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# "UbcH10 is useful in the diagnosis and prognosis of human neoplasms"

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# “UbcH10 is useful in the diagnosis and prognosis of human neoplasms”

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# LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

♦ Berlingieri MT, Pallante P, Sboner A, Barbareschi M, Bianco M, Ferraro A, Mansueto G, Borbone E, Guerriero E, Troncone G, Fusco A. UbcH10 is overexpressed in malignant breast carcinomas. Eur J Cancer. 2007 Dec;43:2729-35.

♦ Troncone G, Guerriero E, Pallante P, Berlingieri MT, Ferraro A, Del Vecchio L, Gorrese M, Mariotti E, Iaccarino A, Palmieri EA, Zeppa P, Palombini L, Fusco A. UbcH10 expression in human lymphomas. Histopathology. 2009 May;54:731-40.

♦ Guerriero E, Ferraro A, Desiderio D, Pallante PL, Berlingieri MT, Iaccarino A, Palmieri EA, Palombini L, Fusco A, Troncone G. UbcH10 Expression on Thyroid Fine Needle Aspirates (Manuscript submitted to Cancer Cytopathology).

# ABSTRACT

The UbcH10 gene belongs to the E2 gene family and encodes for a 19.6 kDa protein involved in ubiquitin-dependent proteolysis. The hybridization of an Affymetrix HG_U95Av2 microarray led us to highlight that this gene is up-regulated by 150 fold in all of the thyroid carcinoma cell lines in comparison to a primary cell culture of normal thyroid origin. To assess the role of UbcH10 in cancer progression, we analyzed its expression and its clinical/pathological relevance in breast and thyroid carcinomas and in lymphoproliferative diseases.

In these tumor types, analysis of UbcH10 expression was performed on both cell lines and clinical samples by quantitative RT-PCR, Western blot, immunohistochemistry and flow cytometry. The effect of UbcH10 protein suppression by RNA interference was also evaluated in different tumor cell lines.

Consistent data were derived from all tumor types. Deregulated UbcH10 expression was clearly associated to a highly malignant phenotype. Implications were both diagnostic and prognostic: UbcH10 specifically associated with the breast tumors more aggressive expressing ErbB2; UbcH10 specifically marked high grade non-Hodgkin lymphomas; high UbcH10 expression increased the suspicion of malignancy on preoperative thyroid biopsies. In several cell lines suppression of UbcH10 expression affected the neoplastic cell growth potential.

All together our results indicate that UbcH10 is a marker of aggressive neoplastic behavior. Assessing its expression on clinical samples may contribute to both cancer diagnosis and prognosis. Moreover, the suppression of its function is to be evaluated as a potential therapeutic tool.

# 1. BACKGROUND

## 1.1 The cell cycle

The fundamental process for the development of cell life is the reproduction. It occurs by an elaborate series of events called the cell cycle, whereby chromosomes and other components are duplicated and then distributed into two daughter cells (Figure 1). A complex network of regulatory proteins governs progression through the steps of the cell cycle (O Morgan 2007).

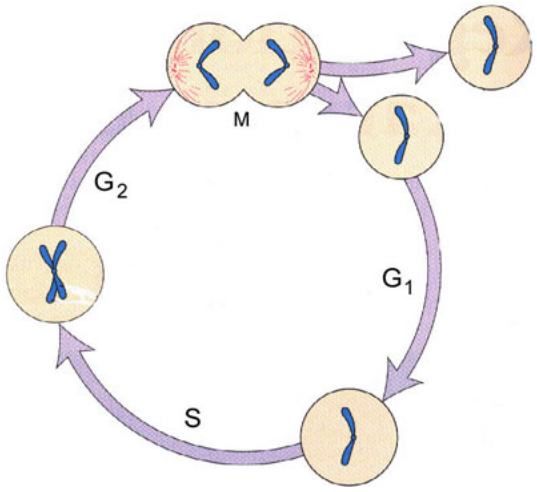


**Figure 1 The cell cycle.** Cell reproduction begins with the duplication of the cell's components, including the exact duplication of each chromosome in S phase. These components are then divided equally between two daughter cells in M phase.

The stages of the cell cycle are typically defined on the basis of chromosomal events. In the early cell cycle (the S phase), the DNA is replicated. The second phase of the cell cycle is the M phase, which is typically composed of two events: nuclear division (mitosis) and cell division (cytokinesis). The period between the end of the M phase and the beginning of the next is called Interphase. Most cell cycles contain additional phases, known as gap phases, between the S and the M phases. Gap phases provide additional time for cell growth, which generally requires much more time than is needed to duplicate and segregate the chromosomes. Gap phases also serve as important regulatory transitions, in which progression to the next cell-cycle stage can be controlled by a variety of intracellular and extracellular signals (O Morgan 2007).

## 1.2 The cell cycle control system

All the events of the cell cycle are under the strict control of a regulatory network called the cell cycle control system. Its central components are a family of enzymes: the Cyclin-dependent kinases (Cdks). Like other protein kinases, Cdks catalyze the covalent attachment of phosphate group (derived from ATP) to protein substrates. This phosphorylation changes the substrate's enzymatic activity or its interaction with other proteins. Cdks activities rise and fall as the cell progresses through the cell cycle. So, for example, an increase in Cdk activity at the beginning of the S phase causes the phosphorylation of proteins that then initiate the DNA synthesis. Cdks are activated by binding to regulatory proteins called cyclins (Figure 2) (Morgan 2007; Murray and Kirschner 1989).

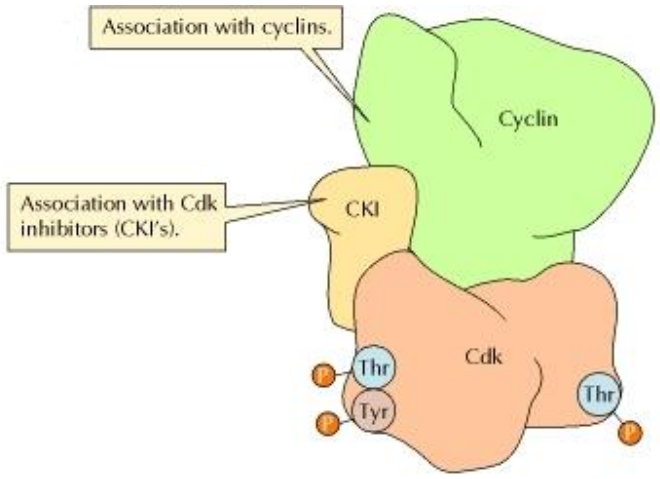


**Figure 2 Cyclin-dependent kinase activation.** The cell cycle control system is based on cyclin-dependent kinases (Cdks) that are activated at specific cell cycle stages by regulatory subunits called cyclins.

Oscillations in Cdk activity during the cell cycle are due primarily to changes in the amounts of cyclins. Different types of cyclins are produced at different cell cycle phases, resulting in the periodic formation of distinct cyclin-Cdk complexes that trigger different cell cycle events that form the core of the cell cycle control system. The regulatory transitions are the checkpoints. The first is called Start or the G1/S checkpoint. When conditions are ideal for cell proliferation, G1/S and S phase cyclin-Cdk complexes are activated, resulting in the phosphorylation of proteins that initiate the DNA replication, the centrosome duplication and other early cell cycle events. G1/S and S phase cyclin-Cdk complexes also promote the activation of the M phase cyclin-Cdk complex, which drives the progression

through the second checkpoint to the entry into mitosis (G2/M checkpoint). The M phase cyclin-Cdks phosphorylate proteins, that promote spindle assembly, bring the cell to methaphase. The third checkpoint is the transition from metaphase to anaphase, which leads to sister chromatid segregation, to completion of mitosis and to cytokinesis. Progression through this checkpoint occurs when the M phase cyclin-Cdk complexes stimulate an enzyme called the anapha- promoting complex (APC), which causes the proteolytic destruction of cyclin and of proteins that hold the sister chromatids together (Hartwell and Weinert 1989). Activation of this enzyme therefore triggers the sister chromatid separation and segregation. Destruction of cyclins is required for the spindle disassembly, for the completation of mitosis, and for cytokinesis (Figure 3).

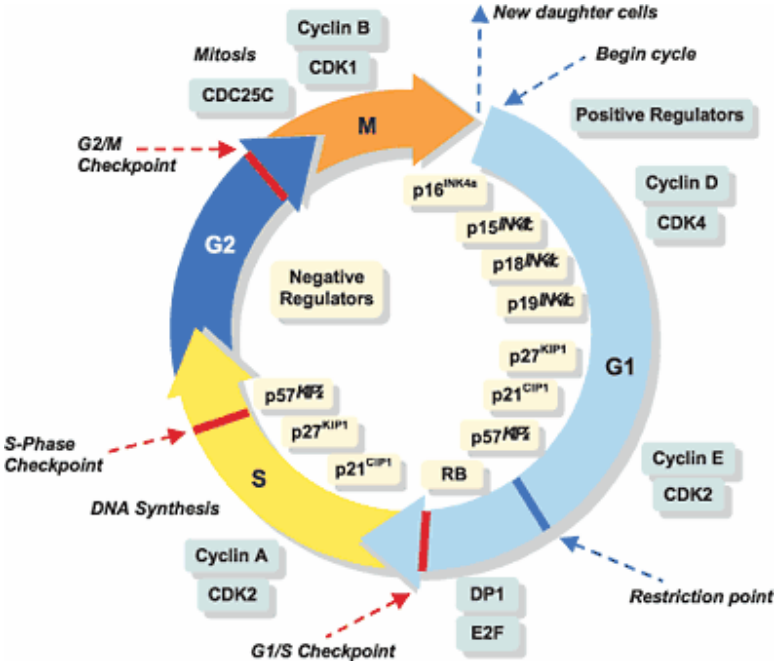


**Figure 3 Overview of cell cycle control.**

The Cdks also associate with inhibitory subunits. These Ckd inhibitors (Ckis) mediate cell cycle arrest in response to various antiproliferative signals (Sherr and Roberts 1995). Temporally regulated degradation of cyclins and Ckis is carried out by the ubiquitin-proteosome system ( Nefsky and Beach 1996). Thus, destruction of the cell cycle regulatory proteins is a critical mechanism by which mitogenic and antimitogenic stimuli control the cell cycle (Baringa 1995; King et al. 1996; Rolfe et al. 1997).

## 1.3 Ubiquitin Proteasome pathway

The ubiquitin proteasome pathway is the principal mechanism for protein catabolism in the mammalian cytosol and nucleus. This highly regulated pathway affects a wide variety of cellular substrates and processes, including cell cycle and division. Defects in this system can result in the pathogenesis of several important human diseases (Glickman and Ciechanover 2002). Degradation of a protein via the ubiquitin proteasome pathway involves two discrete and successive steps: tagging of the substrate protein by the covalent attachment of multiple ubiquitin molecules (conjugation); and the subsequent degradation of the tagged protein by the 26S proteasome (Glickman and Ciechanover 2002). This classical function of ubiquitin is associated with housekeeping functions, with the regulation of protein turnover and with antigenic-peptide generation. More recently, it has become evident that protein modification by ubiquitin also has unconventional (non-degradative) functions such as the regulation of DNA repair and the endocytosis (Bergink and Jentsch 2009; Léon and Haguenauer-Tsapis 2009). These non-traditional functions are dictated by the number of ubiquitin units attached to the proteins (mono- versus poly-ubiquitination) and also by the type of ubiquitin chain linkage that is present. Usually, the attachment of ubiquitin to the ε-amine of lysine residues of target proteins requires a series of ATP-dependent enzymatic steps by E1 (ubiquitin activating), by E2 (ubiquitin conjugating) and by E3 (ubiquitin ligating) enzymes (Figure 4). The biochemical process of ubiquitin conjugation is started by the ATP-dependent E1 enzyme, followed by its transfer to the active site cysteine of E2 enzymes (also called Ubc in yeast, or UbcH for human enzymes). These enzymes perform the second step in the ubiquitin conjugation reactions by forming a thiolester linkage with the C-terminal glycine. E2 enzymes function alone and in conjunction with E3 ligases to catalyze the attachment of ubiquitin to the acceptor lysine residues of target proteins to form isopeptide bonds (Peters et al. 1998; Hilt and Wolfe 2000). Ubiquitin can be conjugated to itself via specific lysine (K6, K11, K27, K29, K33, K48 or K63) residues which results in diverse types of chain linkages. These covalent ubiquitin bonds (isopeptide linkages) can be reversed by specific deubiquitinating enzymes which remove ubiquitin conjugates from proteins and disassemble the ubiquitin chains (Glickman and Ciechanover 2002).

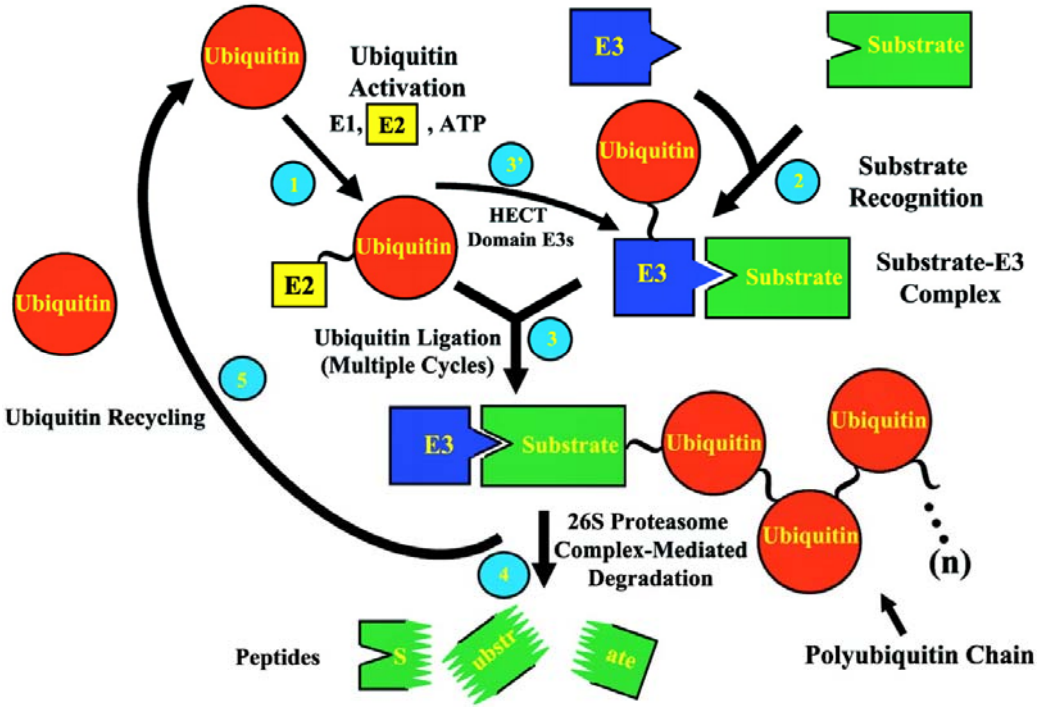


**Figure 4 The ubiquitin proteolytic pathway.** *1*: Activation of ubiquitin by the ubiquitin-activating enzyme E1, an ubiquitin-carrier protein, E2 (ubiquitin-conjugating enzyme, UBC), and ATP. The product of this reaction is a high-energy E2_ubiquitin thiol ester intermediate. *2*: Binding of the protein substrate, via a defined recognition motif, to a specific ubiquitin-protein ligase, E3. *3*: Multiple (*n*) cycles of conjugation of ubiquitin to the target substrate and synthesis of a polyubiquitin chain. E2 transfers the first activated ubiquitin moiety directly to the E3-bound substrate, and in the following cycles,it transfers to previously conjugated ubiquitin moiety. *4*: Degradation of the ubiquitin- tagged substrate by the 26S proteasome complex with release of short peptides. *5*: Ubiquitin is recycled via the activity of deubiquitinating enzymes (DUBs) (Glickman and Ciechanover 2002).

The proteasome is a large multicatalytic protease (26S) that degrades polyubiquitinated proteins to small peptides. It is composed of two subcomplexes: a 20S core particle (CP), that carries the catalytic activity, and a 19S regulatory particle (RP). The catalytic sites are localized to some of the CP subunits. Each extremity of the 20S barrel can be capped by a 19S RP. One important function of the 19S RP is to recognize the ubiquitinated proteins and other potential substrates of the proteasome. After degradation of the substrate, short peptides derived from the substrate, are released, as well as reusable ubiquitin (Bochtler et al. 1999; Glickman 2000; Voges et al. 1999).

## 1.4 UbcH10 and its involvement in cancer

The UbcH10 gene belongs to the E2 gene family and codes for a protein of 19.6 kDa that is involved in the ubiquitin-dependent proteolysis (Hershko et al. 1998; Joazeiro et al. 2000). This enzyme is required for the destruction of the mitotic cyclins and for the cell cycle progression. Multiple transcript variants encoding different isoforms, have been found for this gene.

As mentioned earlier the progression through the cell cycle is accomplished by the degradation of key cell-cycle regulators via ubiquitin proteasome pathway (Pickart 2001; Harper et al. 2002; Peters 2002; De Gramont et al. 2006; Rape et al. 2006). Deregulation of appropriate cell cycle control often results in chromosomal instability, which is a potential trigger for the onset of cancer (Yamasaki and Pagano 2004).

The role of UbcH10 in the cell progression is closely connected to E3-APC that is the central coordinator of the cell-cycle progression in mitosis and in G1 phase (Peters 2002). Their interaction and cooperation within the cell cycle is finely regulated at different levels. Many molecules as cyclin and their inhibitors, mitotic regulators (Emi1) (Lukas et al. 1999; Hsu et al. 2002; Rape and Kirschner 2004) and co-activators (Cdc20 and Cdh1) are involved in these mechanisms (Figure 5) (Burton et al. 2005; Kraft et al. 2005; Yamano et al. 2004; Glotzer et al. 1991; Pfleger and Kirschner 2000; Hsu et al. 2002; Reimann et al. 2001). In particular UbcH10 in conjunction with the APC catalyzes the destruction of the cyclins A and B, playing an important role in the control of the cell exit from mitosis.

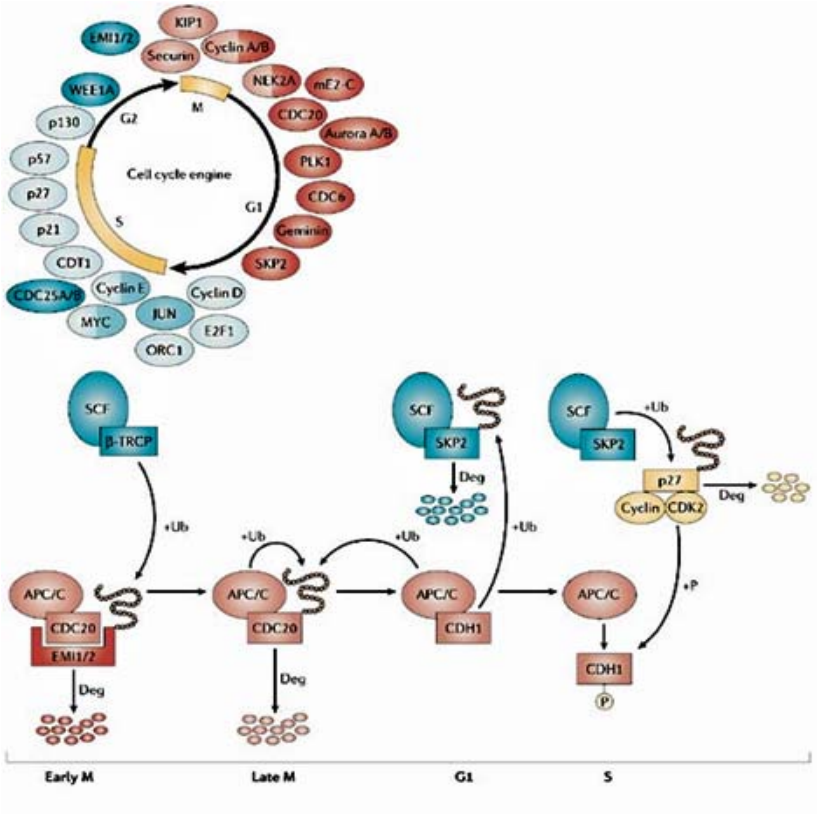


**Figure 5 The Anaphase Promoting Complex (APC).** The anaphase-promoting complex/cyclosome (APC/C) is a ubiquitin ligase that controls the cell-cycle progression by targeting proteins for the destruction from the 26S proteasome (Nakayama and Nakayama 2006).

These results suggest that UbcH10 is potentially involved in the termination of the spindle assembly checkpoint and further implies that aberrant UbcH10 expression impairs the spindle assembly checkpoint resulting in chromosomal instability (Summers et al. 2008; Kriegenburg t al. 2008). So, it is not surprising to find that mutation of the active site cysteine of UbcH10 confers a dominant-negative phenotype that results in metaphase arrest, demonstrating that this protein is essential for the cell cycle progression (Townsley et al. 1997). Although clearly associated with the regulation of the cell cycle, the potential role of UbcH10 in cancer development has only recently been explored. Expression of the mouse ortholog of human UbcH10, mEC-2, was reported up-regulated in NIH3T3 cells transformed by EWS/FLI1 (activated by cdc42, v-ABL or c-myc), but not in a non-transformed NIH3T3 clone expressing EWS/FLI1 (Arvand et al. 1998). Recently, a comparison of expression levels of 17 E2 ubiquitin ligase genes in

normal and tumor tissues identified UbcH10 as the gene most specifically associated with cancer and, consistent with this notion, its over-expression in NIH3T3 cells led to an increased proliferative capacity (Okamoto et al. 2003). Moreover, in expression profiling studies, it was reported that the levels of UbcH10 transcript are highly elevated in different types of cancers compared with the corresponding normal tissues (Welsh et al. 2001; La Tulippe et al. 2002; Wagner et al. 2004, Beringieri et al. 2007; Troncone et al. 2009). It was demonstrated that its over-expression in some carcinomas may be due, at least in part, to genomic amplification of its locus (Wagner et al. 2004). It was also reported that the silencing of UbcH10 by RNA interference (RNAi), in combination with TRAIL/DR5 agonistic antibodies, resulted in enhanced cell death only in neoplastic cells but not in non-malignant human ones (Wagner et al. 2004).

## 1.5 UbcH10 as a potential clinical marker in breast cancer

The recent techniques based on microarray cDNA hybridization have allowed the evaluation of the simultaneous expression of thousands of genes and, therefore, the identification of genes specifically regulated in neoplastic diseases (Michiels et al. 2007). For this purpose we have recently examined the gene expression profile of thyroid carcinoma cell lines compared with normal counterpart. Among the mostly up-regulated genes we identified the UbcH10 gene. We were able to demonstrate that abundant UbcH10 levels were present in the highly invasive, undifferentiated thyroid carcinomas (Pallante et al. 2005). More recently, it was shown that the UbcH10 expression significantly correlates with the tumor grade and the undifferentiated histotype of the ovarian carcinomas. Moreover a significant relationship was observed between the UbcH10 expression and the overall survival (Berlingieri et al. 2007). Therefore, we decided to extend the studies of UbcH10 expression to breast carcinomas representing the first highest incidence neoplasia in women.

## 1.6 Proliferation assessment is relevant in human lymphomas

Proliferation markers are useful in the pathological assessment of Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) (Sanchez-Beato et al.

2003). In particular, the Ki67 protein, whose expression is associated with cell cycle stages G1, S and G2 / M, is routinely used to grade NHLs into low- and high-growth fraction neoplasms (Bryant et al. 2006). The former includes B-cell small lymphocytic lymphoma/ chronic lymphocytic leukaemia (SLL / CLL), splenic marginal zone lymphoma (SMZL), mantle cell lymphoma (MCL) and follicular lymphoma (FL); the low proliferative index of these tumors is associated with a small cell size, advanced disease stages, low clinical aggression and poor response to chemotherapy (Seng et al.1997; Capello et al. 2000). In contrast, diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL) and peripheral T-cell lymphoma (PTCL) have a higher proliferative index, larger cells and an aggressive behavior; PTCL is poorly sensitive to current treatments, whereas DLBCL and BL are potentially responsive to chemotherapy (Sanchez-Beato et al. 2003). It was recently shown that proliferation assessment by the G2/M cell cycle genes is more predictive to treatment response than that based on the Ki67 protein (Obermann et al. 2005; Bjorck et al. 2005). Thus, the search for novel proliferation markers whose expression specifically covers the G2 / M cell cycle window is worth pursuing. A preliminary screening of anatomical UbcH10 expression has shown that it is highest in lymphoid tissue profiling (Wagner et al. 2004). More recently, UbcH10 was also shown to be up-regulated in a small cohort of FL patients by gene expression profiling (Bjorck et al. 2005). However, UbcH10 investigation has not yet been systematically extended to lymphomas, so we screened UbcH10 expression in cell lines and tissues representative of a wide range of indolent and aggressive lymphomas. We also evaluated UbcH10 cell cycle regulation and the effect of suppression of its synthesis on lymphoma cell growth.

