with β-catenin positivity

To further investigate the functional status of GSK-3β in cHL we verified the immunohistochemical expression of pS9 GSK-3β in the neoplastic clone (**Figure 16**). Our data showed that, among the 100 samples, 20 were assessed positive for the inhibitory pGSK-3β with a range of positivity in the neoplastic population from 1% to 58% and a mean expression of 8% (**Table 2**).

We also evaluated β-catenin expression in neoplastic cells **(Figure 17)**. In 6/20 pS9 GSK-3β^{+} cases, β-catenin protein was present in the nuclei of HC and HRSC, with a range of positivity from 2% to 36% and a mean expression of 6% in the malignant cells (**Table 2**).

Interestingly, a statistically significant association between the β-catenin positivity and the pS9 pGSK-3β expression was observed (P = 0.013). Our data demonstrate a GSK-3β-dependent regulation of the β-catenin in cHL, confirming in particular the kinase activity in promoting the β-catenin ubiquitination and proteasomal degradation in the Wnt pathway.

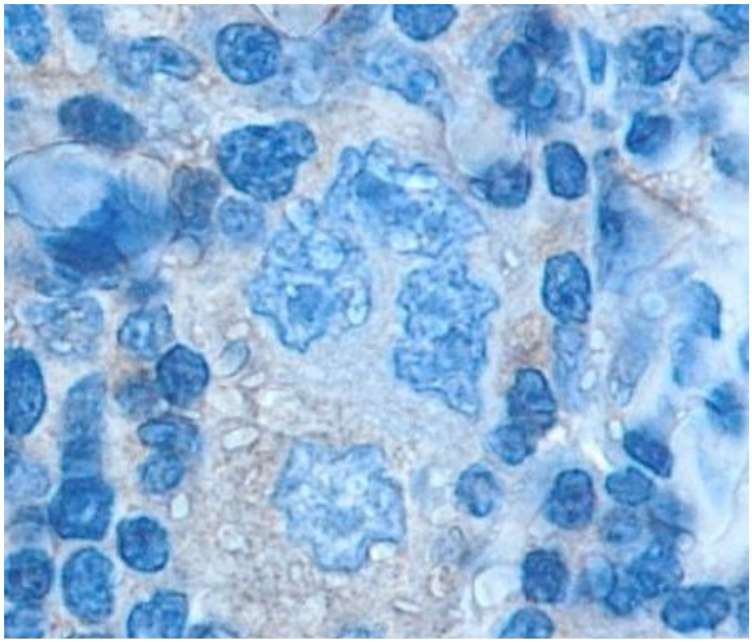

Figure 16. Immunohistochemical analysis shows the positivity of S9 pGSK-3β in HC and HRSC (x400).

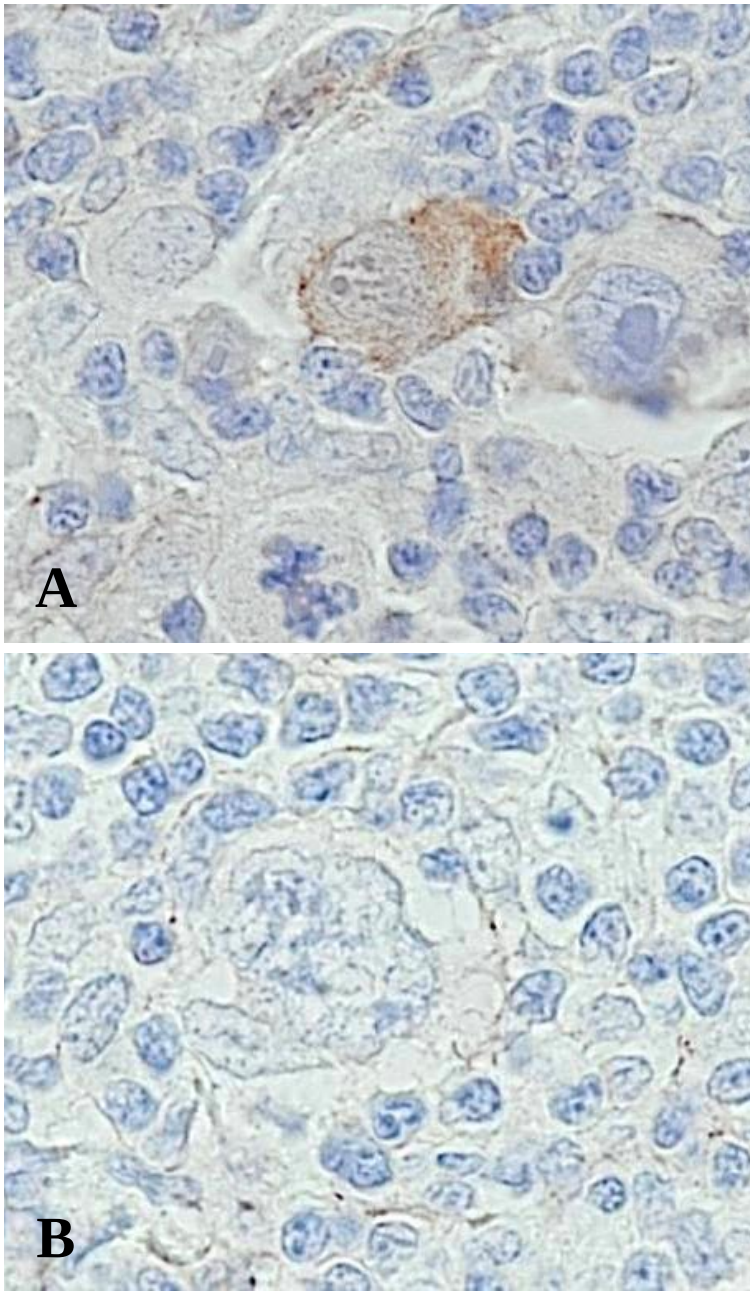

Figure 17. Immunohistochemical analysis shows: A) HRSC espressing β-catenin; B) absence of β-catenin in the neoplastic population (x400).