## 1.7 UbcH10 as a preoperative malignancy candidate marker on thyroid Fine Needle Aspirate (FNA)

In spite of their very high frequency, only rarely thyroid nodules are malignant (Mazzaferri 1993). Fine needle aspiration (FNA) is widely used to identify those nodules whose treatment unequivocally requires surgery (Mazzaferri 1993). However, FNA is not always able to differentiate between benign and malignant lesions (Baloch et al. 2002). This uncertainty is clearly conveyed by the recent National Cancer Institute (NCI) thyroid FNA conference classification (Baloch et al. 2008). The follicular neoplasm category applies to a monotonous population of three-dimensional groups of follicular cells with scarce colloid; the suspicious for malignancy class is recommended when features suggesting malignancy are only focally observed (Baloch et al. 2008). These uncertain diagnosis do not correspond

to a single entity, but rather to a wide range of inflammatory, hyperplastic, and neoplastic histological lesions (Baloch et al. 2008). These latter including follicular adenoma (FA), follicular carcinoma (FTC) and follicular variant of papillary thyroid carcinoma (PTC), do not or only rarely harbour RET/PTC re-arragements or BRAF mutations (Xing 2005). Thus, panels of mRNA and protein cancer markers are needed to refine indeterminate diagnosis (Saggiorato et al. 2005). In this setting the 3-gene mRNA assay, which included cyclin D2 (CCND2), protein convertase 2 (PCSK2), and prostate differentiation factor (PLAB), allowed molecular classification of FTC and FA (Weber et al. 2005; Shibru et al. 2008).

Genes that regulate cell-cycle progression may be differentially expressed in malignant versus benign thyroid nodules (Kebebew et al. 2006). Since UbcH10 is one of the genes most up-regulated in thyroid cancer cell lines and tissues it is conceivable to postulate for this marker a role on FNAs. In this study UbcH10 was applied to follicular neoplasm and suspicious for malignancy thyroid FNA. UbcH10 expression was evaluated both at transcriptional and translational levels. At the mRNA level, its diagnostic performance was compared with those of the most performing components (CCND2, PCSK2) of the 3-gene diagnostic assay; similarly, at the protein level UbcH10 was compared to that of the standard proliferation marker Ki-67.

# 2. AIMS OF THE STUDY

UbcH10 (also known as E2C or UBE2C) is a cell cycle-related protein involved in mitosis completion. Its ubiquitin-conjugating enzymatic activity (E2) is exerted from G2/M to early G1 phase, when UbcH10 together with ubiquitin ligase (E3) transfers ubiquitin to the mitotic cyclins, thereby promoting their degradation by the proteosome. Once mitotic cyclins are ubiquitinated, UbcH10 triggers its own destruction. This event marks mitotic completion and provides the molecular switch that allows cells to bring cell division to an end and proceed to the new round of the DNA duplication.

To assess the role of UbcH10 in cancer progression, we analyzed its expression and its clinical/pathological relevance in breast and thyroid carcinomas and in lymphoproliferative diseases. In these tumor types, we evaluated whether UbcH10 expression differs between normal and neoplastic tissues and whether its status is correlated to relevant clinical parameters. The effect of UbcH10 protein suppression in different tumor cell lines was also evaluated.

The final objective of this study would be the perspective to use UbcH10 as a new additional tool for diagnosis and prognosis of both epithelial and lymphoid neoplasms.

# 3. MATERIALS AND METHODS

## 3.1 Cell culture

Lymphomas cell lines were obtained from the Continuous Cell Lines Collection at CEINGE Biotecnologie Avanzate (Naples, Italy). The HL cell lines used in this study were HDLM2, KM-H2, L-428, L-540 and L-1236. The NHL cell lines were Raji (Burkitt's lymphoma) and Karpas-299 (human anaplastic large cell lymphoma), Hut-102 and Hut-78 (cutaneous T-cell lymphoma). The human breast carcinoma cell lines used in this study were MB231 (metastasis of adenocarcinoma); MDA468 (metastasis of adenocarcinoma); MDA436 (metastasis of adenocarcinoma); MCF7 (metastasis of adenocarcinoma); T47D (metastasis of ductal carcinoma) and ZR 75-1 (metastasis of ductal carcinoma); they were purchased from the American Type Culture Collection (ATCC). All cell lines were grown as already described (Troncone et al. 2009).

## 3.2 Human tissue samples

Neoplastic human breast and lymphoid tissues were obtained from surgical specimens and immediately frozen in liquid nitrogen. Samples were stored frozen until RNA or protein extractions were performed. All the samples were obtained at the Dipartimento di Scienze Biomorfologiche e Funzionali, University of Naples Federico II, upon approval of the University Ethics Committee.

## 3.3 Thyroid FNA samples

The FNAs were carried out at the Dipartimento di Scienze Biomorfologiche e Funzionali (University of Naples Federico II), as described elsewhere (Zeppa et al. 1990; Troncone et al. 2000). All patients of this study provided informed consent and the study was approved by the University Federico II Ethics Committee. Thyroid FNAs are routinely performed by the cytopathologist using 25-gauge needles, under ultrasound guidance aided by the radiologist. From each passage by the nodule the obtained material is smeared into one or two slides. On-site Diff-Quik stained smears are prepared from the first pass for rapid assessment of specimen adequacy. When a neoplastic lesion is suspected from the on-site assessment, additional passes are performed. The rationale behind this study

sample collection method was to ensure first an adequate cytological diagnosis and, then, to exploit the left-over material for UbcH10 analysis. A total of 103 cases with a cytological diagnosis of either follicular neoplasm or suspicious for malignancy were prospectively collected. Histological follow-up was available in 84 cases, that represented this study series. Fifty-eight patients were women and 26 were men (age range 18-75). On review, histology showed dominant hyperplastic nodule in 24 cases, Hashimoto's thyroiditis (HT) in 4 cases, follicular adenoma (FA) in 26 cases, papillary thyroid carcinoma (PTC) in 24 cases, and follicular cell carcinoma (FC) in 6 cases. Thus, on histology, the malignancy rate of our series was 35,7 %, which confirms that the performance of traditional cytology in this setting is not totally reliable (Baloch et al. 2002). This limit is intrinsic to the method and it is not due to our sample collection procedures.

## 3.4 RNA isolation

Total RNA was extracted from tissues and cell cultures using the RNAeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

## 3.5 Reverse transcriptase and qPCR analysis

*Reverse transcription*

1 μg of total RNA from each sample was reverse-transcribed with QuantiTect® Reverse Transcription Kit (Qiagen) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions.

*TaqMan qRT-PCR: selection of primers and probes*

To design a quantitative real-time polymerase chain reaction (qRT-PCR) assay the Human ProbeLibrary system (Exiqon, Vedbaek, Denmark) was used. Using the free ProbeFinder assay design software, which is included in the package, the best probe and primer pair was chosen. To amplify a fraglnent for RT-PCR ofUbcH10 mRNA, its accession number NM_007019 was entered on the assay design page of the ProbeFinder software. The sequences of forward and reverse primers are reported in Table 1 and they correspond to the nucleotides 172-190 for the forward and 443-425 for the reverse. The probe number was 'human #58' (according to the numbering of Exiqon's Human ProbeLibrary kit). The same procedure was used to

choose the probe and primers for the housekeeping gene glucose 6-phosphate dehydrogenase (g6pd; accession number X03674). An amplicon of 106 nucleotides that spanned the third and fourth exons was chosen. The probe number was 'human #05' (according to the numbering of Exiqon's Human ProbeLibry kit) and the primer sequences are reported in Table 1. All fluorogenic probes were dual-labelled with FAM at the 5' end and with a black quencher at the 3' end.

*qRT-PCR*

Relative Quantitative TaqMan PCR was performed in a Chrom04 Detector (MJ Research, Waltham, MA, USA) in 96-well plates using a final volume of 20 )11. For PCR we used 8 )11 of 2.5x RealMasterMix™ Probe ROX (Eppendorf AG, Hamburg, Germany), 200 nM of each primer, 100 nM probe and cDNA generated from 50 ng of total RNA. The conditions used for PCR were 2 min at 95°C, and then 45 cycles of 20 s at 95°C and I min at 60°C. Each reaction was performed in duplicate. The $2^{-\Delta\Delta ct}$ method to calculate the relative expression levels was used (Livak KJ et a1. 2001).

*Syber Green qRT-PCR*

We also carried out qRT-PCR reactions in a final volume of 20 µl using 10 µl of 2x Power SYBR Green PCR Master Mix (Applied Biosystems), 200 nM of each primer and cDNA generated from 50 ng of total RNA. The conditions used for PCR were 10 min at 95°C and then 45 cycles of 30 s at 95°C and 1 min a 60°C. Each reaction was carried out in duplicate, and at the end of the PCR run, a dissociation curve was constructed using a ramping temperature of 0.2°C per sec from 65°C to 95°C. A single melting point was obtained for all the genes analyzed and HGUS amplicons (data not shown). The HGUS gene served as control. The primers used are reported in Table 1.

**Table 1 List of primers**

| Expression | Fwd 5'→3' | Fwd 5'→3' |
|---|---|---|
| CCND2 | GGACATCCAACCCTACATGC | CGCACTTCTGTTCCTCACAG |
| G6PD | ACAGAGTGAGCCCTTCTTCAA | GGAGGCTGCATCATCGTACT |
| HGUS | CTCATTTGGAATTTTGCCGATT | CCCAGTGAAGATCCCCTTTTTA |
| PCSK2 | GAGAAGACGCAGCCTACACC | CTGCAAAGCCATCTTTACCC |
| UbcH10 | TGCCCTGTATGATGTCAGGA | GGGACTATCAATGTTGGGTTCT |

The Mann-Withney U Test was used to determine differences between mRNA expression levels. A P value of $<0.05$ was considered statistically significant. To determine the diagnostic accuracy of this gene assay, we determined the area under the curve (AUC) of the receiver operating characteristic (ROC) curve for each gene individually and in combination by using logistic regression analysis.

## 3.6 Protein extraction, Western blotting, and antibodies

The detailed procedure for protein extraction and Western blotting for UbcH10 has been described in detail in the original article Troncone et al. 2009.

## 3.7 Tissue microarray and conventional immunohistochemistry

*Tissue samples, technique and evaluation*

The detailed procedure for IHC for UbcH10, Ki-67 and Cyclin B1 has been described in detail in the original article Berlingieri et al. 2007 and Troncone et al. 2009.

## 3.8 Immunostaining on Cell Blocks (CBs)

The residual material from one or two passages by the lesion was processed as cell block (CB) and dedicated to UbcH1 0 and Ki-67 immunostainings. The CBs were prepared with the plasma-thrombin clot technique as described elsewhere (Sanchez et al. 2006). The criteria for CB adequacy were the presence of three or more groups of follicular cells or two or more tissue fragments according to Sanchez et al. Only 57 cases (67,8%) had a contributory CB and that were adequate for UbcH10 assessment by immunostaining. Immunocytochemistry on FNA-derived, formalin-fixed, and paraffin-embedded CBs was done as described elsewhere[5]. Briefly, antigen retrieval microwave treatment (0.01 M citrate buffer, pH 6.0) was applied for three cycles of 5 min each at 750 W. Endogenous peroxidase activity was quenched with methanol-hydrogen peroxide (3%) for 15 min. After blocking with unrelated antiserum, slides were incubated with the primary antibodies: rabbit polyclonal anti-UbcH10 (BostonBiochem), diluted 1: 1000; mouse monoclonal anti-Ki67, clone Mib-l (Dako, Glostrup, Denmark) diluted 1: 100. After incubation with the primary antibodies, CB sections were stained with specie-specific biotinylated secondary antibodies, followed by peroxidase-labelled streptavidin (Dako); the signal was developed by using diaminobenzidine chromogen (Dako) as substrate. Incubations with unrelated antibodies or without the specific antibodies were used as negative controls. Single cells were scored for UbcH 10 and Ki-67 expression with a computerized system (Ibas 2000, Kontron, Zeiss, Munich, Germany) as described elsewhere (Troncone et al. 2007). Scoring was done taking into account in the case of Ki67, only nuclear reactivity, whereas for UbcH10 cells showing nuclear and/or cytoplasmic reactivity were considered to be positive. To determine the cut-off value most predicable of malignancy, we calculated the area under the curve (AUC) of the receiver operating characteristic (ROC) for both UbcH10 and Ki-67 by means of the SPSS Inc. (Cary, NC) software package. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were calculated with standard formulae for each stain separately and for the combination of both stains, using benign vs malignant histological diagnosis as the standard.

## 3.9 RNA Interference

The detailed procedure for UbcH10 RNA interference experiments has been described in detail in the original article (Troncone et al. 2009).

## 3.10 Flow cytometry for UbcH10 expression

Peripheral blood lymphocytes and neoplastic cell lines were analysed for expression of the UbcH10 protein by flow cytometry (Becton Dickinson FACS Canto II; New York, NY, USA). Samples were processed as described in Troncone et al. 2009. We evaluated UbcH10 protein levels also during the different phases of the cell cycle using biparametric staining for DNA and for UbcH10 (Troncone et al. 2009).

# 4. RESULTS AND DISCUSSION

## 4.1 UbcH10 gene is overexpressed in breast carcinoma cell lines

We evaluated the expression of UbcH10 by RT-PCR in breast carcinoma cell lines in comparison to the normal tissue. All of the carcinoma cell lines showed a high UbcH10 expression that, conversely, was barely detectable in the normal tissue (Figure 6A). These results were confirmed by Western blot analysis that showed the presence of an abundant band of 19.6 kDa corresponding to the UbcH10 protein, compared to the normal breast tissue (Figure 6B).

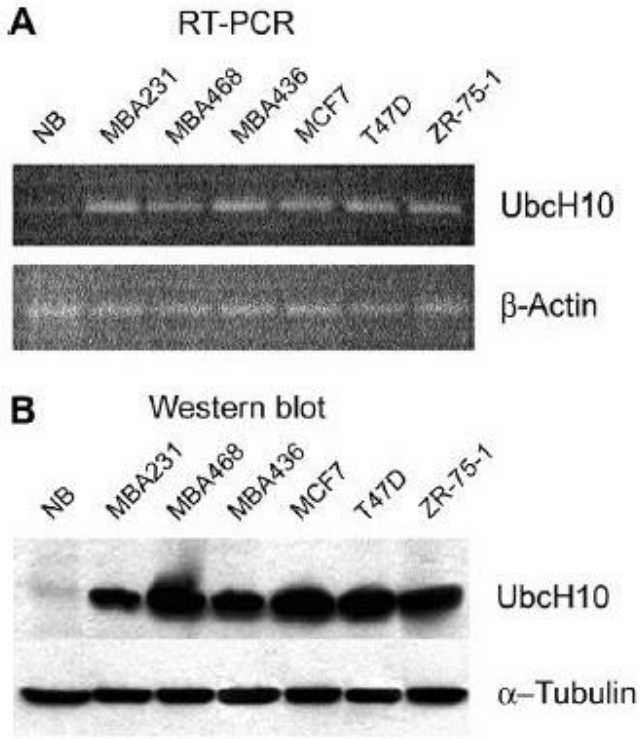


**Figure 6 UbcH10 expression in human breast carcinoma cell lines.** (A) UbcH10 gene expression analysis by RT-PCR in human breast carcinoma cell lines versus the normal breast tissue (NB). β-Actin gene expression was evaluated as control to normalize the amount of the used RNAs. (B) UbcH10 protein expression analysis by Western blot in the same human breast carcinoma cell lines. Blot against α-Tubulin has been performed as control for equal protein loading**.**

## 4.2 Analysis of UbcH10 expression in normal and neoplastic breast tissues by RT-PCR and Western blot analysis

UbcH10 expression was also evaluated by RT-PCR analysis on a panel of matched tumour/normal tissues. As shown in Figure 7A, an amplified band of 115 bp was clearly detected in two ductal, two lobular and one mixed carcinoma samples, but not in one mastopathy and in all the corresponding normal breast tissues. Similar results were obtained when the UbcH10 protein levels were analysed as demonstrated by a representative Western blot shown in Figure 7B. A band corresponding to the UbcH10 protein, in fact, was detected in ductal, lobular and mixed carcinoma tissues, but not in two mastopathies and in normal breast tissues. Equal amounts of total proteins were used for each sample as demonstrated by the same gel analysed with an antibody against a-Tubulin. Therefore, these data show a strong overexpression of UbcH10 in breast malignancies.

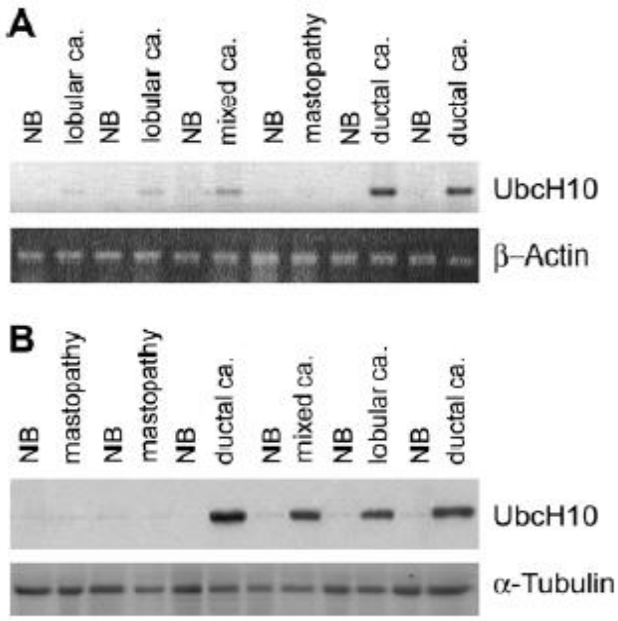


**Figure 7 UbcH10 expression in human breast fresh tumour samples.** (A) RT-PCR analysis of UbcH10 expression in human breast tumour samples versus their normal counterparts. b-Actin expression shows the same amount of RNAs used. NB, normal breast tissue; (B) Western blot analysis of UbcH10 protein expression in a panel of breast neoplasias. The level of a-Tubulin has been used as loading control. NB, normal breast tissue.

## 4.3 Immunostaining pattern of UbcH10 expression in breast cancer

To confirm the UbcH10 overexpression we analyzed 103 malignant and 21 benign cases by immunohistochemical technique because it allows a rapid and sensitive screening of breast pathological tissues and is amenable to regular use as a routine diagnostic test. The immunocytochemical staining pattern of UbcH10 in breast carcinomas differed from that observed in benign breast samples. In fact, the latter were almost always completely negative for UbcH10 expression and the mean of cells expressing UbcH10 was 0.22%. Only occasionally, single UbcH10-labelled breast epithelial cells could be observed by meticulous scrutiny (Figure 8A). Conversely, the UbcH10 staining was always easily detectable in the nuclei of the breast carcinoma cells with a strong staining intensity, mostly evident in cells showing mitotic figures (Figure 8B and 8C).

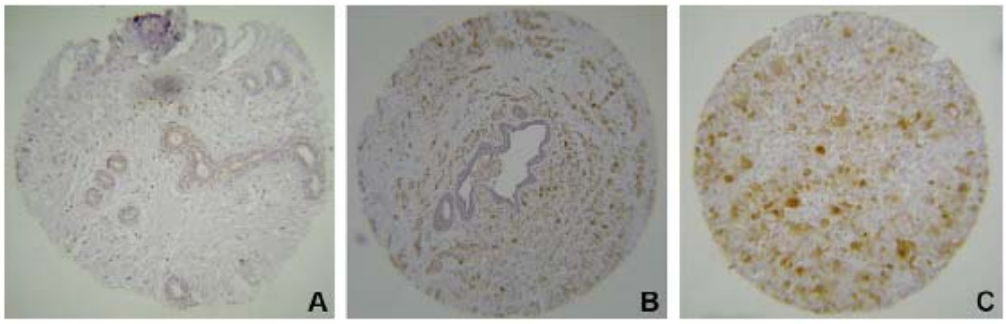


**Figure 8 Immunostaining pattern of UbcH10 expression in breast cancer.** UbcH10 expression in benign (A) (original magnification, 25x) and malignant (B, C) (original magnification, 25x) breast tissues. While benign tissue is lacking UbcH10 expression (A), ductal cancer (C) displays high levels of protein expression. Abundant UbcH10 expression is also shown by lobular cancer (B): note a benign duct negative for UbcH10 expression, whereas infiltrating malignant cells are strongly positive for UbcH10.

In this group the average of UbcH10-positive cells was 11.01%; the differences in the average percentage of UbcH10 stained cells between benign and malignant lesions was highly significant ($p = 0.0001$ Wilcoxon signed rank test). The data obtained in our laboratories assess that UbcH10 expression allow to discriminate benign from malignant breast neoplasias since immunohistochemical studies in breast cancer indicate a significant difference ($p = 0.0001$) in the average percentage of UbcH10 stained cells between benign (0.22%) and malignant lesions (11.01%).

## 4.4 UbcH10 expression and breast cancer clinical-pathological data

Neoplastic breast diseases range from benign fibroadenomas, lobular and ductal, to very aggressive undifferentiated carcinomas (Brierley JD et al. 2003). One of the main differences between lobular and ductal breast carcinomas is the presence of inactivating E-cadherin gene mutations in the former ( De Leeuw et al. 1997). In many other respects, lobular breast carcinomas and low-grade ductal carcinomas exhibit similar geno-phenotypic profiles ( Korkola et al. 2003). The development of p53 dysfunction may be a hallmark of infiltrating ductal carcinomas of intermediate and high grade. Sequential ErbB2/neu and Ras abnormalities define a subset of aggressive high-grade tumours, and the development of Rb dysfunction may define a separate subset of aggressive ductal cancers (Shackeney and Silverman 2003). Moreover, recent results on breast cancer show a direct relationship between the gene expression profile and clinical aggressiveness of the neoplasia (Cleator nd Ashworth 2004). Based on these observations, breast cancer represents a good model for studying epithelial multistep carcinogenesis.

In this study we used Tissue Microarrays (TMAs) provided with relevant clinico-pathological parameters, such as tumour size, node status, grading, hormonal status, proliferation index, p53 and ErbB2 status and survival rates. High UbcH10 expression was associated with ductal histotype ($p = 0.065$; Fisher's exact test), with ErbB2 positivity ($p = 0.092$ Fisher's exact test) and high Ki-67 staining ($p = 0.015$ Fisher's exact test), while no relationship was seen with tumor size and grade, p53 expression, hormonal status (as assessed by ER and PgR tissue staining) and the rates of overall and relapse free survival.

## 4.5 UbcH10 expression is dependent on ErbB2 expression in MCF-7 cell line

The association of UbcH10 staining with ErbB2 amplification suggested the hypothesis that the expression of Ubch10 could be under the control of ErbB2 activity. To validate this hypothesis we suppressed the synthesis of the ErbB2 protein by interference methodology and analysed the UbcH10 expression. As shown in the Western blot of Figure 9, the transfection with siRNA oligonucleotides targeting ErbB2 was able to drastically reduce the ErbB2 protein levels in the MCF-7 cell line. Consistently with our hypothesis, the expression of UbcH10 paralleles ErbB2 levels (72 h), showing an association between UbcH10 expression and ErbB2 amplification.

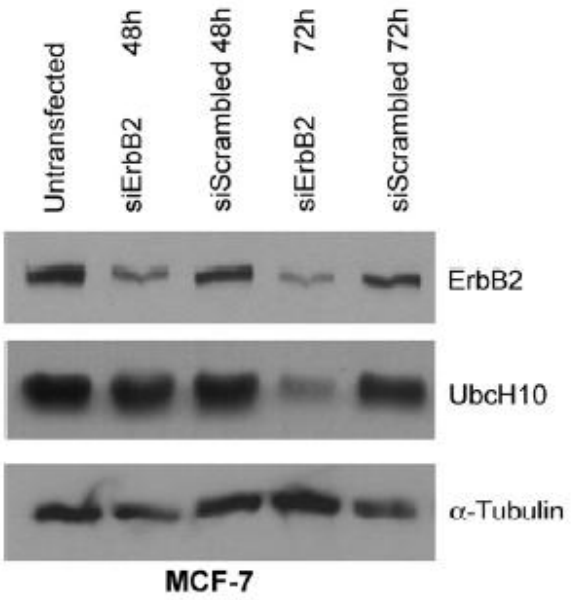


**Figure 9 UbcH10 expression is dependent on ErbB2 expression.** Inhibition of ErbB2 protein expression by siRNA in MCF7 cell line evaluated by Western blot analysis shows, at 72 h after siRNA transfection, a decrease of the UbcH10 expression. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Total cell lysates were prepared and normalised for protein concentration. The expression of a-Tubulin was used to control equal protein loading (30 µg).

Therefore, UbcH10 can be considered one of the effectors of ErbB2 and then its role in breast carcinogenesis may be taken in consideration: this idea is further supported by functional studies demonstrating that the suppression of the UbcH10 expression reduced the growth of a breast carcinoma cell line.

## 4.6 Suppression of the UbcH10 synthesis inhibits breast carcinoma cell growth

We asked whether UbcH10 overexpression had a role in the process of breast carcinogenesis by evaluating the growth rate of one breast carcinoma cell line, in which UbcH10 protein was suppressed by RNA interference. The T47D cell line was treated with siRNA duplexes targeting the UbcH10 mRNA. After transfection we observed an efficient knock-down of the UbcH10 protein levels at 48 h after

treatment (Figure 10A). The analysis of cell growth in the presence or absence of the UbcH10 siRNA duplexes revealed that the block of the UbcH10 protein synthesis significantly inhibits breast carcinoma cell growth. In fact, as shown in Figure 10B, a significant reduction in cell growth rate was observed in T47D cell line treated with UbcH10 siRNA in comparison to the untreated cells or those treated with the control scrambled siRNA.

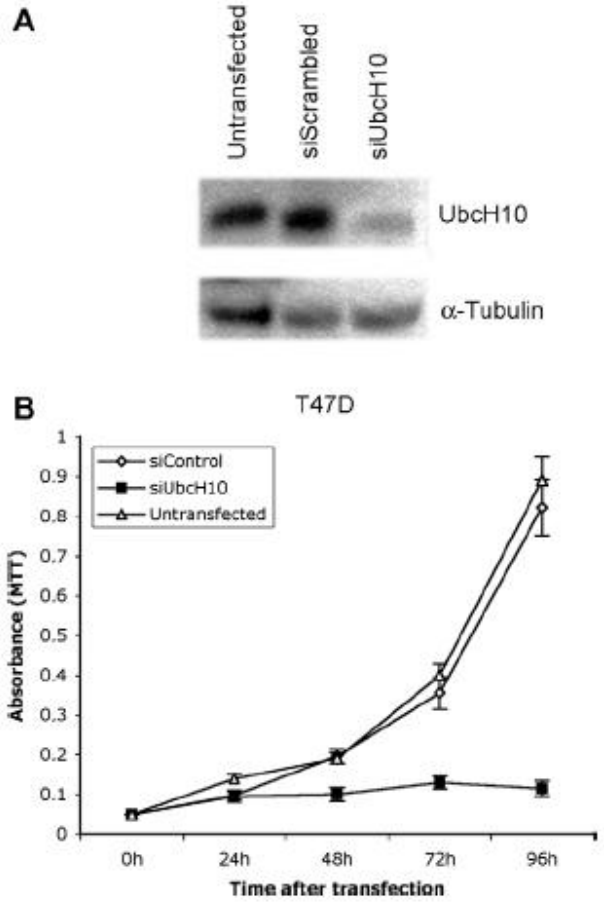


**Figure 10 The block of UbcH10 protein synthesis by RNA interference inhibits the proliferation of breast carcinoma cells.** (A) Inhibition of UbcH10 protein expression by siRNA in T47D cell line evaluated by Western blot analysis. At 48 h after siRNA transfection, total cell lysates were prepared and normalised for protein concentration. The expression of α-Tubulin was used to control equal protein loading (30 μg). (B) Growth curves of T47D cell line after siUbcH10 treatment. T47D cells were transfected with siUbcH10 duplexes (siUbcH10) and the relative number of viable cells was determined by MTT assay. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Absorbance was read at 570 nm and the data are the mean of triplicates.

Our results confirm a critical role of the ubiquitination process in the breast carcinogenesis and in the proliferation of breast cancer cells. In fact, a recent work demonstrates the overexpression of a novel RING-type ubiquitin ligase breast cancer-associated gene 2 (BCA2) in breast carcinomas and its correlation with an increased proliferation, whereas a specific BCA2 small interfering RNA inhibited growth of T47D human breast cancer cells and NIH3T3 mouse cells (Burger et al. 2005). In conclusion, these results show a critical role of UbcH10 in breast carcinogenesis, strongly suggesting the suppression of its function as a possible tool in breast carcinoma therapy.

## 4.7 UbcH10 expression in lymphoma cell lines

Since our and other study have consistently shown that high UbcH10 expression is a marker of aggressive carcinoma behavior (Pallante et al. 2005; Berlingieri et al. 2007; Berlingieri et al. 2007; Wagner et al.. 2004; Lin et al. 2006; Jiang et al. 2008), we have characterized the expression of UbcH10 at mRNA and protein levels in cell lines and tissues from indolent and aggressive lymphoma types. UbcH10 gene expression was assessed in a large array of HL and NHL cell lines. qRT-PCR analysis showed that UbcH10 mRNA fold change values were higher in neoplastic cell lines than in peripheral blood (Figure 11A). Accordingly, a conspicuous 19.6-kDa band was shown by Western blotting only in cells (Figure 11B). Flow cytometry confirmed higher levels of UbcH10 expression versus peripheral blood lymphocytes. However, UbcH10 expression differed widely among cell lines, as shown by the UbcH10 FITC median values, which ranged from 428 to 7152 (Figure 11C).

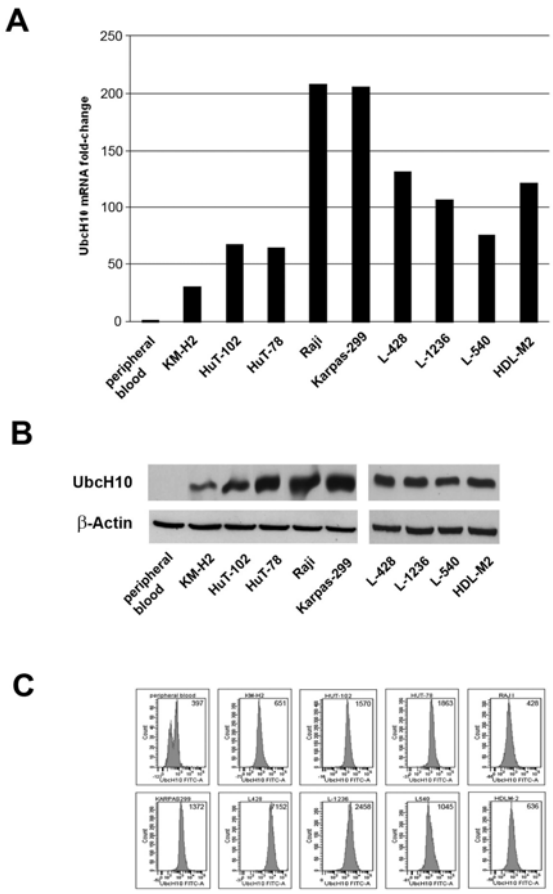


**Figure 11 UbcH10 expression in human lymphoma cell lines.** (A) UbcH10 gene expression analysis by quantitative real-time polymerase chain reaction in human lymphoma cell lines versus normal peripheral blood lymphocytes. UbcH10 mRNA levels were high in all cell lines and barely detectable in peripheral blood lymphocytes. (B) UbcH10 gene expression analysis by Western blot in human lymphoma cell lines versus normal peripheral blood lymphocytes. Note the very clear 19.6-kDa band corresponding to the UbcH10 protein in all cell lines, but not in peripheral blood lymphocytes. Blot against β-actin served to control for equal protein loading. (C) UbcH10 protein expression analysis by flow cytometry in human lymphoma cell lines versus normal peripheral blood lymphocytes. Data within the quadrants are expressed as median UbcH10 fluorescent intensity values.

## 4.8 UbcH10 expression in lymphoid tissues

Both hyperplastic (FH) and neoplastic lymphoid tissues were systematically screened for UbcH10 expression. UbcH10 expression was abundant in FH, as consistently shown by qRT-PCR (Figure 12A) and Western blot (Figure 12B). The pole of the reactive germinal centers harboring mitotic cells was intensely immunoreactive, and a very sharp border resulted from the contrast between the highly positive proliferating centroblasts and the surrounding quiescent mantle zone cells (Figure 13). This pattern is very similar to that shown by Ki67 and by cyclin B1 (Figure 14). Similarly, in the thymus, UbcH10 labeling occurred only in proliferating cortical cells (data not shown). Thus, the intense UbcH10 expression in reactive lymphoid tissue was mainly due to the expansion of proliferative tissue.

The association between UbcH10 expression and proliferation was also evident in lymphomas. In fact, mitotic cells were consistently immunoreactive in each single cylinder (see arrows in Figure 13). In HL, UbcH10 selectively highlighted atypical cells. In particular, Hodgkin and Sternberg–Reed cells were strongly positive (Figure 13). In NHLs, the neoplastic grade closely paralleled UbcH10 expression. qRT-PCR showed higher average levels of expression in DLBCL, PTCL and BL than in SLL ⁄ CLL, MCL and SMZL samples (Figure 12A). Similarly, Western blot analysis revealed more intense expression in high- than in low-grade lymphomas (Figure 12B). This was confirmed by the TMA immunohistochemical data (Figure 8A). UbcH10 positive cells were scarce in CLL ⁄ SLL (median 1.5%), and only mitotic cells and para-immunoblasts were immunoreactive (Figure 13). UbcH10 expression was sporadic in MCL (median 3.0%), homogeneously distributed and limited to mitotic cells (Figure 13). Low UbcH10 expression occurred in SMZL (median 2.5%); the centre of the neoplastic nodules, consisting of small dark cells, remained negative, whereas the outer marginal zone, containing large cells and transformed blasts, was strongly positive. Thus, UbcH10 recapitulated the Ki67 ‘target-like’ staining typical of SMZL(Figure 13) ( Piris et al. 1998). In FL grade II, UbcH10 staining (median 12.0%) was displayed only by centroblasts, whereas centrocytes remained negative. Neoplastic follicles lacked the zonation and the sharp border seen in the germinal centres of FH because positive cells did not concentrate at one pole but were scattered throughout the follicles (Figure 13). Higher (median 20.0%) levels of UbcH10 expression were found in PTCL, in which a large number of cells with irregular, pleomorphic nuclei were labelled (Figure 13). Similarly, in DLBCL, there was abundant nuclear ⁄cytoplasmic UbcH10 reactivity (median 32.5%) in most centroblasts and in mitotic figures (Figure 13). UbcH10 expression was highest in BL cells (median 65.0%); indeed, most BL cells had very abundant nuclear ⁄cytoplasmic reactivity (Figure 13).

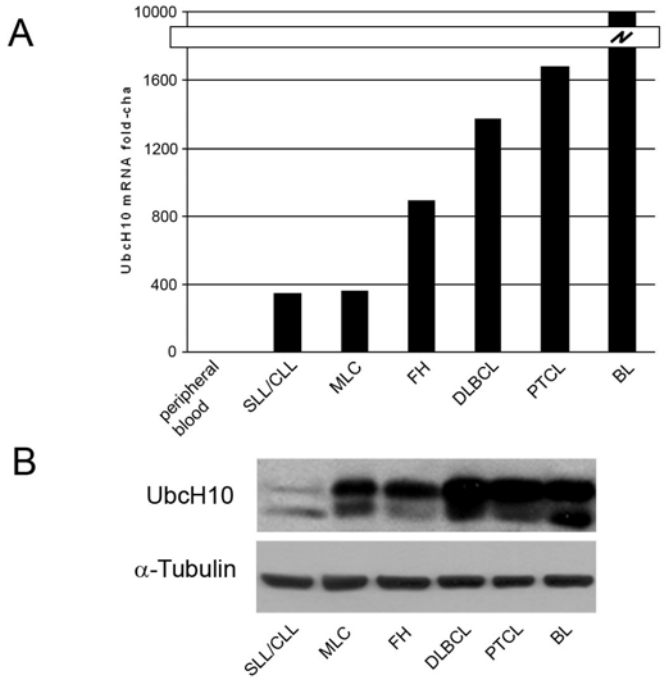


**Figure 12 UbcH10 expression in lymphoid fresh tissue samples.** (A) Quantitative real-time polymerase chain reaction analysis showing higher UbcH10 mRNA fold changes in high-grade [diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL) and peripheral T-cell lymphoma (PTCL)] than in low-grade lymphoma [small lymphocytic lymphoma (SLL)／chronic lymphocytic leukemia (CLL), mantle cell lymphoma (MCL)] and reactive lymph nodes [follicular hyperplasia (FH)]. Each histogram represents the average level of expression for each histotype. (B) Western blot analysis of UbcH10 protein expression showing a more intense band in high grade (DLBCL, BL and PTCL) than in low-grade lymphoma (SLL ／ CLL and MCL) and reactive lymph nodes (FH). A representative sample for each histotype is shown. Blot against α-Tubulin showed as control for equal protein loading.

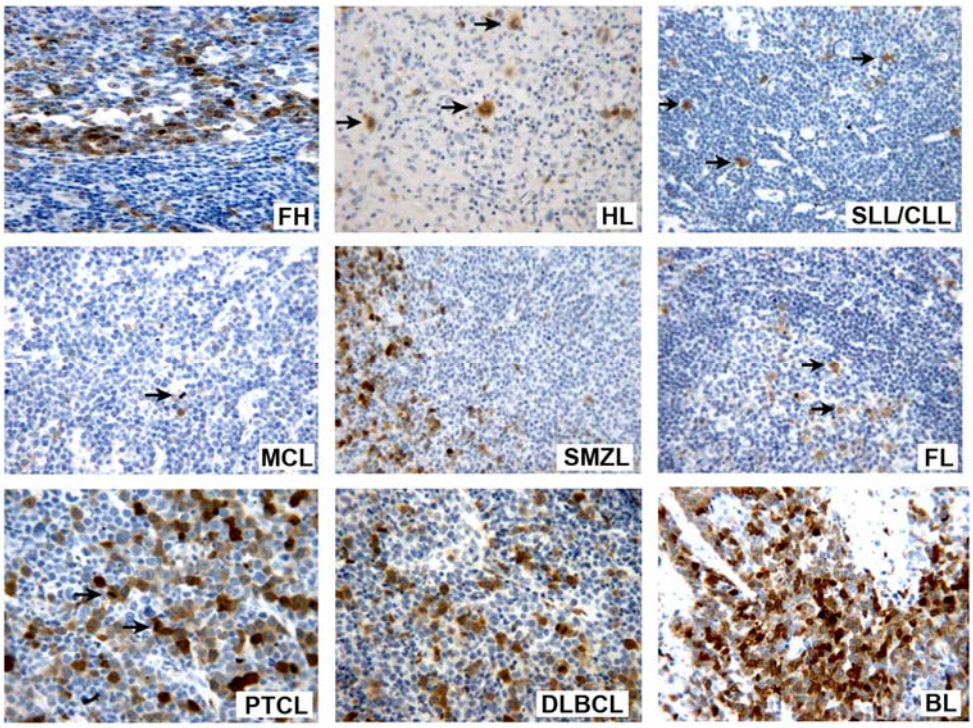


**Figure 13 UbcH10 expression by tissue microarray immunohistochemistry.** Follicular hyperplasia: abundant UbcH10 expression. The immunoreactivity is mainly seen at one pole of the germinal centre (arrows). A sharp border results from the contrast between the highly positive centroblasts and the lack of signal in the surrounding mantle zone. Hodgkin's lymphoma: immunoreactivity for UbcH10 selectively highlights atypical cells. Note the strong nuclear/cytoplasmic reactivity of popcorn cells (arrows) against a clean background of infiltrating inflammatory cells. Small lymphocytic lymphoma /chronic lymphocytic leukemia: sporadic UbcH10 immunoreactivity. Only mitotic prolymphocytes and para-immunoblasts are positive (arrows), whereas small lymphocytes remain negative. Mantle cell lymphoma: low UbcH10 expression. The reactivity is limited to rare mitotic cells (arrow), whereas the neoplastic cells with irregular nuclei are negative. Splenic marginal zone lymphoma: UbcH10 target pattern. The neoplastic nodules show a negative central zone and positive large cells in the outer marginal zone. Follicular lymphoma grade II: neoplastic nodules lack the zonation and the sharp border of reactive germinal centres. Positive centroblasts are evenly scattered throughout the neoplastic follicles (arrows), whereas centrocytes are negative. Peripheral T-cell lymphoma: high UbcH10 expression. A large number of UbcH10+ cells with irregular, pleomorphic nuclei (arrows). Diffuse large B-cell lymphoma: high UbcH10 expression. Note the intense UbcH10 nuclear/cytoplasmic immunoreactivity of the centroblasts and the intense labelling of the mitotic figures. Burkitt's lymphoma: very high UbcH10 expression. The vast majority of neoplastic cells show abundant UbcH10 expression. (Haematoxylin counterstain).

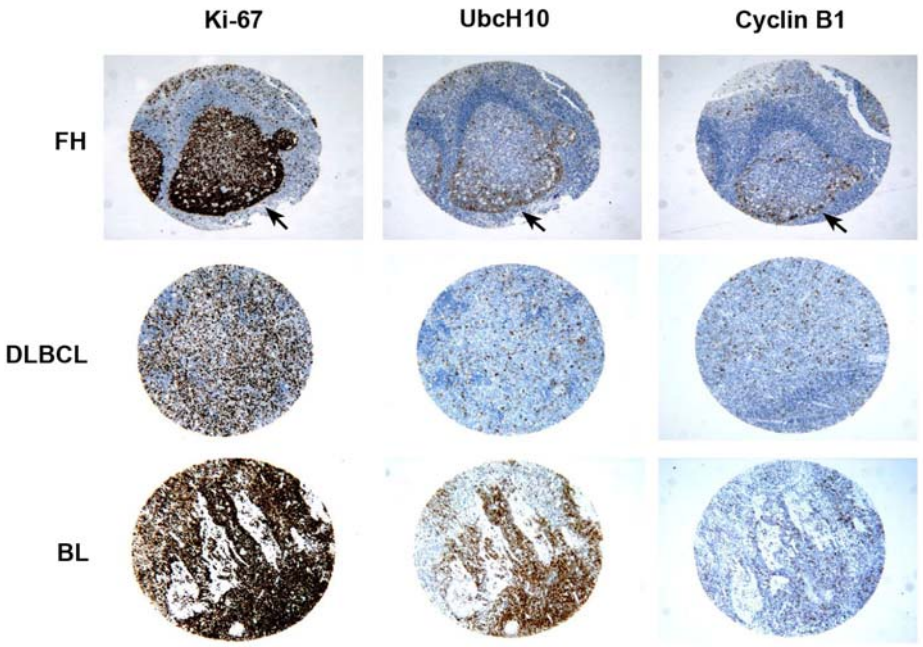


**Figure 14 Ki67, UbcH10 and cyclin B1 expression on tissue microarray consecutive sections.** Follicular lymphoma: Ki67 immunoreactivity highlights a very hyperplastic follicle, in which the light zone has been overrun by the expanded dark zone to yield a nearly 100% growth rate. Only the subpopulation of Ki67 localized at the germinal centre pole and displaying the highest Ki67 labelling (arrow) is also positive (arrow) for UbcH10. A similar distribution (arrow) is shown by cyclin B1. Diffuse large B-cell lymphoma: Ki67 stains a very large percentage of neoplastic cells evenly distributed throughout the tissue core. By contrast, UbcH10 stains a much smaller number of cells, and the pattern of immunoreactivity resembles that of cyclin B1. Burkitt's lymphoma: virtually all neoplastic cells are stained by Ki67; the large majority of cells are also stained by UbcH10. Note that, by immunohistochemistry, UbcH10 distinguishes between DLCBL and BL better than either Ki67 or cyclin B1 (haematoxylin counterstain).

As a general rule, UbcH10 expression was cell-cycle dependent and related to proliferation. Low levels were observed in peripheral blood lymphocytes, in quiescent naive B-cells of the mantle zones of secondary lymphoid follicles, and in low-grade lymphomas. High levels were observed in a wide variety of HL and NHL cell lines, in cells showing mitotic figures, in reactive germinal centre proliferating centroblasts and in aggressive lymphomas. UbcH10 expression increased with pathological grade, and reached a maximum in BL as revealed by qRT-PCR and TMA immunohistochemistry. This confirms the close relationship between UbcH10 overexpression and poor tumour differentiation described in

thyroid (Pallante et al. 2005), ovarian (Berlingieri et al. 2007), breast (Berlingieri et al. 2007), lung (Wagner et al. 2004), bladder (Wagner et al. 2004), liver (Ieta et al. 2007) and brain (Jiang et al. 2008) tumors. UbcH10 immunoreactivity was significantly related to reactivity of the proliferation markers Ki67 ($r = 0.91$; $P < 0.001$) and cyclin B1 ($r = 0.93$; $P < 0.001$; Figure 15B) and occurred in similar lymphoid tissue areas as shown on consecutive TMA sections (Figure 14).

## 4.9 UbcH10 cell cycle regulation

We next investigated the relationship between UbcH10 and other proliferation markers on consecutive TMA sections. There was a significant correlation between UbcH10 and both Ki67 (Spearman's q 0.913; $P < 0.001$) and cyclin B1 (Spearman's q 0.931; $P < 0.001$) (Figure 15B). As evident on consecutive sections, cells stained by Ki67, UbcH10 and cyclin B1 antibodies were distributed in the same tissue areas (Figure 14), in particular, UbcH10 and cyclin B1 labelled a subpopulation of Ki67+ cells. Thus, UbcH10 immunoreactivity was in keeping with a G2/M labelling index, which was similar to that obtained with cyclin B1 (Figure 14). We investigated this concept further by evaluating changes in the levels of UbcH10 expression during cell cycle progression. To this end, we evaluated a wide range of neoplastic lymphoid cell lines by flow cytometry. Similar kinetics was shown by both low and high expressing cell lines. Indeed, in all instances UbcH10 expression steadily increased from late G1 through the S phase, peaked at G2/M, and dramatically decreased in the G1/G0 phase of the cell cycle (Figure 16).

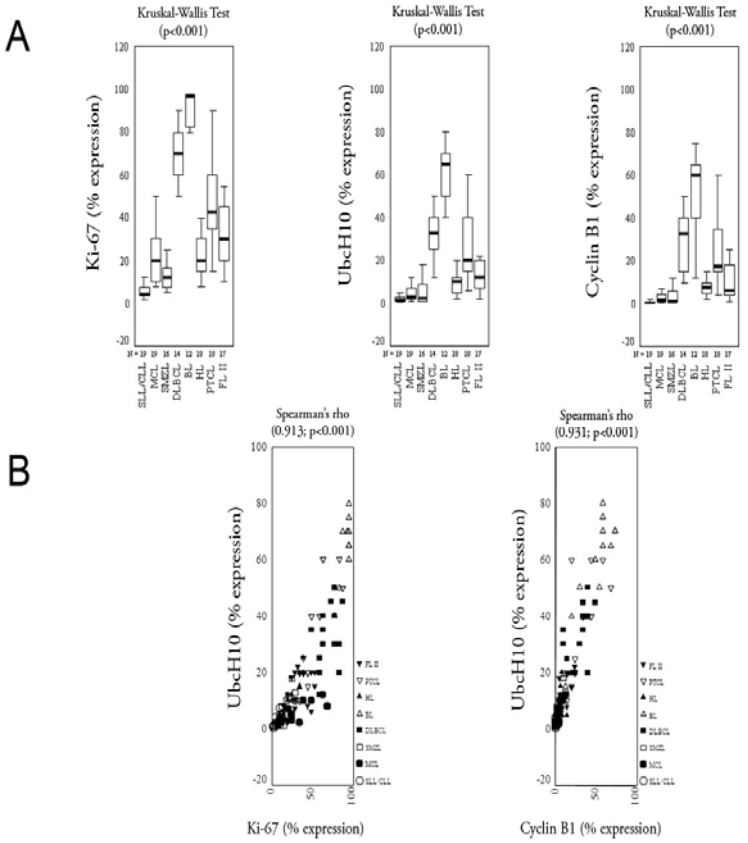


**Figure 15 Statistical analysis of UbcH10, Ki67 and cyclin B1 immunohistochemistry on tissue microarray.** (A) Graphic representation of protein levels evaluated by immunohistochemistry. Data are reported as median, highest and lowest values and interquartile range containing the 50% of values for each diagnostic group. For any protein the differences between the different lymphoma types were analysed by the Kruskal–Wallis one-way anova test. (B) Graphic representation of the non-parametric Spearman's q test used to assess the correlation between UbcH10, Ki67 and cyclin B1 protein expression among the different diagnostic groups.

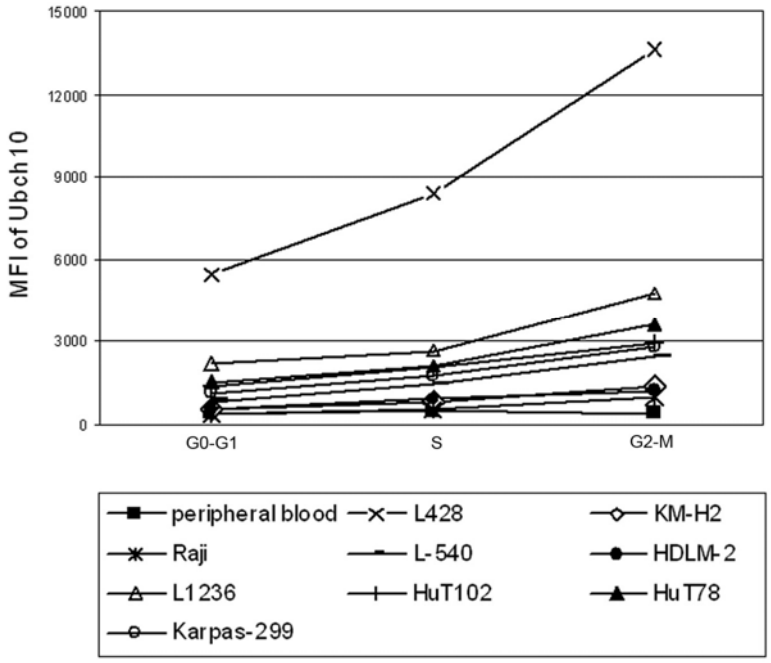


**Figure 16 Median value of fluorescence intensity for UbcH10 immunoreactivity (MFI).** Correlation between the expression of UbcH10 and the phases of cellular cycle in human lymphoma cell lines.