5. Discussion

GSK-3β is a serine/threonine kinase involved in cell cycle progression, differentiation, embryogenesis, migration, metabolism, survival and cellular senescence (Embi N, *et al*., 1980; Rylatt DB, *et al*., 1980). One of the major biological functions of GSK-3β is to inhibit β-catenin by sequestration and promotion of its proteasomal degradation in the Wnt canonical pathway. The inactivation of GSK-3β is followed by β-catenin accumulation and translocation in the nucleus, where it regulates the transcription of numerous genes.

The aberrant expression of GSK-3β has been implicated in the pathogenesis of many disorders such as diabetes, atherosclerosis, neurodegenerative diseases and cancer (Kockeritz L, *et al*., 2006; Frame S, *et al*., 2001).

Several studies reported an overespression of GSK-3β in human glioblastoma, colon, pancreatic, ovarian, kidney and prostate cancer, suggesting its oncogenic role in many solid neoplasms (Miyashita K, *et al*., 2009; Luo J, 2009; Shakoori A, *et al*., 2005; Ougolkov AV, *et al*., 2005; Zhou W, *et al*., 2012; Cao Q, *et al*., 2006; Bilim V, *et al*., 2009; Liao X, *et al*., 2004). Moreover, emerging evidence earmark GSK-3β as a therapeutic target in acute human leukemias (MLL-associated), multiple myeloma and chronic lymphocytic leukemia indicating a multifaceted role of this kinase in normal and malignant hematopoiesis (Wang Z, *et al*., 2008; Piazza F, *et al*., 2008; Ougolkov AV, *et al*., 2007).

In the present study, western blot analysis was performed on cHL cell lines and normal B-cells, evaluating the expression of GSK-3β, its phosphorylated forms and β-catenin. Our results showed an imbalanced between the two phosphorylated forms of GSK-3β in cHL cell lines and an activation of β-catenin signalling. No difference in terms of expression levels for both inhibitory and stimulatory pGSK-3β forms was observed in normal B-cells. Although the underlying mechanisms are yet to be understood, several roles for GSK-3β in normal and tumoral cells could advantageous for cancer treatment strategies that target this kinase.

Based on this first observation, we also carried out an immunohistochemical analysis in normal B cells from reactive lymph nodes and cHL patients. Our results showed a diffuse cytoplasmic positivity of GSK-3β in both CB and CC of the germinal follicular centres and in 65% of HC and HRSC.

Stimulatory pGSK3β was expressed in 100% of cases with a mean expression of 56% of positive malignant cells in which the kinase was detected in both cytoplasm and nuclei of the malignant cells. Conversely, in centrocytes, centroblasts and mantle cells of normal B counterpart, a restricted cytoplasmic localization was observed. Moreover, only 20 samples were assessed positive for inhibitory pGSK-3β with a range of positivity from 1% to 58% and a mean expression of 8%.

These data suggest an altered physiological turnover and shuttling in/out of the nucleus of the kinase.

Aberrant nuclear accumulation of GSK-3β was reported in bladder cancer, where it was associated to a worse prognosis and contributed to urhotelial cancer cell proliferation and survival, identifying GSK-3β as potential therapeutic target (Naito S, *et al*., 2010). Interestingly, Ougolkov AV *et al.* found that nuclear accumulation of GSK-3β was a marker of poorly differentiated pancreatic adenocarcinoma, where it influenced NF-kB-mediated gene transcription, and revealed that GSK-3β inhibition decreased cancer cell proliferation and survival (Ougolkov AV, *et al*., 2007). Furthermore, the same group demonstrated that GSK-3β accumulated in the nuclei of human chronic lymphocytic leukaemia B cells and that pharmacological inhibition of the enzyme suppressed the NF-kB transcriptional activity, decreasing the expression of antiapoptotic proteins (XIAP, BCL2) and inducing apoptosis in neoplastic cells (Ougolkov AV, *et al*., 2007).

Regarding the β-catenin expression, it was present only in 12% of cases. Interestingly, a statistically significant association between the nuclear β-catenin positivity and the inhibitory pGSK-3β expression was observed in these samples.

Our results demonstrated that cHL cell lines were not a good "*in vitro*" model because the expression data of the investigated proteins were not reproducible in "*ex vivo*" model. Moreover β-catenin signaling resulted activated instead only in rare cases, β-catenin can be detected in HC and HRSC, suggesting that β-catenin signaling has no relevance in biology of cHL. This subset of patients should be further investigated in a larger series, to verify if β-catenin activation is associated to distinctive biologic and clinical features.

The "*ex vivo"* investigation suggest that GSK-3β plays a previously unrecognized important role in regulating the nuclear activity of β-catenin in cHL cells by affecting its binding to the promoters of a subset of related-target genes.

GSK-3β, identified initially as a kinase involved in the glycogen metabolism, has been recognized as an important mediator of the inflammatory processes with strong implications in cancer development. GSK-3β is an intriguing protein that seems to be involved in the pathogenesis of cHL, but many questions remain unanswered and the role of GSK-3β and its potential application in this disease become an interesting aspect to clarify. These findings may be relevant for future clinical studies, identifying GSK-3β as a potential therapeutic target for cHL.

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