Thus, UbcH10 expression was mostly associated with the G2/M phase. Although Ki67 covers the whole cell cycle from early G1 on (Bryant et al. 2006), it is conceivable that only in G2/M phase are UbcH10 levels high enough to be detected by immunohistochemistry. In fact, in all the TMA cores of non-neoplastic lymphoid tissue or lymphoma, the proportion of cells expressing UbcH10 never exceeded that of Ki67, being similar to the proportion of cyclin B1-stained cells (Figures 14 and 15A). Thus, aggressive B-cell lymphomas, which are constituted mainly by cycling cells, are better typed into DLBCL and BL by UbcH10 than by Ki67, whose expression is uniformly too intense in both neoplasms (Figures 14 and 15A). Taken together, these data indicate that UbcH10 is a novel lymphoid proliferation marker, whose overexpression in aggressive lymphomas probably reflects the tumor related increases in cell proliferation.

## 4.10 Suppression of UbcH10 synthesis inhibits lymphoma cell growth

Since UbcH10 expression and lymphoid cell proliferation were closely related, we next evaluated whether suppression of the synthesis of the UbcH10 protein by RNA interference affected the growth rate of the human anaplastic large cell lymphoma Karpas-299 cell line. This cell line was treated with siRNA duplexes targeting the UbcH10 mRNA. Twenty-four hours after transfection, there was an efficient knock-down of UbcH10 protein levels (Figure 17A). Analysis of cell growth in the presence or absence of the UbcH10 siRNA duplexes revealed that the block of UbcH10 protein synthesis significantly inhibited lymphoma cell growth. In fact, as shown in Figure 17B, cell growth rate was significantly lower in cell lines treated with UbcH10 siRNA than in untransfected cells or in those treated with the control scrambled siRNA.

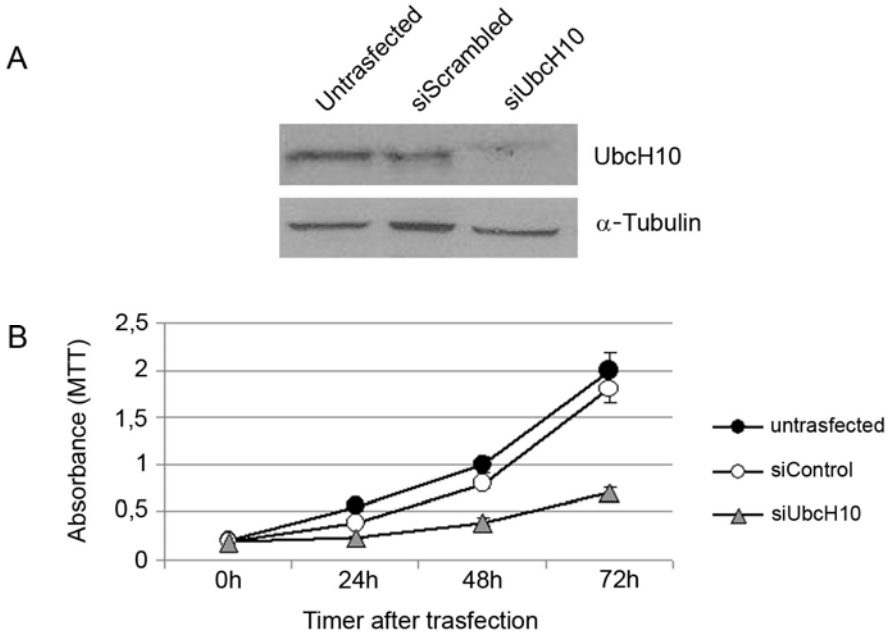


**Figure 17 The block of UbcH10 protein synthesis by RNA interference inhibits the proliferation of the lymphoma cells.** (A) Inhibition of UbcH10 protein expression by small interfering (si) RNA in the Karpas-299 cell line evaluated by Western blot analysis (24 h). The expression of a-tubulin was used to control for equal protein loading 30 lg. (B) Growth curves of the Karpas-299 cell line after siUbcH10 treatment. Karpas-299 cells were transfected with si-UbcH10 duplexes (siUbcH10) and the relative number of viable cells was determined by MTT assay. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Absorbance was read at 570 nm and the data are the mean of triplicates.

These results indicate that UbcH10 plays a critical role in lymphoid cell proliferation. Previous studies have shown that UbcH10 is essential for cell cycle progression ( Lukas et al. 2004). In this study we have shown that blocking of UbcH10 protein synthesis significantly inhibited growth of an NHL cell line. It was recently reported that oesophageal adenocarcinoma cells strongly expressing UbcH10 were highly sensitive to treatment with the proteasome inhibitor MG-262 (Lin et al. 2006). Although no specific UbcH10 inhibitors are currently available for clinical use, targeting the ubiquitin-proteosome system may block the degradation of ubiquitinated mitotic cyclins, thereby preventing the effect exerted by UbcH10 on cell cycle progression. Further investigations are required to explore the possibility that inhibition of UbcH10 could be a therapeutic approach in lymphomas.

## 4.11 UbcH10 expression on FNAs

*UbcH10 and Ki-67 imunohistochemical expression on CBs derived from follicular neoplasm and suspicious for malignancy FNAs.*

UbcH10 and Ki-67 immunohistochemical expression were assessed on CB derived from FNAs diagnosed as either follicular neoplasm or suspicious for malignancy; representative examples are reported in Figure 18.

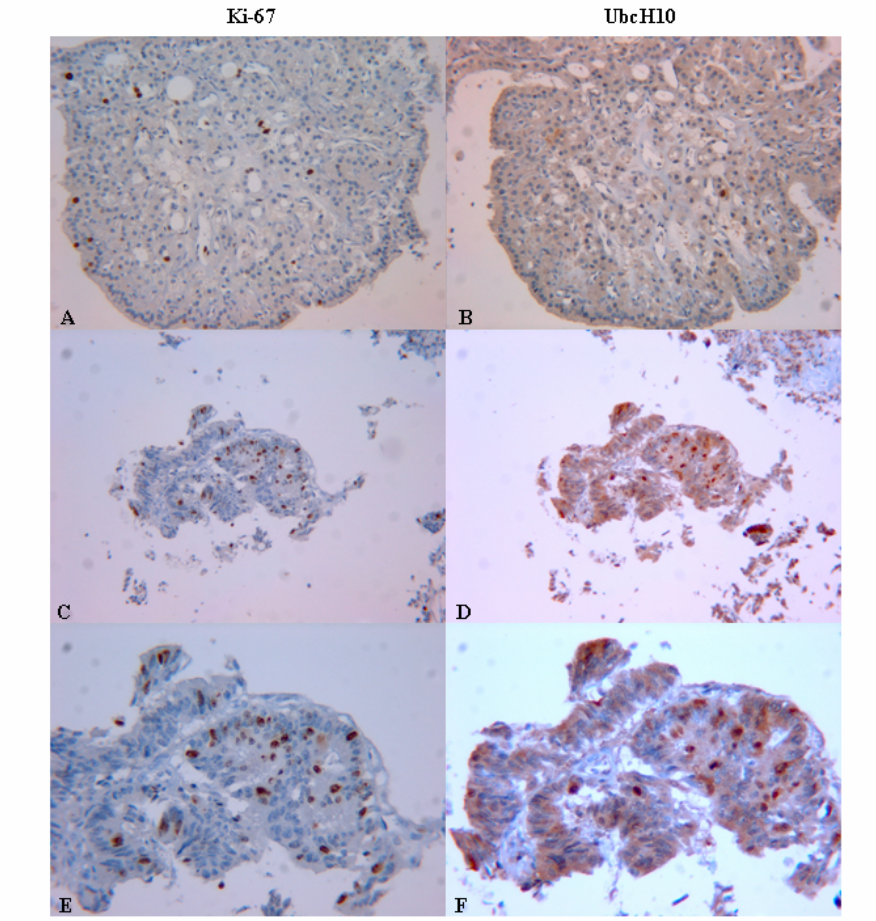


**Figure 18 UbcH10 and Ki-67 expression in FNA derived CBs corresponding to histologically diagnosed FA (A and B) and PTC (C-F).** Both Ki-67 and UbcH10 labeled more cells in PTC rather than in FA (Hematoxylin counterstained 10x.). At higher magnification, note that UbcH10 and Ki-67 share a similar staining pattern as shown on parallel (E and F) CB sections (Hematoxylin counterstained 20x).

The expression of both UbcH10 and Ki-67 was significantly higher in malignant than in benign thyroid lesions (Table 2).

**Table 2 UbcH10 and Ki-67 immunohistochemical expression values.**

| % expression | Benign histology n=38 | Malignant histology n=19 | Mann-Whitney U test P value |
|---|---|---|---|
| UbcH10 | 0,0 (0,0-2,0) | 2,3 (0,0-5,0) | <0,001 |
| Ki-67 | 1,0 (0,0-5,0) | 4,3 (0,0-6,3) | <0,001 |

UbcH10 and Ki-67 immunohistochemical expression values (median and range) on CBs derived from FNAs whose corresponding histological specimens were grouped as either benign or malignant.

To our knowledge, this is the first study evaluating UbcH10 on FNA samples. Thus, it was important to set meaningful cut off values to score UbcH10 immunostaining. The value more predicable of malignancy was determined using the ROC analysis. The best compromise between sensitivity and specificity was reached at a cut-off value of 1,25% for UbcH10 (AUC=0,964; P<0,001) and at a cut-off value of 3,05 for Ki-67 (AUC=0,967; P<0,001). Resulting specificity, sensitivity, PPV, NPV, and diagnostic accuracy of UbcH10 and Ki-67 and of their combinations are reported in Table 3.

**Table 3 Diagnostic performance of UbcH10, Ki-67 and their combination.**

| | Cut-off | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|---|
| UbcH10 | ≥1,25% | 100% | 89% | 83% | 100% | 93% |
| Ki-67 | ≥3,05% | 100% | 95% | 90% | 100% | 96% |
| UbcH10 & Ki-67 | ≥1,25 & ≥3,05 | 100% | 95% | 90% | 100% | 96% |
| UbcH10 or Ki-67 | ≥1,25 or ≥3,05 | 100% | 89% | 83% | 100% | 93% |

Diagnostic performance of UbcH10, Ki-67 and their combination in FNAs diagnosed as either follicular neoplasm or suspicious for malignancy. Data analysis based on the individual most efficient positive cut-off values for stained thyroid cells derived from ROC analysis.

Both markers were found to be highly sensitive (100%), and there were no false-negative cases (NPV, 100%). However, staining was less specific for UbcH10 (89%) than for Ki-67 (95%); the PPV was 83% for UbcH10 and 90% for Ki-67.

Although this performance was remarkable, even on CBs whose corresponding histology was malignant only a few cells stained for UbcH10 (Figure 18D and F). This is conceivable since UbcH10 expression is limited to cells encompassing the G2/M cell cycle phase, as we previously showed (Troncone et al. 2009); interestingly, the proportion of cells expressing UbcH10 never exceeded that of Ki67, which concurs with the notion that Ki67 covers the whole cell cycle from early G1 on (Bryant et al. 2006). Thus, the UbcH10 cut-off value derived from the ROC analysis was too low to allow a reliable microscopic scoring. Moreover, the association of UbcH10 to Ki-67 (samples that were positive for both proteins) did not improve specificity (95%) and accuracy (96%). Consequently, UbcH10 immunostaining on FNAs is not feasible and does not improve Ki-67 performance.

*Quantitative analysis of UbcH10, CCND2 and PCSK2 mRNA levels derived from follicular neoplasm and suspicious for malignancy FNAs*

In this study the usefulness of UbcH10 mRNA levels detection from follicular neoplasm and suspicious for malignancy FNA was evaluated. Data were compared to CCND2 and PCSK2 assessment. Histology represented the gold standard; FNAs were divided into two groups: one associated to benign histology and the other to malignancy. FNAs associated to malignant histology showed significantly different UbcH10 (P= 0.02) and CCND2 (P= 0.002) mRNA levels (Table 4); conversely, PCSK2 mRNA levels were less discriminative (P= 0.23).

**Table 4 For each gene individually and in combination we reported the P value calculated using Mann-Withney U Test and the area under the curve (AUC).**

| | P value | AUC |
|---|---|---|
| UbcH10 | 0.02 | 0.74 |
| CCND2 | 0.002 | 0.81 |
| PCSK2 | 0.22 | 0.62 |
| UbcH10/CCND2 | - | 0.84 |

Each gene average level of expression in FNAs associated to benign and malignant histology is schematically shown in Figure 19.

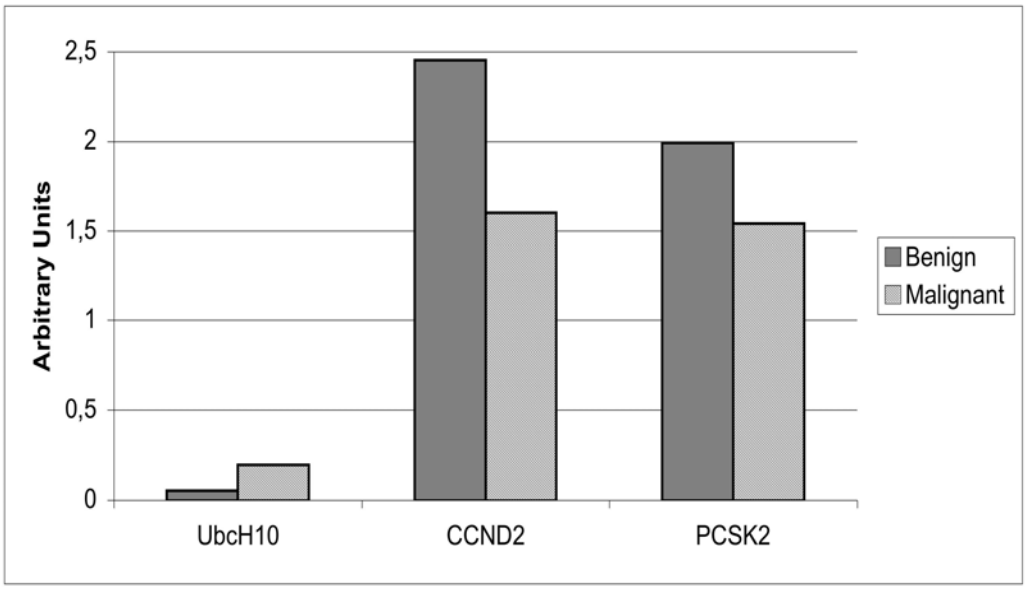


**Figure 19 Quantitative analysis of UbcH10, CCND2 and PCSK2 mRNA levels derived from FNAs associated to benign and malignant histology.** Average level of expression of UbcH10, CCND2 and PCSK2 genes in benign and malignant lesions. Arbitrary Units are obtained by the following formula: $2^{-\Delta C\ t}$ where ΔCt =Ct HGUS - Ct target (Shibru et al.2008).

ROC curve results are shown in Figure 20 A-B. The AUC for UbcH10 and CCND2 to distinguish benign from malignant thyroid neoplasm tissue samples were respectively 0.74 and 0.81 (Figure 20A). Their combination increased the diagnostic accuracy, with an AUC of 0.84 (Figure 20B). The AUC for PCSK2 was 0.62 (Figure 20A).

Thus, UbcH10 expression assessment by qRT-PCR is effective and the results are easy to be interpreted, thanks to a constitutive reference gene (Shibru et al. 2008; Samija et al. 2008). CCND2 and PCSK2, the most performing components of the 3-gene assay, were used for comparison (Weber et al. 2005; Shibru et al. 2008). The UbcH10 diagnostic accuracy was similar to that CCND2 and higher than PCSK2. Moreover, the UbcH10-CCND2 combination further increased the qRT-PCR diagnostic accuracy.

Thyroid cancer markers are more effective when they are evaluated in panels rather than individually; our data suggest that a panel composition should include UbcH10, thus it is a useful marker in neoplasms diagnosis. Using gene expression profiling, we found that UbcH10 was one of the genes most up-regulated in thyroid cancer cell lines (Pallante et al. 2005); then, we confirmed this observation on histological samples: UbcH10 is barely detectable in normal thyroid tissues, goiters and adenomas, whereas its increase was shown in papillary and follicular carcinomas (Pallante et al. 2005). UbcH10 is a prognostic marker for thyroid (Pallante et al. 2005), ovarian (Berlingieri et al. 2007), breast (Berlingieri et al. 2007) and lymphoid (Troncone et al. 2009) neoplasms. Similar findings have been reported by others for carcinomas arising from the lung (Wagner et al. 2004), bladder (Wagner et al. 2004), gastrointestinal tract (Takahashi et al. 2006; Ieta et al. 2007), liver (Ieta et al. 2007), gallbladder (Washiro et al. 2008), prostate (La Tulippe et al. 2002), oesophagus (Lin J et al. 2006) and brain (Jiang et al. 2008 ). In addition, UbcH10 is a very sensitive and specific marker of circulating breast tumour cells (Chen et al. 2006). Thus, it is widely held that UbcH10 overexpression is associated to cancer. Conversely, other candidate thyroid cancer markers are supported by less consistent data. In one study, CCND2 was underexpressed in malignant samples (Weber et al. 2005) and upregulated in others (Shibru et al. 2008). In this study we observed that CCND2 is downregulated in malignant samples.

Thus here we show that UbcH10 can effectively be translated into a clinically useful marker for making patient care decisions, without interfering with conventional cytology. In summary, our prospective study of routinely collected follicular neoplasm/suspicious for malignancy FNA suggested that quantitative RT-PCR analysis of UbcH10 expression level, rather than immunohistochemistry, is the elective method to increase the suspicion of malignancy in this controversial area of diagnostic cytology.

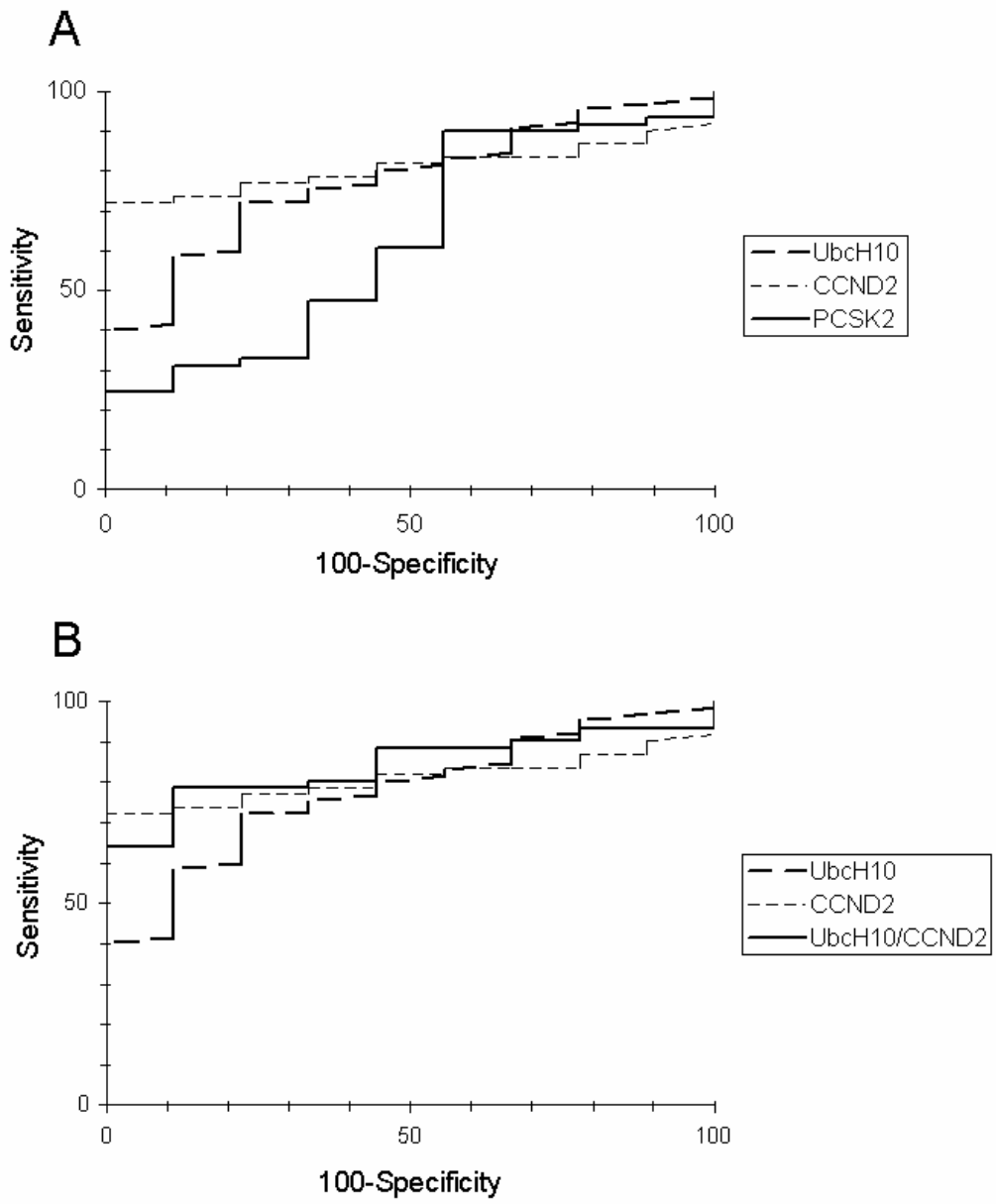


**Figure 20 AUC for individual UbcH10, CCND2 and PCSK2 expression and their combination by using logistic regression analysis.** Panel A shows the ROC curve for any single gene. Panel B shows the ROC curves for singly and combined UbcH10 and CCND2 genes.

# 5. CONCLUSIONS

UbcH10 is essential for G2/M cell cycle progression, as it plays a pivotal role in mitotic cyclins ubiquitination. Previous studies found that its expression was up-regulated in several cancer cell lines and tissues. Moreover UbcH10 expression was, also *in vivo*, consistently associated to cell proliferation. In this study we evaluated UbcH10 in three different diagnostic settings: breast cancer, lymphomas and thyroid FNAs.

Breast cancer represents a good model for studying epithelial multistep carcinogenesis, and, therefore, we aimed to evaluate the detection of the UbcH10 expression as a possible tool in the diagnosis of this tumor. In this setting we demonstrated that high UbcH10 expression is associated to those aggressive neoplasms. In breast carcinoma cell lines the suppression of the ErbB2 expression induced a reduction of UbcH10 level and, moreover, the block of UbcH10 protein synthesis by RNA interference resulted in cell growth inhibition.

Proliferation markers are useful to grade non-Hodgkin's lymphoma into low- and high-growth fraction neoplasms. Thus UbcH10, whose expression specifically covers the G2/M cell cycle window, is worth investigating. Also in lymphomas UbcH10 expression was related to the grade of malignancy, being low in indolent tumors and high in a variety of NHL cell lines and in aggressive lymphomas. As observed in breast cancer, blocking of UbcH10 synthesis by RNA interference inhibited cell growth. Flow cytometry of lymphoma cell lines confirmed that UbcH10 expression is cell-cycle dependent, steadily increasing in S phase, peaking in G2/M phase and dramatically decreasing in G0/G1 phases.

UbcH10 is one of the genes most up-regulated in thyroid cancer cell lines and tissues. Often pre-operative thyroid biopsies yield uncertain diagnoses. In these settings, testing for UbcH10 may have significance. We demostred that UbcH10 qRT-PCR analysis, rather than immunohistochemistry, was useful to increase the suspicion of malignancy on thyroid FNA samples.

In conclusion, all together, these results indicate a broad role of UbcH10 as neoplastic marker of different neoplasm types. UbcH10 may have both a diagnostic and a prognostic significance. Moreover, the suppression of its function is a possible tool in antineoplastic therapy.

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# UbcH10 is overexpressed in malignant breast carcinomas

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ABSTRACT

Our group has recently demonstrated the overexpression of the UbcH10 gene in undifferentiated thyroid carcinomas. Subsequently, a clear correlation between UbcH10 overexpression and a reduced survival in ovarian carcinoma patients has been described indicating UbcH10 as a valid prognostic marker in this neoplastic disease.

Here we have extended the analysis of the UbcH10 expression to neoplastic breast diseases. We demonstrated, by tissue micro-arrays immunohistochemical studies, a significant difference ($p = 0.0001$) in the mean percentage of UbcH10 stained cells between benign (0.22%) and malignant (11.01%) neoplastic lesions. High UbcH10 expression was associated with intense Ki-67 staining ($p = 0.015$) and ErbB2 positivity ($p = 0.092$).

The suppression of the ErbB2 expression in breast carcinoma cell lines induces a reduction of UbcH10 level. Consistently, the inhibition of breast carcinoma cell growth was achieved following the block of UbcH10 protein synthesis by RNA interference. Therefore, these results suggest the perspective of a therapy of aggressive breast carcinomas based on the suppression of the UbcH10 function.



## 1. Introduction

Human neoplasias derive from the accumulation of genetic alterations inside the cell. This results in drastic changes of the protein levels involved in cell growth control, signal transduction and cellular regulatory system in specific and characteristic manner.[1] The recent techniques based on microarray cDNA hybridisation have allowed the evaluation of the simultaneous expression of thousands of genes and, therefore, the identification of genes specifically regulated in neoplastic diseases.[2] To this purpose we have recently examined the gene expression profile of thyroid carcinoma cell lines compared with normal counterpart. Among the mostly up-regulated genes we identified the UbcH10 gene.[3] The UbcH10 gene belongs to the E2 gene family and codes for a protein of 19.6 kDa that is involved in the ubiquitin-dependent proteolysis. In this pathway, ubiquitin-conjugating enzyme (E2), together with ubiquitin ligase (E3), transfers ubiquitin to

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specific substrate proteins.[4,5] We were able to demonstrate that abundant UbcH10 levels were present in the highly invasive, undifferentiated thyroid carcinomas.[3] More recently, we have shown that UbcH10 expression significantly correlates with the tumour grade and the undifferentiated histotype of the ovarian carcinomas and a significant relationship was observed between UbcH10 expression and overall survival.[6]

Therefore, we decided to extend the studies of UbcH10 expression to breast carcinomas, this representing the first highest incidence neoplasia in women.[7]

Here, we report that the expression levels of UbcH10, evaluated by semiquantitative RT-PCR and immunohistochemical analysis, were increased in breast carcinomas compared to benign breast tissues. Since an association was found between ErbB2 and UbcH10 expression, we have blocked ErbB2 synthesis by RNA interference: this resulted in the reduction of UbcH10 expression, further validating a critical role of UbcH10 overexpression in the progression step of breast carcinogenesis.

## 2. Materials and methods

### 2.1. Cell culture

The human breast carcinoma cell lines used in this study were MB231 (metastasis of adenocarcinoma); MDA468 (metastasis of adenocarcinoma); MDA436 (metastasis of adenocarcinoma); MCF7 (metastasis of adenocarcinoma); T47D (metastasis of ductal carcinoma) and ZR 75-1 (metastasis of ductal carcinoma); they were purchased from the American Type Culture Collection (ATCC). They were grown in DMEM (Gibco Laboratories, Carlsbad, CA) containing 10% foetal calf serum (Gibco Laboratories), glutamine (Gibco Laboratories) and ampicillin/streptomycin (Gibco Laboratories). Cells were incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37 °C.

### 2.2. Human tissue samples

Neoplastic human breast tissues were obtained from surgical specimens and immediately frozen in liquid nitrogen. Samples were stored frozen until RNA or protein extractions were performed.

Breast carcinoma samples were collected at the Dipartimento di Anatomia Patologica e Citopatologia, Facoltà di Medicina e Chirurgia, Università di Napoli 'Federico II', by Dr. G. Troncone.

### 2.3. RNA isolation

Total RNA was extracted from tissues and cell cultures using the RNAeasy mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

### 2.4. Reverse transcriptase - PCR analysis

Five micrograms of total RNA from each sample, digested with DNAseI (Invitrogen), were reverse-transcribed using random hexanucleotides and MuLV reverse transcriptase (Applied Biosystems, Foster City, CA). Semiquantitative PCR was carried out on cDNA using the GeneAmp PCR System 9600 (Applied Biosystems). RNA PCR Core Kit (Applied Biosystems) was used to perform semiquantitative PCR reactions. For the UbcH10 gene, after a first denaturing step (94 °C for 3 min), PCR amplification was performed for 25 cycles (94 °C for 30 s, 57 °C for 30 s, 72 °C for 30 s). The sequences of forward and reverse primers were: forward 5′-GTCTGGCGATAAAGGGAT-3′ and reverse 5′-GGAGAGCAGAATGGTCCT-3′ corresponding to the nucleotides 172–190 and 443–425 respectively. The human ß-actin gene primers, amplifying a 109 bp cDNA fragment, were used as control: ß-actin-forward, 5′-TCGTGCGTGACATTAAGGAG-3′; ß-actin-reverse, 5′-GTCAGGCAGCTCGTA-GCTCT-3′. To ensure that RNA samples were not contaminated with DNA, negative controls were obtained by performing the PCR on samples that were not reverse-transcribed, but otherwise identically processed. For semiquantitative PCR, reactions were optimised for the number of cycles to ensure product intensity within the linear phase of amplificaton. The PCR products were separated on a 2% agarose gel, stained with ethidium bromide and scanned using a Typhoon 9200 scanner.

### 2.5. Protein extraction, Western blotting and Antibodies

Cells were washed once in cold PBS and lysed in a lysis buffer containing 50 mM HEPES, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg aprotinin, 0.5 mM sodium orthovanadate, 20 mM sodium pyrophosphate. The lysates were clarified by centrifugation at 14,000 rpm × 10 min. Protein concentrations were estimated by a Bio-Rad assay (Bio-Rad), and boiled in Laemmli buffer (Tris-HCl pH 6.8 0,125 M, SDS 4%, glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0.002%) for 5 min before electrophoresis. Proteins were subjected to SDS-PAGE (15% polyacrylamide) under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon-P Millipore Corp., Bedford, MA); complete transfer was assessed using prestained protein standards (Bio-Rad). After blocking with TBS-BSA (25 mM Tris, pH 7.4, 200 mM NaCl, 5% bovine serum albumin), the membrane was incubated with the primary antibody against UbcH10 (Boston Biochem Inc., Cambridge, MA) for 60 min (at room temperature). To ascertain that equal amounts of protein were loaded, the Western blots were incubated with antibodies against the α-tubulin protein (Sigma). Membranes were then incubated with the horseradish peroxidase-conjugated secondary antibody (1:3.000) for 60 min (at room temperature) and the reaction was detected with a Western blotting detection system (ECL; Amersham Biosciences, United Kingdom).

### 2.6. Immunostaining of TMA: Technique and Evaluation

UbcH10 breast immunoreactivity has been evaluated in two tissue micro-arrays (TMAs), constructed at the Department of Histopathology, S. Chiara Hospital, Trento, Italy. Original diagnosis were reviewed according to standard criteria[8] and clinical, long term follow-up and immunohistochemical data available for all tumours were collected by means of

TMABoost, an integrated system for the management of tissue microarray experiments.[9] The breast TMA included 23 benign samples and 115 invasive breast carcinomas, with two cores sampled for each case.

Briefly, xylene dewaxed and alcohol rehydrated TMA paraffin sections were placed in Coplin jars filled with a 0.01 M tri-sodium citrate solution, and heated for 3 min in a conventional pressure cooker.[3] After heating, slides were thoroughly rinsed in cool running water for 5 min. They were then washed in Tris-Buffered Saline (TBS) pH 7.4 before incubating overnight with the specific rabbit polyclonal antibody α-UbcH10 (BostonBiochem) diluted 1:1000. After incubation with the primary antibody, tissue sections were stained with biotinylated anti-rabbit immunoglobulins, followed by peroxidase labelled streptavidine (Dako, Carpinteria, CA, USA); the signal was developed by using diaminobenzidine (DAB) chromogen as substrate. Incubations, both omitting and preadsorbing the specific antibody, were used as negative controls.

Special care was taken to evaluate UbcH10 expression only on well preserved tissue cores. In fact, whenever possible, the labelling of mitotic figures was adopted as the required criteria to ensure the validity of staining, as shown previously.[3,6] Two benign and 12 malignant cases, in which both cores were not adequately stained, were excluded from the study. Thus, the percentage of UbcH10 expression was evaluated in 21 benign and 103 malignant cases; the latter were classified including ductal ($n = 69$), lobular ($n = 13$) and special histotypes ($n = 21$), such as mucinous, medullary and tubular types. The percentage of UbcH10 stained cells was jointly evaluated by two pathologists (MB, GT) at the double headed microscope. Values relative to each tumour samples, present in duplicate, were derived by combining the percentage of any single cores.

### 2.7. UbcH10 expression in breast cancer: Clinical-pathological analysis on TMA

To analyse tissue microarrays, pooling methods and cut-off values have to be chosen.[10] Ideally, these choices should be driven by prior biological knowledge. To our best knowledge, there is no biological evidence in favour of a certain pooling method; therefore, we employed maximum, minimum and mean as pooling methods for the replicates. Statistical analysis was carried out for each pooling method. We dichotomised biomarker expression against its median values, so as to ease biological interpretation. Finally, to account for the heterogeneity of protein expression across tumour tissues, cases ($n = 29$) with only one core section with valid staining were excluded by the statistical analysis. This resulted in 74 cases of breast cancer in which comparisons were made between UbcH10 expression and clinicalpathological data (histotype, grading, T, N, etc.), as well as with other biomarkers (i.e. p53, ER, PR and others).

### 2.8. Statistical methods

The association of clinicopathological and biological data with UbcH10 expression was examined using the following tests: Wilcoxon signed rank test or $\chi 2$ or Kruskall–Wallis test on numerical expression data and with Fisher's exact test on categorised data. Statistical analysis was performed using the R statistical package[11] and SPSS ver. 11.5 for Windows. Each comparison was performed accounting for the different pooling methods. Kaplan–Meier survival analysis with log-rank test was carried out for both overall and relapse free survival. A $p$-value less that 0.05 was considered statistically significant for each analysis.

### 2.9. RNA interference

For small interfering RNA (siRNA) experiments, the following double-strand RNA oligos specific for UbcH10 coding region were used: 5′-AACCTGCAAGAAACCTACTCA-3′ as previously described.[12] As negative control we used a corresponding scrambled sequence as follows: 5′-AACTAACACTAGCTCAAGACC-3′.

For ErbB2 siRNA experiments we used a Hs_ERBB2 HP Validated siRNA from Qiagen (Catalog Number SI02223571) and as a control a Nonsilencing Control siRNA from Qiagen (Catalog Number 1022076).

All of the siRNA were transfected using Human/Mouse Starter Kit (Qiagen) according to the manufacturer's recommendations. siRNAs were used at a final concentration of 100 nM and $12 \times 10^5$ cells/well were plated in 6-well format plates. Proteins were extracted at 48 h and 72 h after siRNA treatment and the levels of the proteins were evaluated by Western blot.

## 3. Results

### 3.1. UbcH10 gene is overexpressed in breast carcinoma cell lines

We evaluated the expression of UbcH10 by RT-PCR in breast carcinoma cell lines in comparison to the normal tissue. All of the carcinoma cell lines showed a high UbcH10 expression that, conversely, was barely detectable in the normal tissue (Fig. 1A). These results were confirmed by Western blot analysis that showed the presence of an abundant band of 19.6 kDa corresponding to the UbcH10 protein, compared to the normal breast tissue (Fig. 1B).

### 3.2. Analysis of UbcH10 expression in normal and neoplastic breast tissues by RT-PCR and Western blot analysis

UbcH10 expression was also evaluated by RT-PCR analysis on a panel of matched tumour/normal tissues. As shown in Fig. 2A, an amplified band of 115 bp was clearly detected in two ductal, two lobular and one mixed carcinoma samples, but not in one mastopathy and in all the corresponding normal breast tissues. Similar results were obtained when the UbcH10 protein levels were analysed as demonstrated by a representative Western blot shown in Fig. 2B. In fact, a band corresponding to the UbcH10 protein was detected in ductal, lobular and mixed carcinoma tissues, but not in two mastopathies and in normal breast tissues. Equal amounts of total proteins were used for each sample as demonstrated by the same gel analysed with an antibody against α-Tubulin. Therefore, these data show a strong overexpression of UbcH10 in breast malignancies.

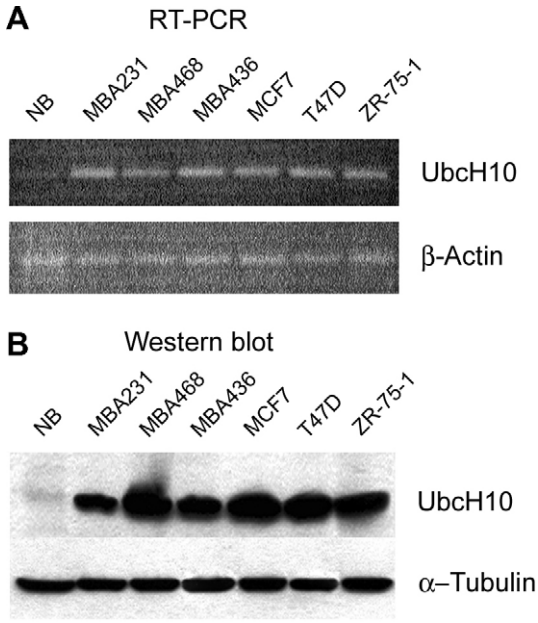


**Fig. 1 – UbcH10 expression in human breast carcinoma cell lines. (A) UbcH10 gene expression analysis by RT-PCR in human breast carcinoma cell lines versus the normal breast tissue (NB). β-Actin gene expression was evaluated as control to normalise the amount of the used RNAs. (B) UbcH10 protein expression analysis by Western blot in the same human breast carcinoma cell lines. Blot against α-Tubulin has been performed as control for equal protein loading.**

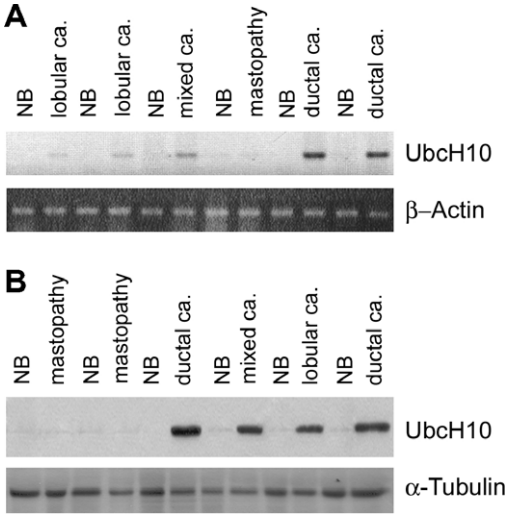


**Fig. 2 – UbcH10 expression in human breast fresh tumour samples. (A) RT-PCR analysis of UbcH10 expression in human breast tumour samples versus their normal counterparts. β-Actin expression shows the same amount of RNAs used. NB, normal breast tissue; (B) Western blot analysis of UbcH10 protein expression in a panel of breast neoplasias. The level of α-Tubulin has been used as loading control. NB, normal breast tissue.**

### 3.3. *Immunostaining pattern of UbcH10 expression in breast cancer*

To confirm the UbcH10 overexpression we analysed 103 malignant and 21 benign cases by immunohistochemical technique because it allows a rapid and sensitive screening of breast pathological tissues and is amenable to regular use as a routine diagnostic test. The immunocytochemical staining pattern of UbcH10 in breast carcinomas differed from that observed in benign breast samples. In fact, the latter were almost always completely negative for UbcH10 expression and the mean of cells expressing UbcH10 was 0.22%. Only occasionally, single UbcH10-labelled breast epithelial cells could be observed by meticulous scrutiny (Fig. 3A). Conversely, the UbcH10 staining was always easily detectable in the nuclei of the breast carcinoma cells with a strong staining intensity, mostly evident in cells showing mitotic figures (Fig. 3B and 3C). In this group the mean of UbcH10-positive cells was 11.01%; the differences in the mean percentage of UbcH10 stained cells between benign and malignant lesions was highly significant ($p = 0.0001$ Wilcoxon signed rank test).

### 3.4. *UbcH10 expression and clinical-pathological data*

In this study we used Tissue Microarrays (TMAs) provided with relevant clinico-pathological parameters, such as tumour size, node status, grading, hormonal status, proliferation index, p53 and ErbB2 status and survival rates. High UbcH10 expression was associated with ductal histotype ($p = 0.065$; Fisher's exact test), with ErbB2 positivity ($p = 0.092$ Fisher's exact test) and high Ki-67 staining ($p = 0.015$ Fisher's exact test), while no relationship was seen with tumour size and grade, p53 expression, hormonal status (as assessed by ER and PgR tissue staining) and the rates of overall and relapse free survival.

### 3.5. *UbcH10 expression is dependent on ErbB2 expression*

The association of UbcH10 staining with ErbB2 amplification suggested the hypothesis that the expression of Ubch10 could be under the control of ErbB2 activity. To validate this hypothesis we suppressed the synthesis of the ErbB2 protein by interference methodology and analysed the UbcH10 expression. As shown in the Western blot of Fig. 4, the transfection with siRNA oligonucleotides targeting ErbB2 was able to drastically reduce the ErbB2 protein levels in the MCF-7 cell line. Consistently with our hypothesis, the expression of UbcH10 paralleles ErbB2 levels (72 h).

### 3.6. *Suppression of the UbcH10 synthesis inhibits breast carcinoma cell growth*

We asked whether UbcH10 overexpression had a role in the process of breast carcinogenesis by evaluating the growth rate of one breast carcinoma cell line, in which UbcH10 protein was suppressed by RNA interference. The T47D cell line was treated with siRNA duplexes targeting the UbcH10 mRNA. After transfection we observed an efficient knock-down of

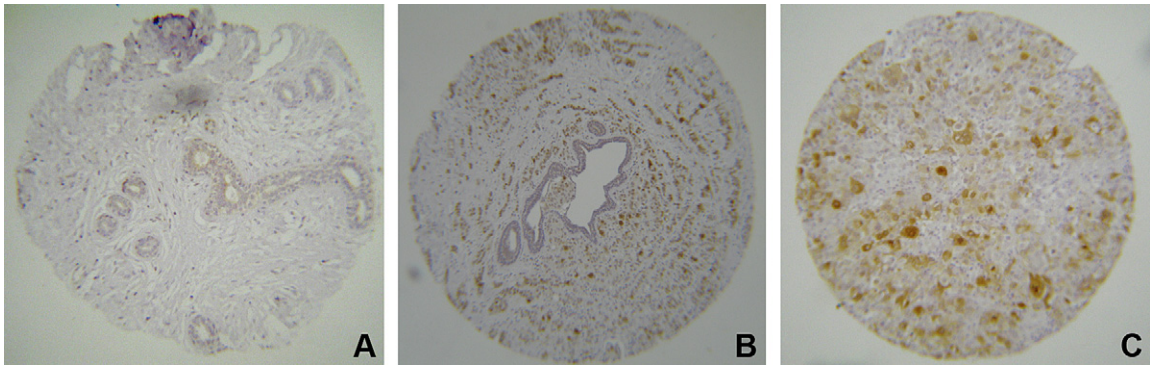


**Fig. 3 – Immunostaining pattern of UbcH10 expression in breast cancer. UbcH10 expression in benign (A) (original magnification, 25×) and malignant (B, C) (original magnification, 25×) breast tissues. While benign tissue is lacking UbcH10 expression (A), ductal cancer (C) displays high levels of protein expression. Abundant UbcH10 expression is also shown by lobular cancer (B): note a benign duct negative for UbcH10 expression, whereas infiltrating malignant cells are strongly positive for UbcH10.**

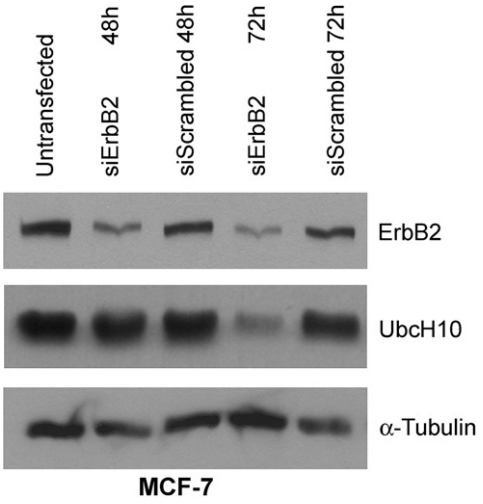


**Fig. 4 – UbcH10 expression is dependent on ErbB2 expression. Inhibition of ErbB2 protein expression by siRNA in MCF7 cell line evaluated by Western blot analysis shows, at 72 h after siRNA transfection, a decrease of the UbcH10 expression. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Total cell lysates were prepared and normalised for protein concentration. The expression of α-Tubulin was used to control equal protein loading (30 μg).**

the UbcH10 protein levels at 48 h after treatment (Fig. 5A). The analysis of cell growth in the presence or absence of the UbcH10 siRNA duplexes revealed that the block of the UbcH10 protein synthesis significantly inhibits breast carcinoma cell growth. In fact, as shown in Fig. 5B, a significant reduction in cell growth rate was observed in T47D cell line treated with UbcH10 siRNA in comparison to the untreated cells or those treated with the control scrambled siRNA.

These results indicate a critical role of UbcH10 in neoplastic breast cell proliferation.

## 4. Discussion

It has already been determined that approximately 20–30% of breast cancers arise in women who have inherited mutations in cancer susceptibility genes such as BRCA1, BRCA2 and other DNA repair genes.[13] Conversely, the vast majority of breast cancers are sporadic, presumably resulting from the accumulation of genetic damage over lifetime.[13]

Neoplastic breast diseases range from benign fibroadenomas, lobular and ductal, to very aggressive undifferentiated carcinomas.[14] One of the main differences between lobular and ductal breast carcinomas is the presence of inactivating E-cadherin gene mutations in the former.[15] In many other respects, lobular breast carcinomas and low-grade ductal carcinomas exhibit similar geno-phenotypic profiles.[16] The development of p53 dysfunction may be a hallmark of infiltrating ductal carcinomas of intermediate and high grade. Sequential ErbB2/neu and Ras abnormalities define a subset of aggressive high-grade tumours, and the development of Rb dysfunction may define a separate subset of aggressive ductal cancers.[17] Moreover, recent results on breast cancer show a direct relationship between the gene expression profile and clinical aggressiveness of the neoplasia.[18]

Based on these observations, breast cancer represents a good model for studying epithelial multistep carcinogenesis, and, therefore, our study aimed to evaluate the detection of the UbcH10 expression as a possible tool to be used in the diagnosis of breast carcinomas.

The data obtained in our laboratories assess that UbcH10 expression allow to discriminate benign from malignant breast neoplasias since immunohistochemical studies in breast cancer indicate a significant difference ($p = 0.0001$) in the mean percentage of UbcH10 stained cells between benign (0.22%) and malignant lesions (11.01%). Interestingly, an association was found between UbcH10 expression and ErbB2 amplification. This prompted us to verify whether there was a functional correlation between these two events. Indeed, a drastic decrease in UbcH10 expression followed the block of ErbB2 protein synthesis. Therefore, UbcH10 can be considered

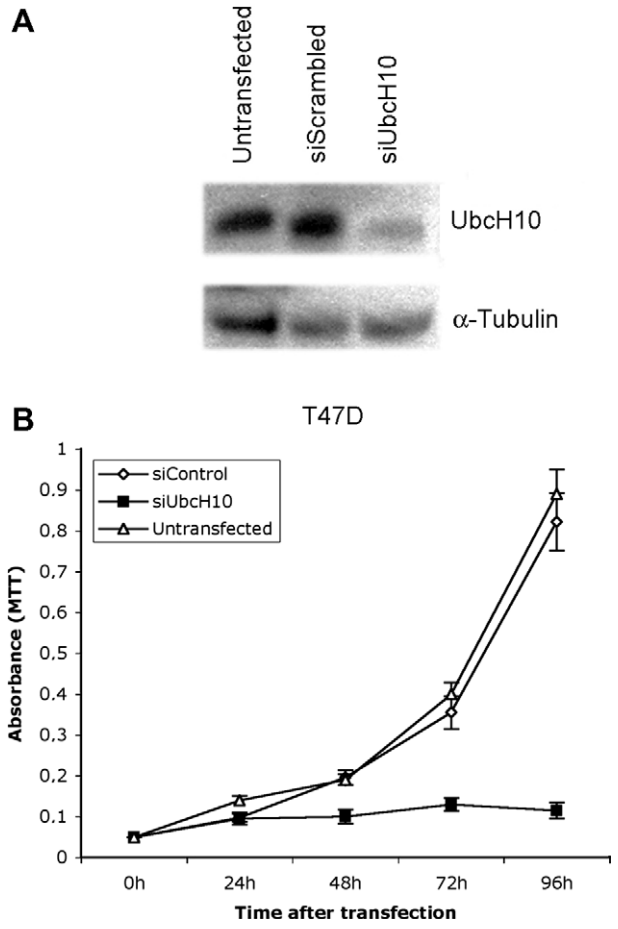


**Fig. 5 – The block of UbcH10 protein synthesis by RNA interference inhibits the proliferation of breast carcinoma cells. (A) Inhibition of UbcH10 protein expression by siRNA in T47D cell line evaluated by Western blot analysis. At 48 h after siRNA transfection, total cell lysates were prepared and normalised for protein concentration. The expression of α-Tubulin was used to control equal protein loading (30 μg). (B) Growth curves of T47D cell line after siUbcH10 treatment. T47D cells were transfected with siUbcH10 duplexes (siUbcH10) and the relative number of viable cells was determined by MTT assay. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Absorbance was read at 570 nm and the data are the mean of triplicates.**

one of the effectors of ErbB2 and then its role in breast carcinogenesis may be taken in consideration: this idea is further supported by functional studies demonstrating that the suppression of the UbcH10 expression reduced the growth of a breast carcinoma cell line. Therefore, it is likely that UbcH10 overexpression has a role in breast carcinogenesis by influencing the hyperproliferative status of the most malignant cells.

Our results confirm a critical role of the ubiquitination process in the breast carcinogenesis and in the proliferation of breast cancer cells. In fact, a recent work demonstrates the overexpression of a novel RING-type ubiquitin ligase breast cancer-associated gene 2 (BCA2) in breast carcinomas and its correlation with an increased proliferation, whereas a specific BCA2 small interfering RNA inhibited growth of T47D human breast cancer cells and NIH3T3 mouse cells.[19]

In conclusion, these results show a critical role of UbcH10 in breast carcinogenesis, strongly suggesting the suppression of its function as a possibile tool in breast carcinoma therapy.

## Conflict of interest statement

None declared.

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# UbcH10 expression in human lymphomas

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## UbcH10 expression in human lymphomas

*Aims*: The UbcH10 ubiquitin-conjugating enzyme plays a key role in regulating mitosis completion. We have previously reported that UbcH10 overexpression is associated with aggressive thyroid, ovarian and breast carcinomas. The aim of this study was to investigate UbcH10 expression in human lymphomas.

*Methods and results*: Cell lines and tissue samples of Hodgkin's lymphoma (HL) and of non-Hodgkin's lymphoma (NHL) were screened for UbcH10 expression at transcriptional and translational levels. UbcH10 expression was related to the grade of malignancy. In fact, it was low in indolent tumours and high in a variety of HL and NHL cell lines and in aggressive lymphomas. It was highest in Burkitt's lymphoma, as shown by quantitative real-time polymerase chain reaction and by tissue microarray immunohistochemistry. Flow cytometry of cell lines confirmed that UbcH10 expression is cell-cycle dependent, steadily increasing in S phase, peaking in $G_2/M$ phase and dramatically decreasing in $G_0/G_1$ phases. We also showed that UbcH10 plays a relevant role in lymphoid cell proliferation, since blocking of its synthesis by RNA interference inhibited cell growth.

*Conclusions*: Taken together, these results indicate that UbcH10 is a novel lymphoid proliferation marker encompassing the cell cycle window associated with exit from mitosis. Its overexpression in aggressive lymphomas suggests that UbcH10 could be a therapeutic target in this setting.

Keywords: HL, immunohistochemistry, NHL, RT-PCR, TMA, UbcH10 (E2C; Ube2c), Western blot

Abbreviations: BL, Burkitt's lymphoma; BSA, bovine serum albumin; CLL, chronic lymphocytic leukaemia; DLBCL, diffuse large B-cell lymphoma; FH, follicular hyperplasia; FITC, fluorescein isothiocyanate; FL, follicular lymphoma; HL, Hodgkin's lymphoma; MCL, mantle cell lymphoma; NGS, normal goat serum; NHL, non-Hodgkin's lymphoma; PBS, phosphate-buffered saline; PTCL, peripheral T-cell lymphoma; qRT-PCR, quantitative real-time polymerase chain reaction; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis; siRNA, small interfering RNA; SLL, small lymphocytic lymphoma; SMZL, splenic marginal zone lymphoma; TBS, Tris-buffered saline; TMA, tissue microarray

## Introduction

Proliferation markers are useful in the pathological assessment of Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL).[1] In particular, the Ki67 protein, whose expression is associated with cell cycle stages $G_1$, S and $G_2/M$, is routinely used to grade NHLs into low- and high-growth fraction neoplasms.[2] The

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former includes B-cell small lymphocytic lymphoma/chronic lymphocytic leukaemia (SLL/CLL), splenic marginal zone lymphoma (SMZL), mantle cell lymphoma (MCL) and follicular lymphoma (FL); the low proliferative index of these tumours is associated with a small cell size, advanced disease stages, low clinical aggression and poor response to chemotherapy.[3,4] In contrast, diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL) and peripheral T-cell lymphoma (PTCL) have a higher proliferative index, larger cells and aggressive behaviour; PTCL is poorly sensitive to current treatments, whereas DLBCL and BL are potentially responsive to chemotherapy.[1] It was recently shown that proliferation assessment by the $G_2$/M cell cycle genes is more predictive of treatment response than that based on the Ki67 protein.[5,6] Thus, the search for novel proliferation markers whose expression specifically covers the $G_2$/M cell cycle window is worth pursuing.

UBCH10 (also known as E2C or UBE2C) is a cell-cycle-related protein involved in mitosis completion.[7] Its ubiquitin-conjugating enzymatic activity (E2) is exerted from $G_2$/M to early $G_1$ phase, when UbcH10 together with ubiquitin ligase (E3) transfers ubiquitin to the mitotic cyclins, thereby promoting their degradation by the proteosome.[7] Once mitotic cyclins are ubiquitinated, UbcH10 triggers its own destruction.[8] This event marks mitotic completion and provides the molecular switch that allows cells to bring cell division to an end and proceed to the new round of DNA duplication.[9] Thus, UbcH10 is essential for cell cycle progression, and mutation of its active site cysteine confers a dominant-negative phenotype.[10]

In recent years, our group has been investigating the role of UbcH10 in carcinogenesis.[11–13] Using gene expression profiling, we found that UbcH10 was one of the genes most up-regulated in thyroid cancer cell lines.[11] We then showed that UbcH10 is a prognostic marker for thyroid,[11] ovarian[12] and breast carcinomas.[13] Similar findings have subsequently been reported for carcinomas arising from the lung,[14] bladder,[14] gastrointestinal tract,[14,15] liver,[16] gallbladder,[17] prostate,[18] oesophagus[19] and brain.[20] Moreover, UbcH10 is a very sensitive and specific marker of circulating breast tumour cells.[21]

A preliminary screening of anatomical UbcH10 expression has shown that it is highest in lymphoid tissue profiling.[14] More recently, UbcH10 was also shown to be up-regulated in a small cohort of FL patients by gene expression profiling.[6] However, UbcH10 investigation has not yet been systematically extended to lymphomas. The present study was undertaken to screen UbcH10 expression in cell lines and tissues representative of a wide range of indolent and aggressive lymphomas. We also evaluated UbcH10 cell cycle regulation and the effect of suppression of its synthesis on lymphoma cell growth.

## Materials and methods

### CELL CULTURE

Cell lines were obtained from the Continuous Cell Lines Collection at CEINGE Biotecnologie Avanzate (Naples, Italy). The HL cell lines used in this study were HDLM2, KM-H2, L-428, L-540 and L-1236. The NHL cell lines were Raji (Burkitt's lymphoma) and Karpas-299 (human anaplastic large cell lymphoma), Hut-102 and Hut-78 (cutaneous T-cell lymphoma). All cell lines were grown in Dulbecco's Modified Eagle's Medium (Gibco Laboratories, Carlsbad, CA, USA) containing 10% fetal calf serum (Gibco Laboratories), glutamine (Gibco Laboratories) and ampicillin/streptomycin (Gibco Laboratories). Cells were incubated in a humidified atmosphere of 95% air and 5% $CO_2$ at 37°C.

### TISSUE SAMPLES

Fresh surgical specimens of follicular hyperplasia (FH; $n = 5$), CLL/SLL ($n = 5$), MCL ($n = 2$), DLBCL ($n = 5$), BL ($n = 2$) and PTCL ($n = 2$) were obtained at the Dipartimento di Scienze Biomorfologiche e Funzionali, University of Naples Federico II, upon approval of the University Ethics Committee. Samples were immediately frozen in liquid nitrogen after removal and kept at −80°C until required for RNA and protein extraction.

### RNA ISOLATION

Total RNA was extracted from cell cultures and tissues using the RNAeasy mini kit (Qiagen Inc., Valencia, CA, USA) according to the manufacturer's instructions. The extracted RNA was dissolved in diethylpyrocarbonate-treated water, and its concentration and purity were assessed by measurement of optical density at 260/280 nm. The integrity of the RNA was assessed by denaturing agarose gel electrophoresis.

### cDNA PREPARATION

One microgram of total RNA of each sample was reverse-transcribed with QuantiTect® Reverse Transcription (Qiagen Inc.) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions.

### QUANTITATIVE REAL-TIME POLYMERASE CHAIN REACTION (RT-PCR) SELECTION OF PRIMERS AND PROBES

To design a quantitative real-time polymerase chain reaction (qRT-PCR) assay the Human ProbeLibrary™ system (Exiqon, Vedbaek, Denmark) was used. Using the free ProbeFinder assay design software, which is included in the package, the best probe and primer pair was chosen. To amplify a fragment for RT-PCR of UbcH10 mRNA, its accession number NM_007019 was entered on the assay design page of the ProbeFinder software. The sequences of forward and reverse primers were: forward 5′-TGCCCTGTATGATGTCAGGA-3′ and reverse 5′-GGGCTATCAATGTTGGGTTCT-3′, corresponding to the nucleotides 172–190 and 443–425, respectively. The probe number was 'human #58' (according to the numbering of Exiqon's Human ProbeLibrary kit). The same procedure was used to choose the probe and primers for the housekeeping gene glucose 6-phosphate dehydrogenase (*g6pd*; accession number X03674). An amplicon of 106 nucleotides that spanned the third and fourth exons was chosen. The probe number was 'human #05' (according to the numbering of Exiqon's Human ProbeLibry kit) and the primer sequences were: *g6pd* forward 5′-ACAGAG-TGAGCCCTTCTTCAA-3′; *g6pd* reverse 5′-GGAGGCT-GCATCATCGTACT-3′. All fluorogenic probes were dual-labelled with FAM at the 5′ end and with a black quencher at the 3′ end.

Relative Quantitative TaqMan PCR was performed in a Chromo4 Detector (MJ Research, Waltham, MA, USA) in 96-well plates using a final volume of 20 μl. For PCR we used 8 μl of 2.5× RealMasterMix™ Probe ROX (Eppendorf AG, Hamburg, Germany), 200 nM of each primer, 100 nM probe and cDNA generated from 50 ng of total RNA. The conditions used for PCR were 2 min at 95°C, and then 45 cycles of 20 s at 95°C and 1 min at 60°C. Each reaction was performed in duplicate. The $2^{-\Delta\Delta CT}$ method to calculate the relative expression levels was used.[22]

### PROTEIN EXTRACTION AND WESTERN BLOT

Cells and tissues were washed once in cold phosphate-buffered saline (PBS) and lysed in a lysis buffer containing 50 mM *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid, 150 mM NaCl, 1 mM ethylenediamine tetraaceticacid, 1 mM ethylene glycol tetraaceticacid, 10% glycerol, 1% Triton-X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg aprotinin, 0.5 mM sodium orthovanadate and 20 mM sodium pyrophosphate. The lysates were clarified by centrifugation at 16 000 ***g*** × 10 min. Protein concentrations were estimated by a BioRad assay (BioRad, Hercules, CA, USA) and boiled in Laemmli buffer (Tris–HCl 0.125 M pH 6.8, sodium dodecyl sulphate (SDS) 4%, glycerol 20%, 2-mercaptoethanol 10%, bromophenol blue 0.002%) for 5 min before electrophoresis. Proteins were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) (15% polyacrylamide) under reducing condition. After electrophoresis, proteins were transferred to nitrocellulose membranes (Immobilon-P Millipore Corp., Bedford, MA, USA); complete transfer was assessed using prestained protein standards (BioRad). After blocking with Tris-buffered saline (TBS)–bovine serum albumin (BSA) (25 mM Tris, pH 7.4, 200 mM NaCl, 5% BSA), the membrane was incubated with the rabbit polyclonal antibody against UbcH10 (BostonBiochem, Boston, MA, USA) diluted 1:500 for 60 min (at room temperature) and then incubated with the horseradish peroxidase-conjugated secondary antibody (1:3000) for 60 min (at room temperature); the reaction was detected with a Western blotting detection system (ECL; Amersham Biosciences, Little Chalfont, UK). To ascertain that equal amounts of protein were loaded, membranes were incubated with antibodies against the β-actin protein (Sigma, St Louis, MO, USA).

### TISSUE MICROARRAY IMMUNOHISTOCHEMISTRY: TECHNIQUE AND EVALUATION

UbcH10 immunoreactivity was compared with the standard marker of cell proliferation Ki67 and with the $G_2$/M phase indicator cyclin B1[23] on consecutive sections of the TA80 tissue microarrays (TMAs) produced in the Immunocytochemistry Unit at the Centro Nacional de Investigaciones Oncologicas, Madrid, Spain. This TMA included 177 samples consisting of 17 cylinders of reactive lymphoid tissue (including lymphadenitis and thymus) and 160 cylinders representative of the most frequent lymphomas, for all of which the diagnosis and classification had been confirmed by central review of standard tissue sections.[24]

Briefly, xylene-dewaxed and alcohol-rehydrated TMA paraffin sections were placed in Coplin jars filled with a 0.01 M tri-sodium citrate solution and microwaved. After heating, slides were thoroughly rinsed in cool running water for 5 min. They were then washed in TBS pH 7.4 before incubating overnight with the following antibodies: anti-UbcH10 (BostonBiochem), diluted 1:1000; mouse monoclonal anti-Ki67, clone Mib-1 (Dako, Glostrup, Denmark) diluted 1:100; and mouse monoclonal anti-cyclin B1, clone 7A9 (Novocastra, Newcastle, UK) diluted 1:10. After incubation with the

 

primary antibody, tissue sections were stained with specie-specific biotinylated secondary antibodies, followed by peroxidase-labelled streptavidin (Dako); the signal was developed by using diaminobenzidine chromogen (Dako) as substrate. Incubations with unrelated antibodies or without the specific antibodies were used as negative controls.

Special care was taken to evaluate UbcH10, Ki67 and cyclin B1 expression only on well-stained tissue cores. In fact, for each single cylinder and for each single antibody, the labelling of the mitotic figures was the criterion to ensure the validity of immunoreactivity, as previously shown.[11–13] Twenty-seven cases of lymphoma, whose cylinders had been damaged during the array procedure or lacked the appropriate reactivity of mitotic figures for any of the three antibodies, were not considered valid for scoring and were excluded from the study. Immunoreactivity was optimal for all three antibodies in 133 out of 160 cases, namely: 19 CLL/SLL, 19 MCL, 17 FL grade II, 16 SMZL, 14 DLBCL, 12 BL, 18 T-cell lymphomas and 18 HL. UbcH10, Ki67 and cyclin B1 reactivity was evaluated by two pathologists (G.T. and L.P.) with a double-headed microscope and the percentage of immunoreactive cells was scored in each single cylinder by assessing at least 100 cells. In the case of Ki67, only nuclear reactivity was considered to be specific, whereas for UbcH10 and cyclin B1, cells showing nuclear and/or cytoplasmic reactivity were considered to be positive. In each lymphoma type, protein expression was reported as median, highest or lowest value and interquartile range containing 50% of values, and between-type differences were analysed by the Kruskal–Wallis one-way ANOVA test. Correlation between UbcH10, Ki67 and cyclin B1 protein expression among diagnostic groups was evaluated by the non-parametric Spearman's ρ test. A *P*-value $<0.05$ was considered to be statistically significant.

### FLOW CYTOMETRY FOR UbcH10 EXPRESSION

Peripheral blood lymphocytes and neoplastic cell lines were analysed for expression of the UbcH10 protein by flow cytometry (Becton Dickinson FACS Canto II; New York, NY, USA). Samples were fixed in 70% cold ethanol and washed in cold PBS before being incubated with the specific UbcH10 protein rabbit polyclonal antibody (BostonBiochem) diluted 1:10 in PBS containing 0.5% Tween 20 and 1% normal goat serum (NGS). The cells were then washed in cold PBS and in PBS containing 0.5% Tween 20 and 1% NGS for 10 min at room temperature before being incubated with a fluorescein isothiocyanate (FITC)-conjugated antirabbit secondary antibody at room temperature (Becton Dickinson). We evaluated UbcH10 protein levels during the different phases of the cell cycle using biparametric staining for DNA and for UbcH10 and the same protocol reported above, followed by incubation with 2.5 μg/ml propidium iodide and 1 mg/ml small interfering RNA (siRNA) in PBS overnight at 4°C.

### RNA INTERFERENCE

For siRNA experiments, the following double-strand RNA oligo specific UbcH10 coding region was used: 5′-AACCTGCAAGAAACCTACTCA-3′ as previously described.[11–13] As a negative control, a corresponding scrambled sequence was used as follows: 5′-AACTAA-CACTAGCTCAAGACC-3′. All siRNA duplexes were purchased from Qiagen and were transfected using Oligofectamine (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's recommendations. Small interfering RNAs were used at a final concentration of 100 nM and $12 \times 10^5$ cells/well were plated in six-well format plates. Proteins were extracted 24 and 48 h after siRNA treatment and the levels of the proteins were evaluated by Western blot.

## Results

### UbcH10 EXPRESSION IN LYMPHOMA CELL LINES

UbcH10 gene expression was assessed at both mRNA and protein levels in a large array of HL and NHL cell lines. qRT-PCR analysis showed that UbcH10 mRNA fold change values were higher in neoplastic cell lines than in peripheral blood (Figure 1A). Accordingly, a conspicuous 19.6-kDa band was shown by Western blotting only in cells (Figure 1B). Flow cytometry confirmed higher levels of UbcH10 expression versus peripheral blood lymphocytes. However, UbcH10 expression differed widely among cell lines, as shown by the UbcH10 FITC median values, which ranged from 428 to 7152 (Figure 1C).

### UbcH10 EXPRESSION IN LYMPHOID TISSUES

Both hyperplastic (FH) and neoplastic lymphoid tissues were systematically screened for UbcH10 expression. UbcH10 expression was abundant in FH, as consistently shown by qRT-PCR (Figure 2A) and Western blot (Figure 2B). The pole of the reactive germinal centres harbouring mitotic cells was intensely immunoreactive, and a very sharp border resulted from the contrast between the highly positive proliferating

centroblasts and the surrounding quiescent mantle zone cells (Figure 3). This pattern is very similar to that shown by Ki67 and by cyclin B1 (Figure 4). Similarly, in the thymus, UbcH10 labelling occurred only in proliferating cortical cells (data not shown). Thus, the intense UbcH10 expression in reactive lymphoid tissue was mainly due to the expansion of proliferative tissue.

The association between UbcH10 expression and proliferation was also evident in lymphomas. In fact, mitotic cells were consistently immunoreactive in each single cylinder (see arrows in Figure 3). In HL, UbcH10 selectively highlighted atypical cells. In particular, Hodgkin and Sternberg–Reed cells were strongly positive (Figure 3). In NHLs, the neoplastic grade closely paralleled UbcH10 expression. qRT-PCR showed higher average levels of expression in DLBCL, PTCL and BL than in SLL/CLL, MCL and SMZL samples (Figure 2A). Similarly, Western blot analysis revealed more intense expression in high- than in low-grade lymphomas (Figure 2B). This was confirmed by the TMA immunohistochemical data (Figure 5A). UbcH10$^{+}$ cells were scarce in CLL/SLL (median 1.5%), and only mitotic cells and para-immunoblasts were immunoreactive (Figure 3). UbcH10 expression was sporadic in MCL (median 3.0%), homogeneously distributed and limited to mitotic cells (Figure 3). Low UbcH10 expression occurred in SMZL (median 2.5%); the centre of the neoplastic nodules, consisting of small dark cells, remained negative, whereas the outer marginal zone, containing large cells and transformed blasts, was strongly positive. Thus, UbcH10 recapitulated the Ki67 'target-like' staining typical of SMZL

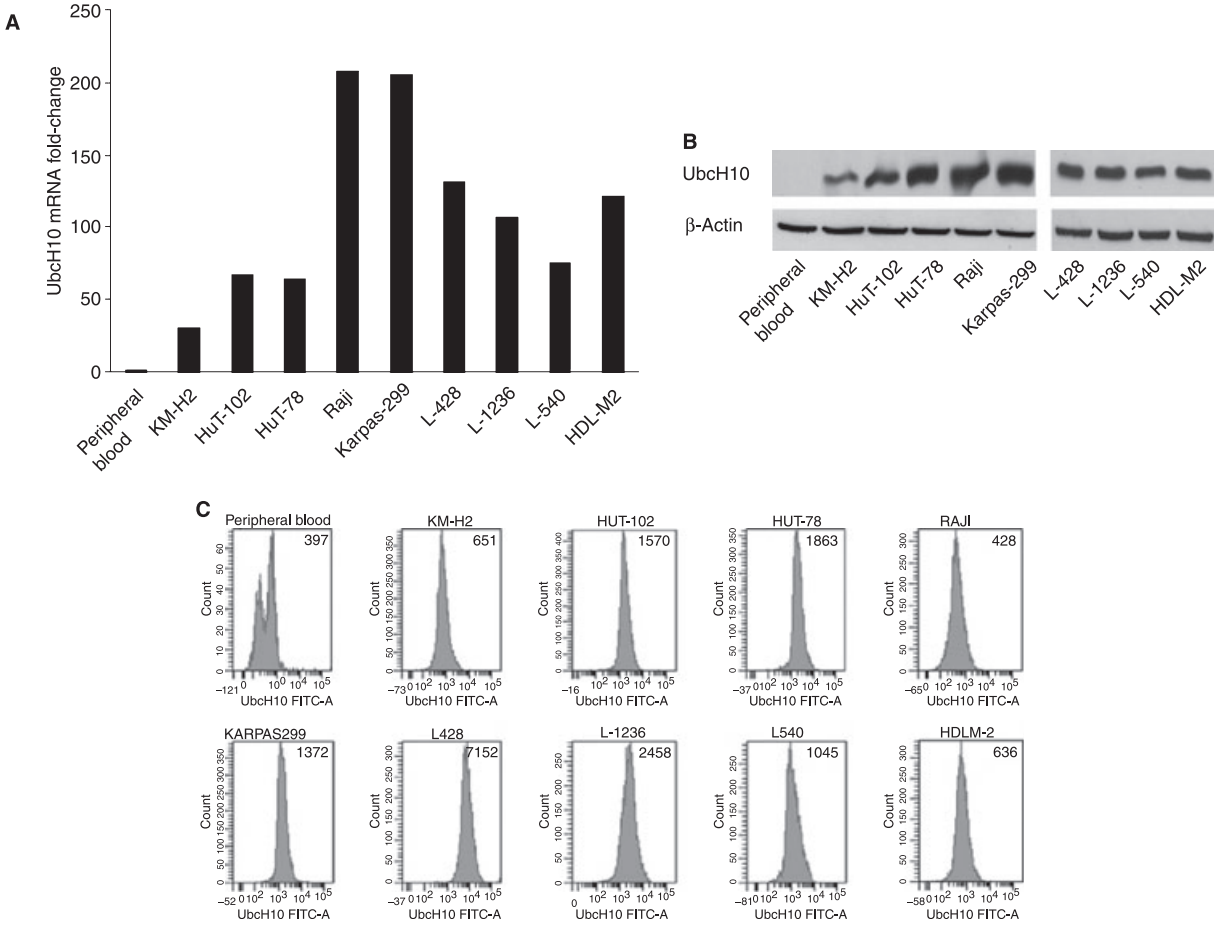


**Figure 1.** UbcH10 expression in human lymphoma cell lines. **A**, UbcH10 gene expression analysis by quantitative real-time polymerase chain reaction in human lymphoma cell lines versus normal peripheral blood lymphocytes. UbcH10 mRNA levels were high in all cell lines and barely detectable in peripheral blood lymphocytes. **B**, UbcH10 gene expression analysis by Western blot in human lymphoma cell lines versus normal peripheral blood lymphocytes. Note the very clear 19.6-kDa band corresponding to the UbcH10 protein in all cell lines, but not in peripheral blood lymphocytes. Blot against β-actin served to control for equal protein loading. **C**, UbcH10 protein expression analysis by flow cytometry in human lymphoma cell lines versus normal peripheral blood lymphocytes. Data within the quadrants are expressed as median UbcH10 fluorescent intensity values.

 

(Figure 3).[25] In FL grade II, UbcH10 staining (median 12.0%) was displayed only by centroblasts, whereas centrocytes remained negative. Neoplastic follicles lacked the zonation and the sharp border seen in the germinal centres of FH because positive cells did not concentrate at one pole but were scattered throughout the follicles (Figure 3). Higher (median 20.0%) levels of UbcH10 expression were found in PTCL, in which a large number of cells with irregular, pleomorphic nuclei were labelled (Figure 3). Similarly, in DLBCL, there was abundant nuclear/cytoplasmic UbcH10 reactivity (median 32.5%) in most centroblasts and in mitotic figures (Figure 3). UbcH10 expression was highest in BL cells (median 65.0%); indeed, most BL cells had very abundant nuclear/cytoplasmic reactivity (Figure 3).

### UbcH10 cell cycle regulation

We next investigated the relationship between UbcH10 and other proliferation markers on consecutive TMA sections. There was a significant correlation between UbcH10 and both Ki67 (Spearman's ρ 0.913; $P < 0.001$) and cyclin B1 (Spearman's ρ 0.931; $P < 0.001$) (Figure 5B). As evident on consecutive sections, cells stained by Ki67, UbcH10 and cyclin B1 antibodies were distributed in the same tissue areas (Figure 4), In particular, UbcH10 and cyclin B1 labelled a subpopulation of $Ki67^+$ cells. Thus, UbcH10 immunoreactivity was in keeping with a $G_2/M$ labelling index, which was similar to that obtained with cyclin B1 (Figure 4). We also investigated UbcH10 kinetics during cell cycle progression by flow cytometry (Figure 6). Similar kinetics was shown by both low- and high-expressing cell lines. Indeed, in all instances UbcH10 expression steadily increased from late $G_1$ through the S phase, peaked at $G_2/M$, and dramatically decreased in the $G_1/G_0$ phase of the cell cycle.

### Suppression of UbcH10 synthesis inhibits lymphoma cell growth

Since UbcH10 expression and lymphoid cell proliferation were closely related, we next evaluated whether suppression of the synthesis of the UbcH10 protein by RNA interference affected the growth rate of the human anaplastic large cell lymphoma Karpas-299 cell line. This cell line was treated with siRNA duplexes targeting the UbcH10 mRNA. Twenty-four hours after transfection, there was an efficient knock-down of UbcH10 protein levels (Figure 7A). Analysis of cell growth in the presence or absence of the UbcH10 siRNA duplexes revealed that the block of UbcH10 protein synthesis significantly inhibited lymphoma cell growth. In fact, as shown in Figure 7B, cell growth rate was significantly lower in cell lines treated with UbcH10 siRNA than in untransfected cells or in those treated with the control scrambled siRNA. These results indicate that UbcH10 plays a critical role in lymphoid cell proliferation.

## Discussion

In recent years, much attention has focused on the expression of the UbcH10 gene in human neoplasms; large series of different carcinoma types have been evaluated thanks to the availability of commercial

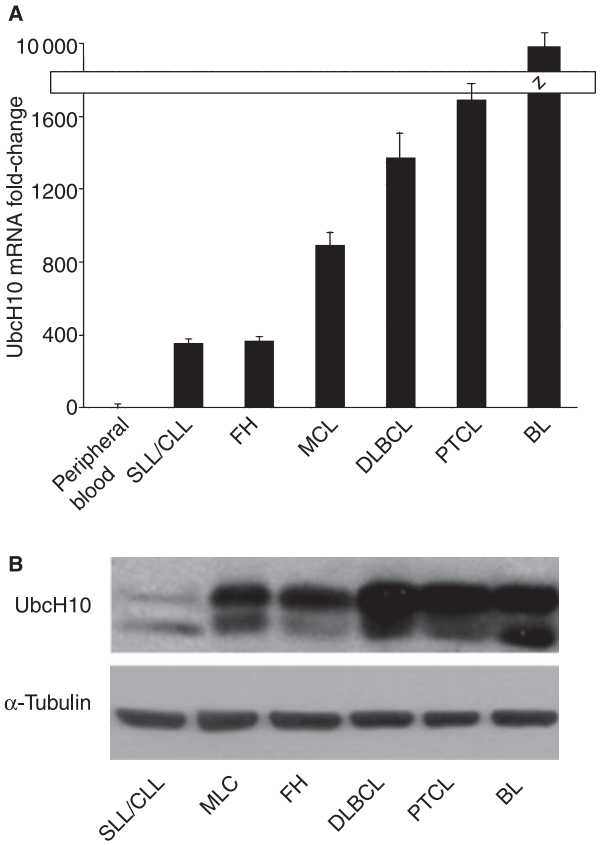


**Figure 2.** UbcH10 expression in lymphoid fresh tissue samples. **A**, Quantitative real-time polymerase chain reaction analysis showing higher UbcH10 mRNA fold changes in high-grade [diffuse large B-cell lymphoma (DLBCL), Burkitt's lymphoma (BL) and peripheral T-cell lymphoma (PTCL)] than in low-grade lymphoma [small lymphocytic lymphoma (SLL)/chronic lymphocytic leukaemia (CLL), mantle cell lymphoma (MCL)] and reactive lymph nodes [follicular hyperplasia (FH)]. Each histogram represents the average level of expression for each histotype. **B**, Western blot analysis of UbcH10 protein expression showing a more intense band in high-grade (DLBCL, BL and PTCL) than in low-grade lymphoma (SLL/CLL and MCL) and reactive lymph nodes (FH). A representative sample for each histotype is shown. Blot against α-tubulin showed as control for equal protein loading.

 

antibodies suitable for staining paraffin-embedded sections.[11–14,19,20] The results have consistently shown that high UbcH10 expression is a marker of aggressive carcinoma behaviour.

Here we have characterized the expression of UbcH10 at mRNA and protein levels in cell lines and tissues from indolent and aggressive lymphoma types. As a general rule, UbcH10 expression was cell-cycle-dependent and related to proliferation. Low levels were observed in peripheral blood lymphocytes, in quiescent naive B-cells of the mantle zones of secondary lymphoid follicles, and in low-grade lymphomas. High levels were observed in a wide variety of HL and NHL cell lines, in cells showing mitotic figures, in reactive germinal centre proliferating centroblasts and in aggressive lymphomas. UbcH10 expression increased with pathological grade, and reached a maximum in BL as revealed by qRT-PCR and TMA immunohistochemis-

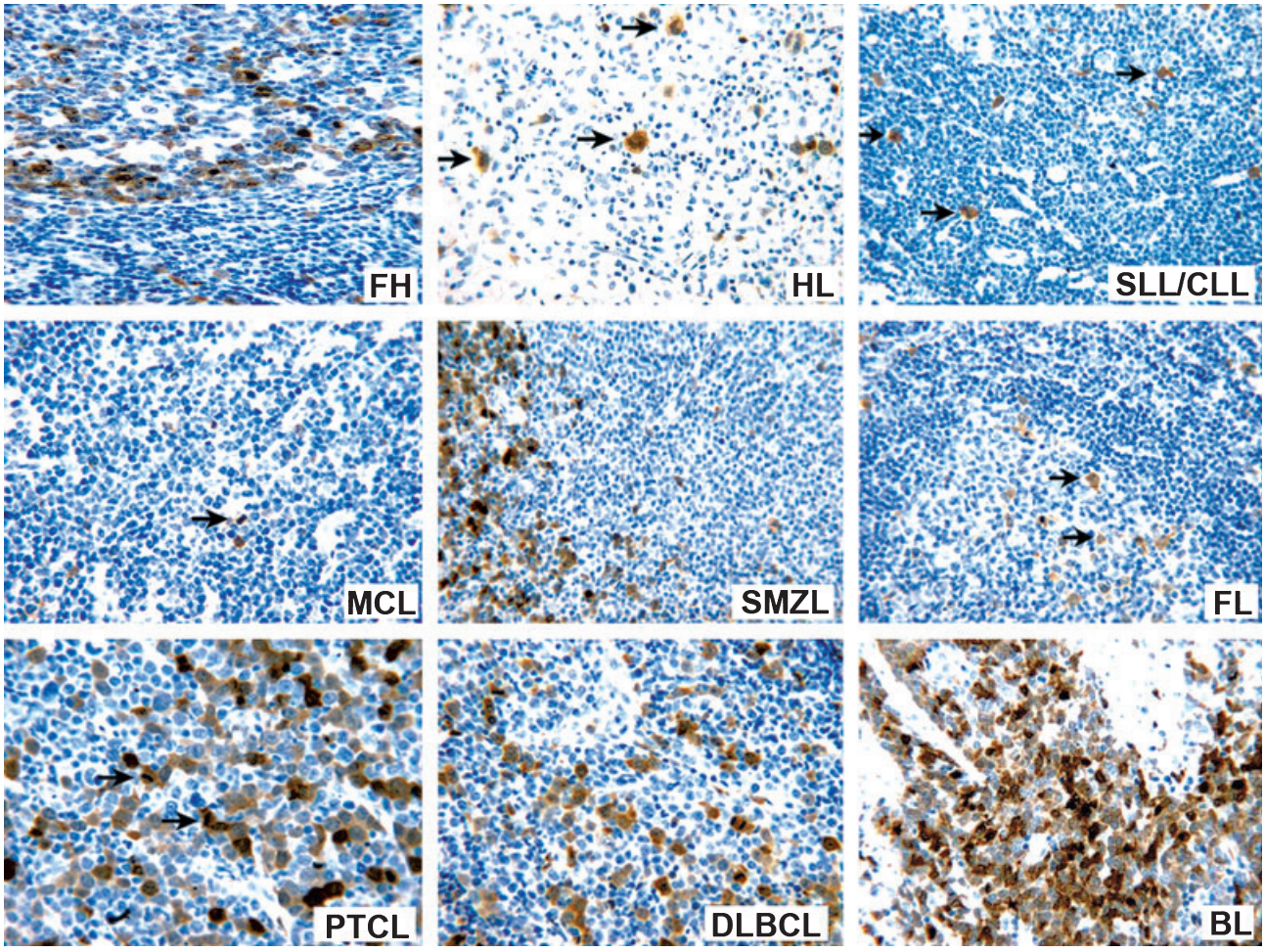


**Figure 3.** UbcH10 expression by tissue microarray immunohistochemistry. Follicular hyperplasia: abundant UbcH10 expression. The immunoreactivity is mainly seen at one pole of the germinal centre (arrows). A sharp border results from the contrast between the highly positive centroblasts and the lack of signal in the surrounding mantle zone. Hodgkin's lymphoma: immunoreactivity for UbcH10 selectively highlights atypical cells. Note the strong nuclear/cytoplasmic reactivity of popcorn cells (arrows) against a clean background of infiltrating inflammatory cells. Small lymphocytic lymphoma/chronic lymphocytic leukaemia: sporadic UbcH10 immunoreactivity. Only mitotic prolymphocytes and para-immunoblasts are positive (arrows), whereas small lymphocytes remain negative. Mantle cell lymphoma: low UbcH10 expression. The reactivity is limited to rare mitotic cells (arrow), whereas the neoplastic cells with irregular nuclei are negative. Splenic marginal zone lymphoma: UbcH10 target pattern. The neoplastic nodules show a negative central zone and positive large cells in the outer marginal zone. Follicular lymphoma grade II: neoplastic nodules lack the zonation and the sharp border of reactive germinal centres. Positive centroblasts are evenly scattered throughout the neoplastic follicles (arrows), whereas centrocytes are negative. Peripheral T-cell lymphoma: high UbcH10 expression. A large number of $UbcH10^{+}$ cells with irregular, pleomorphic nuclei (arrows). Diffuse large B-cell lymphoma: high UbcH10 expression. Note the intense UbcH10 nuclear/cytoplasmic immunoreactivity of the centroblasts and the intense labelling of the mitotic figures. Burkitt's lymphoma: very high UbcH10 expression. The vast majority of neoplastic cells show abundant UbcH10 expression. (Haematoxylin counterstain).

 

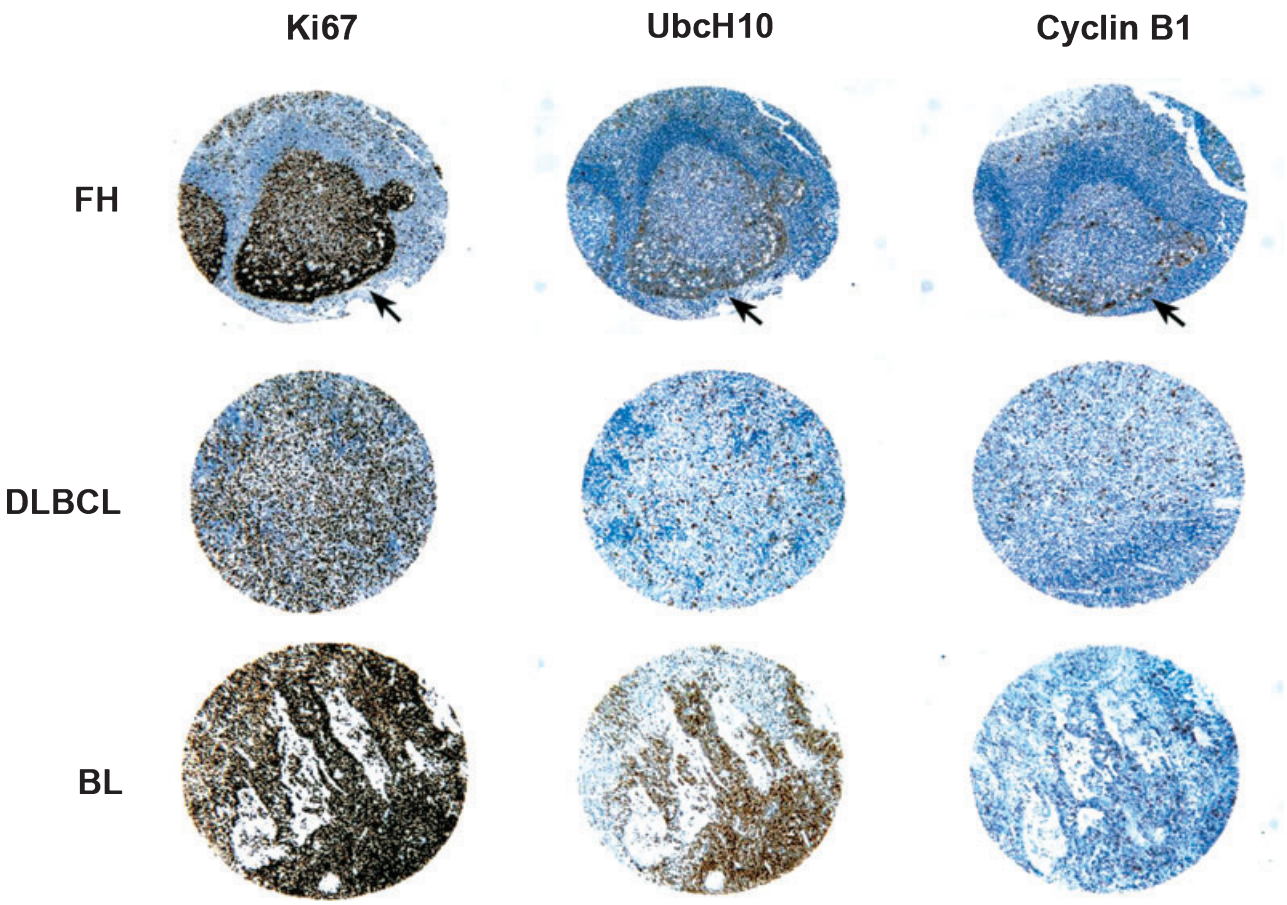


**Figure 4.** Ki67, UbcH10 and cyclin B1 expression on tissue microarray consecutive sections. Follicular lymphoma: Ki67 immunoreactivity highlights a very hyperplastic follicle, in which the light zone has been overrun by the expanded dark zone to yield a nearly 100% growth rate. Only the subpopulation of Ki67 localized at the germinal centre pole and displaying the highest Ki67 labelling (arrow) is also positive (arrow) for UbcH10. A similar distribution (arrow) is shown by cyclin B1. Diffuse large B-cell lymphoma: Ki67 stains a very large percentage of neoplastic cells evenly distributed throughout the tissue core. By contrast, UbcH10 stains a much smaller number of cells, and the pattern of immunoreactivity resembles that of cyclin B1. Burkitt's lymphoma: virtually all neoplastic cells are stained by Ki67; the large majority of cells are also stained by UbcH10. Note that, by immunohistochemistry, UbcH10 distinguishes between DLCBL and BL better than either Ki67 or cyclin B1 (haematoxylin counterstain).

try. This confirms the close relationship between UbcH10 overexpression and poor tumour differentiation described in thyroid,[11] ovarian,[12] breast,[13] lung,[14] bladder[14] liver[16] and brain[20] tumours. UbcH10 immunoreactivity was significantly related to reactivity of the proliferation markers Ki67 ($r = 0.91$; $P < 0.001$) and cyclin B1 ($r = 0.93$; $P < 0.001$; Figure 5B) and occurred in similar lymphoid tissue areas as shown on consecutive TMA sections (Figure 4). Taken together, these data indicate that UbcH10 is a novel lymphoid proliferation marker, whose overexpression in aggressive lymphomas probably reflects the tumour-related increases in cell proliferation.

We investigated this concept further by evaluating changes in the levels of UbcH10 expression during cell cycle progression. To this end, we evaluated a wide range of neoplastic lymphoid cell lines by flow cytometry. UbcH10 levels steadily increased in S phase, peaked at $G_2/M$ phase and dramatically decreased in $G_1/G_0$ phases (Figure 6). Thus, UbcH10 expression was mostly associated with the $G_2/M$ phase. Although Ki67 covers the whole cell cycle from early $G_1$ on,[2] it is conceivable that only in $G_2/M$ phase are UbcH10 levels high enough to be detected by immunohistochemistry. In fact, in all the TMA cores of non-neoplastic lymphoid tissue or lymphoma, the proportion of cells expressing UbcH10 never exceeded that of Ki67, being similar to the proportion of cyclin B1-stained cells (Figures 4 and 5A). Thus, aggressive B-cell lymphomas, which are constituted mainly by cycling cells, are better typed into DLBCL and BL by UbcH10 than by Ki67, whose expression is

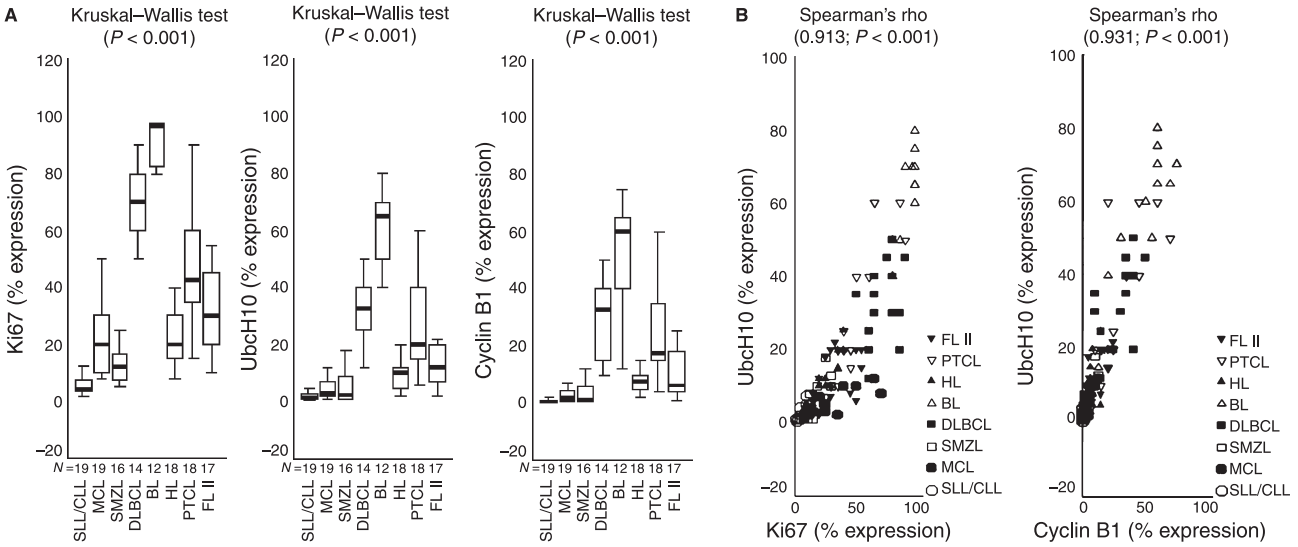


**Figure 5.** Statistical analysis of UbcH10, Ki67 and cyclin B1 immunohistochemistry on tissue microarray. **A**, Graphic representation of protein levels evaluated by immunohistochemistry. Data are reported as median, highest and lowest values and interquartile range containing the 50% of values for each diagnostic group. For any protein the differences between the different lymphoma types were analysed by the Kruskal–Wallis one-way ANOVA test. **B**, Graphic representation of the non-parametric Spearman's ρ test used to assess the correlation between UbcH10, Ki67 and cyclin B1 protein expression among the different diagnostic groups.

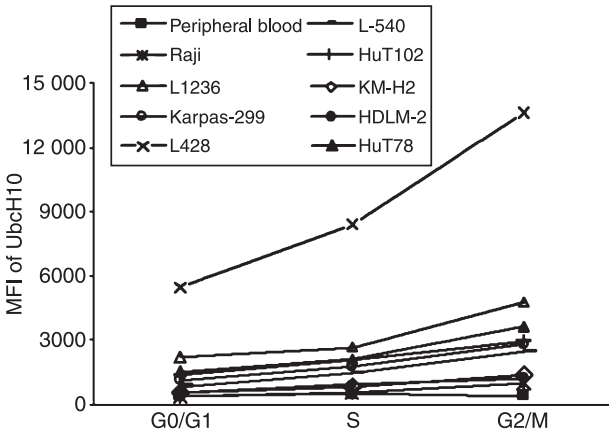


**Figure 6.** Median value of fluorescence intensity for UbcH10 immunoreactivity (MFI). Correlation between the expression of UbcH10 and the phases of cellular cycle in human lymphoma cell lines.

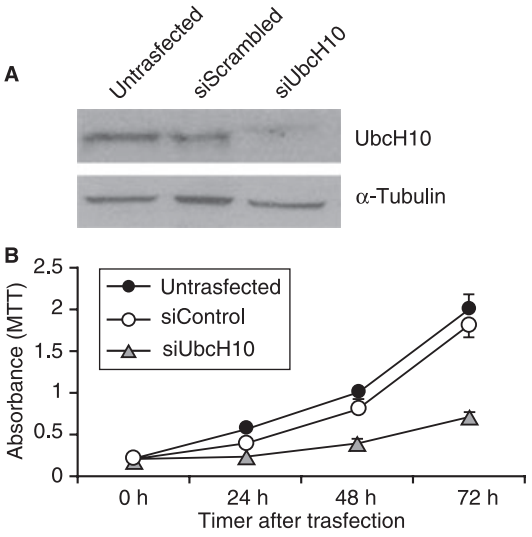


**Figure 7.** The block of UbcH10 protein synthesis by RNA interference inhibits the proliferation of the lymphoma cells. **A**, Inhibition of UbcH10 protein expression by small interfering (si) RNA in the Karpas-299 cell line evaluated by Western blot analysis (24 h). The expression of α-tubulin was used to control for equal protein loading 30 μg. **B**, Growth curves of the Karpas-299 cell line after siUbcH10 treatment. Karpas-299 cells were transfected with si-UbcH10 duplexes (siUbcH10) and the relative number of viable cells was determined by MTT assay. Cells transfected with a scrambled duplex (siScrambled) and untransfected cells (Untransfected) were used as negative controls. Absorbance was read at 570 nm and the data are the mean of triplicates.

uniformly too intense in both neoplasms (Figures 4 and 5A).

Previous studies have shown that UbcH10 is essential for cell cycle progression.[7] This finding prompted us to carry out functional studies to investigate whether UbcH10 could be a therapeutic target. We have shown that blocking of UbcH10 protein synthesis significantly inhibited growth of an NHL cell line. It was recently reported that oesophageal adenocarcinoma cells

 

strongly expressing UbcH10 were highly sensitive to treatment with the proteasome inhibitor MG-262.[19] Although no specific UbcH10 inhibitors are currently available for clinical use, targeting the ubiquitin-proteosome system may block the degradation of ubiquitinated mitotic cyclins, thereby preventing the effect exerted by UbcH10 on cell cycle progression. Further investigations are required to explore the possibility that inhibition of UbcH10 could be a therapeutic approach in lymphomas.

## Competing interests

None to declare.

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# UbcH10 Expression on Thyroid Fine Needle Aspirates

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# UbcH10 Expression on Thyroid Fine Needle Aspirates

Running title: UbcH10 on Thyroid FNAs

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**Condensed abstract**: We have recently identified UbcH10 as a novel cancer marker useful in the diagnosis and prognosis of several human neoplasms, including thyroid cancer. Here we show that quantitative RT-PCR analysis, rather than immunohistochemistry, of UbcH10 expression is useful to increase the suspicion of malignancy in follicular neoplasm/suspicious for malignancy routine thyroid fine-needle aspirate.

## ABSTRACT

**Background.** Thyroid fine-needle aspiration (FNA) samples belonging to the follicular neoplasm/suspicious for malignancy classes are controversial. We identified UbcH10 as a marker useful in the diagnosis of several neoplasms, including thyroid cancer. Here, analysis of UbcH10 expression by quantitative RT-PCR and immunohistochemistry was applied to FNAs. **Methods.** A series of 84 follicular neoplasm/suspicious for malignancy FNAs with histological follow-up (30 malignant) was prospectively collected. UbcH10 imunostaining was carried out on cell blocks and compared to that of the proliferation marker Ki-67. At the mRNA level, UbcH10 was compared with CCND2 and PCSK2 expression, these latter being the most performing components of the previously reported 3-gene assay; to determine the diagnostic accuracy the area under the curve (AUC) of the receiver operating characteristic (ROC) curve for each gene individually and in combination was evaluated. **Results.** UbcH10 and Ki-67 shared a similar pattern; although UbcH10 expression was higher in malignant than in benign lesions (P<0,001), staining was sporadic and the cut-

off value derived by the ROC analysis was too low (1,25%) for routine application. Conversely, UbcH10 expression assessment by qRT-PCR was effective. UbcH10 mRNA levels associated to malignant histology were significantly higher than those associated to benign histology (P= 0.02). The AUC was 0.74 for UbcH10, 0.81 for CCDN2, 0.62 for PCSK2 and 0,84 for UbcH10 and CCND2 combination. **Conclusions.** UbcH10 qRT-PCR analysis, rather than immunohistochemistry, is useful to increase the suspicion of malignancy in thyroid FNAs. UbcH10 may be added as a panel component in qRT-PCR based assays.

## INTRODUCTION

In spite of their very high frequency, only rarely thyroid nodules are malignant [1]. Fine needle aspiration (FNA) is widely used to identify those nodules whose treatment unequivocally requires surgery [1]. However, FNA is not always able to differentiate between benign and malignant lesions [2]. This uncertainty is clearly conveyed by the recent National Cancer Institute (NCI) thyroid FNA conference classification [3]. The follicular neoplasm category applies to a monotonous population of three-dimensional groups of follicular cells with scarce colloid; the suspicious for malignancy class is recommended when features suggesting of malignancy are only focally observed [3]. These uncertain diagnosis do not correspond to a single entity, but rather to a wide range of inflammatory, hyperplastic, and neoplastic histological lesions [3]. These latter including follicular adenoma (FA), follicular carcinoma (FTC) and follicular variant of papillary thyroid carcinoma (PTC), do not or only rarely harbour RET/PTC re-arragements or BRAF mutations [4]. Thus, panels of mRNA and protein cancer markers are needed to refine indeterminate diagnosis [5]. In this setting the 3-gene mRNA assay, which included cyclin D2 (CCND2), protein convertase 2 (PCSK2), and prostate differentiation factor (PLAB), allowed molecular classification of FTC and FA[6, 7].

Genes that regulate cell-cycle progression may be differentially expressed in malignant versus benign thyroid nodules[8]. UbcH10 (alias E2C or UBE2C) is a cell-cycle-related protein involved in mitosis completion[9]. Its ubiquitin-conjugating enzymatic activity (E2) is exerted from G2/M to early

G1 phase, when UbcH10 together with ubiquitin ligase (E3) transfers ubiquitin to the mitotic cyclins thereby promoting their degradation by the proteosome [9]. Once mitotic cyclins are ubiquinated, UbcH10 triggers its own destruction [10]. This event marks mitotic completion and provides the molecular switch that allows cells to bring cell division to an end and to proceed to the new round of DNA duplication [11]. Thus UbcH10 is essential in cell cycle progression [9]. Using gene expression profiling, we found that UbcH10 was one of the genes most up-regulated in thyroid cancer cell lines [12]. Next, we showed that UbcH10 is barely detectable in normal thyroid tissues, goiters and adenomas, whereas its increases in papillary and follicular reaching the highest level expression in anaplastic carcinomas [12]. In addition, UbcH10 is a robust marker of cell proliferation, as we have also observed in ovarian,[13] breast,[13, 14] and lymphoid [15] neoplasms.

UbcH10 has not been assessed on cytological samples yet. Here, UbcH10 was applied to follicular neoplasm and suspicious for malignancy thyroid FNA. UbcH10 expression was evaluated both at transcriptional and translational levels. At the mRNA level, its diagnostic performance was compared with those of the most performing components (CCND2, PCSK2) of the 3-gene diagnostic assay; similarly, at the protein level UbcH10 was compared to that of the standard proliferation marker Ki-67.

## MATERIAL AND METHODS

*Sample collection*

In our institution thyroid FNAs are routinely performed by the cytopathologist using 25-gauge needles, under ultrasound guidance aided by the radiologist.[16] From each passage by the nodule the obtained material is smeared onto one or two slides. On-site Diff-Quik stained smears are prepared from the first pass for rapid assessment of specimen adequacy. When a neoplastic lesion is suspected from the on-site assessment, additional passes are performed.

The rationale behind this study sample collection method was to ensure first an adequate cytological diagnosis and, then, to exploit the left-over material for UbcH10 analysis. All patients of this study provided informed consent and the study was approved by the University of Naples Federico II Ethics Committee. A total of 103 cases with a cytological diagnosis of either follicular neoplasm or suspicious for malignancy were prospectively collected. Eighty-four cases, for which histological follow-up was available, represented this study series. Fifty-eight patients were women and 26 were men (age range 18-75). On review, histology showed dominant hyperplastic nodule in 24 cases, Hashimoto's thyroiditis (HT) in 4 cases, FA in 26 cases, PTC in 24 cases, and FTC in 6 cases.

*Immunostaining*

The residual material from one or two passages by the lesion was processed as cell block (CB) and dedicated to UbcH10 and Ki-67 immunostainings. The CBs were prepared with the plasma-thrombin clot technique, as described elsewhere [17]. The criteria for CB adequacy were the presence of three or more groups of follicular cells or two or more tissue fragments according to Sanchez *et al* [17]. Only 57 cases (67,8%) had a contributory CB and that were adequate for UbcH10 assessment by immunostaining.

Immunocytochemistry on FNA-derived, formalin-fixed, and paraffin-embedded CBs was done as described elsewhere [5]. Briefly, antigen-retrieval microwave treatment (0·01 M citrate buffer, pH 6·0) was applied for three cycles of 5 min each at 750 W. Endogenous peroxidase activity was quenched with methanol-hydrogen peroxide (3%) for 15 min. After blocking with unrelated antiserum, slides were incubated with the primary antibodies: rabbit polyclonal anti-UbcH10 (BostonBiochem, Cambridge, MA, USA), diluted 1:1000; mouse monoclonal anti-Ki67, clone Mib-1 (Dako, Glostrup, Denmark) diluted 1:100. After incubation with the primary antibodies, CB sections were stained with specie-specific biotinylated secondary antibodies, followed by peroxidase-labelled streptavidin (Dako); the signal was developed by using diaminobenzidine chromogen (Dako) as substrate. Incubations with unrelated antibodies or without the specific antibodies were used as negative controls.

Single cells were scored for UbcH10 and Ki-67 expression with a computerized system (Ibas 2000, Kontron, Zeiss, Munich, Germany) as described elsewhere [18]. Scoring was done taking into account in the case of Ki67, only nuclear reactivity, whereas for UbcH10 cells showing nuclear and/or cytoplasmic reactivity were considered to be positive. The Mann-Withney U Test was used to determine UbcH10 and Ki-67 expression level differences between FNAs associated to benign and malignant histology. A p value of $<.05$ was considered statistically significant. To determine the cut-off value most predicable of malignancy, we calculated the area under the curve (AUC) of the receiver operating characteristic (ROC) for both UbcH10 and Ki-67 by means of the SPSS Inc. (Cary, NC) software package [16]. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and diagnostic accuracy were calculated with standard formulae for each stain separately and for the combination of both stains, using benign *vs* malignant histological diagnosis as the standard.

*RNA Extraction and cDNA Synthesis*

The residual material from one passage by the lesion was immediately transferred to a vial containing TRIZOL® solution (Invitrogen, Carlsbad, CA, USA) and stored at -80ºC until RNA extraction. Total RNAs were extracted from FNAs using TRIZOL® solution (Invitrogen), according to the manufacture's instructions. The pureness and concentration of RNA was assessed by NanoDrop® ND-1000 spectrophotometer (NanoDrop®

Technologies, DE, USA). 1 μg of total RNA of each sample was reverse-transcribed with the QuantiTect® Reverse Transcription (QIAGEN, Valencia, CA, USA) using an optimized blend of oligo-dT and random primers according to the manufacturer's instructions.

*Real Time Quantitative RT-PCR and Gene Expression*

We adopted the same methodology previously validated on thyroid FNAs by Shibru *et al*, by using human β-glucoronidase (HGUS) as a constitutive reference gene [7]. Only 62 cases (73,8%) showing successful HGUS amplification were selected for further assessment. To design a qRT-PCR assay we used Primer Express Software v2.0 (Applied Biosystem, Foster city, CA, USA). The primer for CCND2 forward were: 5'GGACATCCAACCCTACATGC3, reverse 5'CGCACTTCTGTTCCTCACAG3; for PCSK2 were: forward 5'GAGAAGACGCAGCCTACACC3', reverse 5'CTGCAAAGCCATCTTTACCC3'; for UbcH10 were: forward 5'TGCCCTGTATGATGTCAGGA3', reverse 5'GGGACTATCAATGTTGGGTTCT3'; for HGUS were: forward 5'CTCATTTGGAATTTTGCCGATT3', reverse 5'CCCAGTGAAGATCCCCTTTTTA3'. Relative Quantitative PCR was performed in 7900 HT SDS Applied Biosytem, in 384-well plates using a final volume of 10 μl. For PCR we used 5 μl of 2x Power SYBER Green PCR Master Mix (Applied Biosytem) 200 nM of each primer, cDNA generated from

100 ng of total RNA. The conditions used for PCR were 10 min at 95°C, and then 45 cycles of 20 sec at 95°C and 1 min at 60°C. Each reaction was performed in duplicate. To normalized real-time PCR data we used the method proposed by Shibru *et al* [7]. The Mann-Withney U Test was used to determine differences between mRNA expression levels. A p value of <.05 was considered statistically significant. To determine the diagnostic accuracy of this gene assay, we determined the AUC of the ROC curve for each gene individually and in combination by using logistic regression analysis.

**RESULTS**

*UbcH10 and Ki-67 imunohistochemical expression on CBs derived from follicular neoplasm and suspicious for malignancy FNAs.*

UbcH10 and Ki-67 expression were assessed on CB derived from FNAs diagnosed as either follicular neoplasm or suspicious for malignancy; representative examples are reported in Figure 1. The expression of both UbcH10 and Ki-67 was significantly higher in malignant than in benign thyroid lesions (Table 1) However, even in CBs corresponding to malignant histology, UbcH10 stained only a minority of the aspirated cells; the number of cells expressing UbcH10 never exceeded that of Ki67. As reported earlier, we determined which cut-off value was more predictive of malignancy. The best compromise between sensitivity and specificity was reached at a cut-off value of 1,25% for UbcH10 (AUC=0,964; p<0,001) and at a cut-off value of 3,05 for Ki-67 (AUC=0,967; p<0,001). Resulting specificity, sensitivity, PPV, NPV,

and diagnostic accuracy of UbcH10 and Ki-67 and of their combinations are reported in Table 2. Both markers were found to be highly sensitive (100%), and there were no false-negative cases (NPV, 100%). However, staining was less specific for UbcH10 (89%) than for Ki-67 (95%); the PPV was 83% for UbcH10 and 90% for Ki-67. The association of both markers (samples that were positive for both proteins) did not improve specificity (95%) and accuracy (96%).

*Quantitative analysis of UbcH10, CCND2 and PCSK2 mRNA levels derived from follicular neoplasm and suspicious for malignancy FNAs.*

In this study the usefulness of UbcH10 mRNA levels detection from follicular neoplasm and suspicious for malignancy FNA was evaluated. Data were compared to CCND2 and PCSK2 assessment. Histology represented the gold standard; FNAs were divided into two groups: one associated to benign histology and the other to malignancy. FNAs associated to malignant histology showed significantly different UbcH10 ($p = 0.02$) and CCND2 ($p = 0.002$) mRNA levels (Table 3); conversely, PCSK2 mRNA levels were less discriminative ($p = 0.23$). Each gene average level of expression in FNAs associated to benign and malignant histology is schematically shown in Figure 2.

ROC curve results are shown in Figure 3 A-B. The AUC for UbcH10 and CCND2 to distinguish benign from malignant thyroid neoplasm tissue samples were respectively 0.74 and 0.81 (Figure 3A). Their combination

increased the diagnostic accuracy, with an AUC of 0.84 (Figure 3B). The AUC for PCSK2 was 0.62 (Figure 3A).

**DISCUSSION**.

This study demonstrates that high UbcH10 expression increases the efficiency of cytology to detect cancer on follicular neoplasm/suspicious for malignancy FNAs. On histology, the malignancy rate of our series was 35,7%, which confirms that the performance of traditional cytology in this setting is not totally reliable [2]. This limit is intrinsic to the method and it is not due to our sample collection procedures. Designing this study special care was taken to validate UbcH10 evaluation in daily practice; thus, we first ensured an adequate cytological diagnosis, without altering the "informativeness" of the material for microscopy and, then, exploited the left-over material for UbcH10 analysis. Thus, only residual material from needle passes was processed as either CB or employed for RNA extraction. Contributory CBs were obtained in 67,8% of the cases; similarly, the 73,8% of the cases, showing successful HGUS amplification, yielded mRNA informative for cancer marker evaluation. Thus here we show that UbcH10 can effectively be translated into a clinically useful marker for making patient care decisions, without interfering with conventional cytology.

The expression of UbcH10 both at protein (by immunohistochemistry) and mRNA level (by Real Time RT-PCR) was analysed. To our knowledge, this is the first study evaluating UbcH10 on FNA samples. Thus, it was

important to set meaningful cut off values to score UbcH10 immunostaining. The value more predicable of malignancy was determined using the ROC analysis. Resulting specificity, sensitivity, PPV, NPV, and diagnostic accuracy of UbcH10 were remarkable. However, even on CBs whose corresponding histology was malignant only a few cells stained for UbcH10 (Figure 1D and F). This is conceivable since UbcH10 expression is limited to cells encompassing the G2 / M cell cycle phase, as we previously showed; [15] interestingly, the proportion of cells expressing UbcH10 never exceeded that of Ki67, which concurs with the notion that Ki67 covers the whole cell cycle from early G1 on.[19] Thus, the UbcH10 cut-off value derived by the ROC analysis was too low to allow a reliable microscopic scoring. Moreover, the association of UbcH10 to Ki-67 (samples that were positive for both proteins) did not improve specificity and accuracy. Consequently, UbcH10 immunostaining on FNAs is not feasible and does not improve Ki-67 performance.

Conversely, UbcH10 expression assessment by qRT-PCR is effective. FNAs associated to malignancy had UbcH10 expression levels higher than those associated with benign histology. The results were easy to be interpreted, thanks to a constitutive reference gene.[7, 20] CCND2 and PCSK2, the most performing components of the 3-gene assay, were used for comparison [6, 7] The UbcH10 diagnostic accuracy was similar to that CCND2 and higher than PCSK2. Moreover, the UbcH10-CCND2 combination further increased the qRT-PCR diagnostic accuracy.

Thyroid cancer markers are more effective when they are evaluated in panels rather than individually; our data suggest that a panel composition should include UbcH10. A large body of evidences suggests UbcH10 plays a diagnostic role. Using gene expression profiling, we found that UbcH10 was one of the genes most up-regulated in thyroid cancer cell lines[12]; then, we confirmed this observation on histological samples: UbcH10 is barely detectable in normal thyroid tissues, goiters and adenomas, whereas its increases in papillary and follicular carcinomas[12]. UbcH10 is a prognostic marker for thyroid[12], ovarian[13] and breast [14]and lymphoid [15] neoplasms. Similar findings have been reported by others for carcinomas arising from the lung[21], bladder[21], gastrointestinal tract [22, 23], liver,[23] gallbladder[24], prostate[25], oesophagus [26] and brain.[27]. In addition, UbcH10 is a very sensitive and specific marker of circulating breast tumour cells[28]. Thus, it is widely held that UbcH10 overexpression is associated to cancer. Conversely, other candidate thyroid cancer markers are supported by less consistent data. In one study, CCND2 was underexpressed in malignant samples[6] and upregulated in an other[7]. In this study we observed that CCND2 is downregulated in malignancy.

In summary, our prospective study of routinely collected follicular neoplasm/suspicious for malignancy FNA suggested that quantitative RT-PCR analysis, rather than immunohistochemistry, of UbcH10 expression as an elective method to increase the suspicion of malignancy in this controversial area of diagnostic cytology.

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## FIGURE LEGENDS

**Figure 1. UbcH10 and Ki-67 expression in FNA derived CBs corresponding to histologically diagnosed FA (A and B) and PTC (C-F).** Both Ki-67 and UbcH10 labelled more cells in PTC rather than in FA (Hematoxylin counterstained 10x.)**.** At higher magnification, note that UbcH10 and Ki-67 share a similar staining pattern as shown on parallel (E and F) CB sections (Hematoxylin counterstained 20x)**.**

**Figure 2**. **Quantitative analysis of UbcH10, CCND2 and PCSK2 mRNA levels derived from FNAs associated to benign and malignant histology**. Average of expression level of UbcH10, CCND2 and PCSK2 genes in benign and malignant lesions. Arbitrary Units are obtained by the following formula: $2^{-\Delta C\,t}$ where $\Delta Ct$ =Ct HGUS - Ct target [7].

**Figure 3**. **AUC for individual UbcH10, CCND2 and PCSK2 expression and their combination by using logistic regression analysis.** Panel A shows the ROC curve for any single gene. Panel B shows the ROC curves for singly and combined UbcH10 and CCND2 genes.

**Table 1**: UbcH10 and Ki-67 immunohistochemical expression values (median and range) on CBs derived from FNAs whose corresponding histological specimens were grouped as either benign or malignant.

| **% expression** | **Benign histology** n=38 | **Malignant histology** n=19 | **Mann-Whitney U test** P value |
|---|---|---|---|
| UbcH10 | 0,0 (0,0-2,0) | 2,3 (0,0-5,0) | <0,001 |
| Ki-67 | 1,0 (0,0-5,0) | 4,3 (0,0-6,3) | <0,001 |

**Table 2**: Diagnostic performance of UbcH10, Ki-67 and their combination in FNAs diagnosed as either follicular neoplasm or suspicious for malignancy. Data analysis based on the individual most efficient positive cut-off values for stained thyroid cells derived from ROC analysis.

| | **Cut-off** | **Sensitivity** | **Specificity** | **PPV** | **NPV** | **Accuracy** |
|---|---|---|---|---|---|---|
| UbcH10 | ≥1,25% | 100% | 89% | 83% | 100% | 93% |
| Ki-67 | ≥3,05% | 100% | 95% | 90% | 100% | 96% |
| UbcH10 & Ki-67 | ≥1,25 & ≥3,05 | 100% | 95% | 90% | 100% | 96% |
| UbcH10 or Ki-67 | ≥1,25 or ≥3,05 | 100% | 89% | 83% | 100% | 93% |

**Table 3**: For each gene individually and in combination we reported the p-value calculated using Mann-Withney U Test and the area under the curve (AUC).

| | **P value** | **AUC** |
|---|---|---|
| UbcH10 | 0.02 | 0.74 |
| CCND2 | 0.002 | 0.81 |
| PCSK2 | 0.22 | 0.62 |
| UbcH10/CCND2 | - | 0.84 |

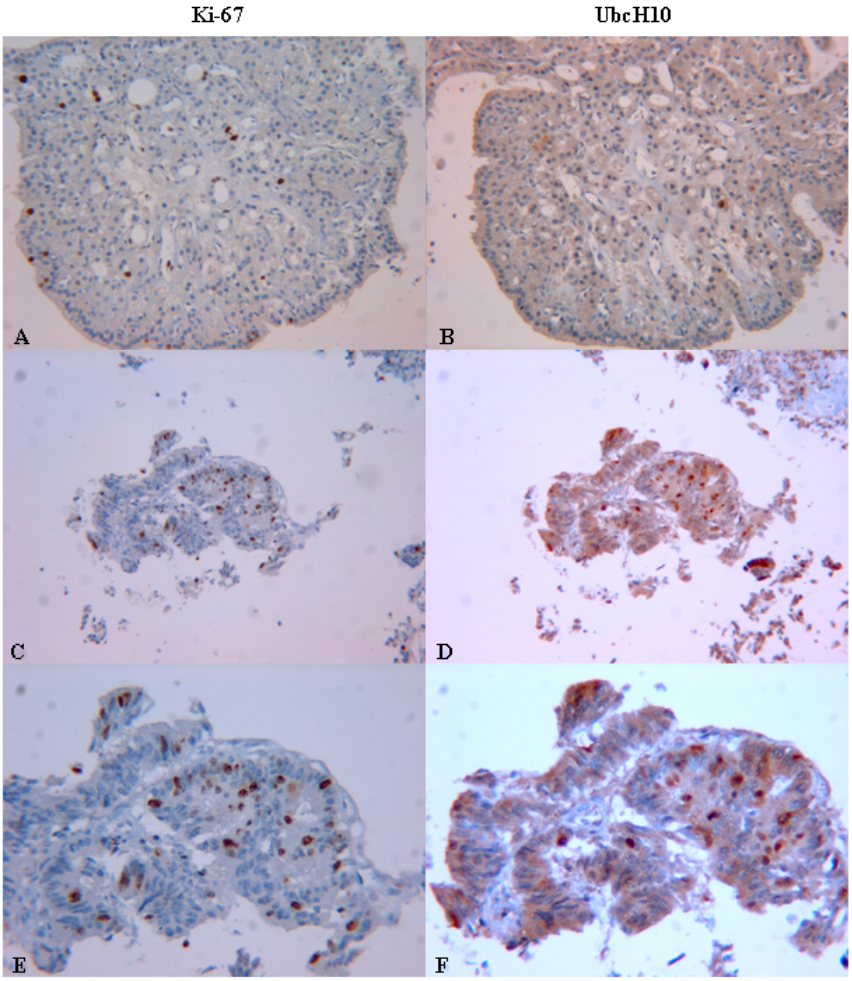


**Figure 1**

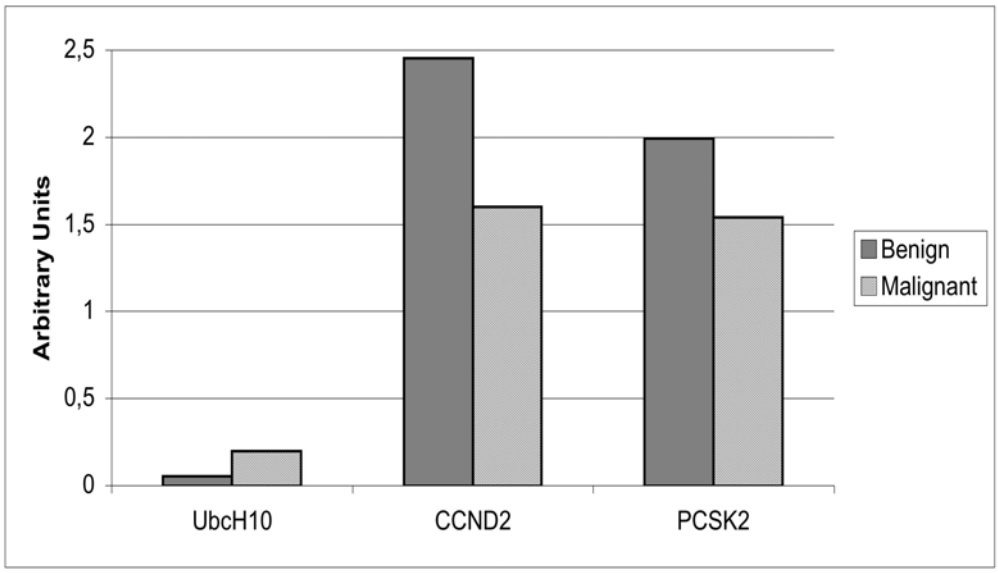
2,5
2
1,5
1
0,5
0
Arbitrary Units
UbcH10
CCND2
PCSK2
Benign
Malignant

**Figure 2**

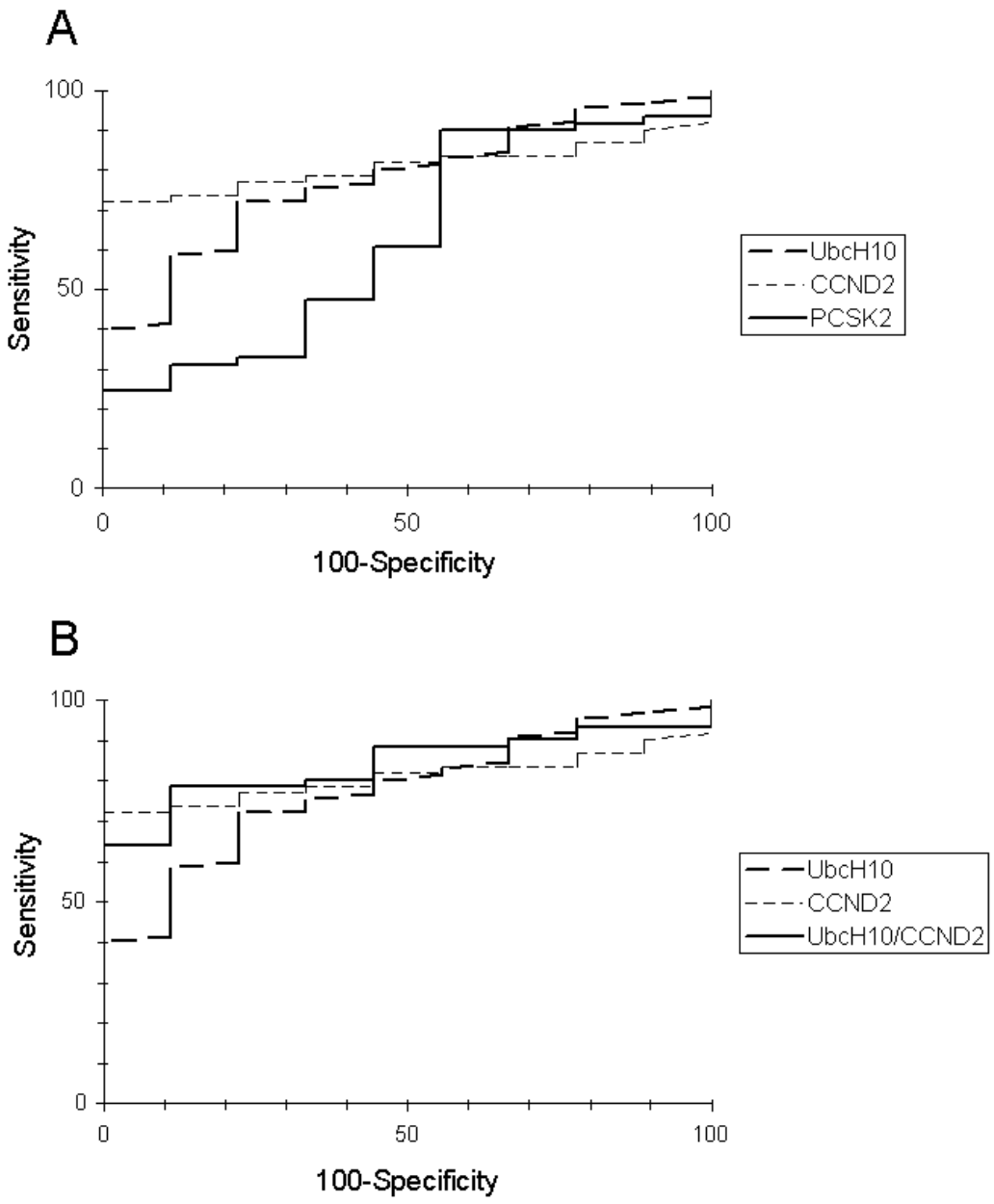
A
100
50
0
Sensitivity
0
50
100
100-Specificity
UbcH10
CCND2
PCSK2
B
100
50
0
Sensitivity
0
50
100
100-Specificity
UbcH10
CCND2
UbcH10/CCND2

**Figure 3**