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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 12×10^5 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.

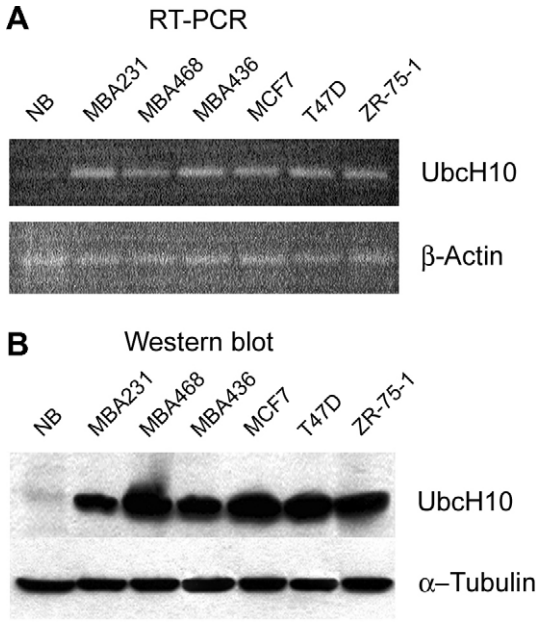

Fig. 1 – UbcH10 expression in human breast carcinoma cell lines. (A) UbcH10 gene expression analysis by RT-PCR in human breast carcinoma cell lines versus the normal breast tissue (NB). β-Actin gene expression was evaluated as control to normalise the amount of the used RNAs. (B) UbcH10 protein expression 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.

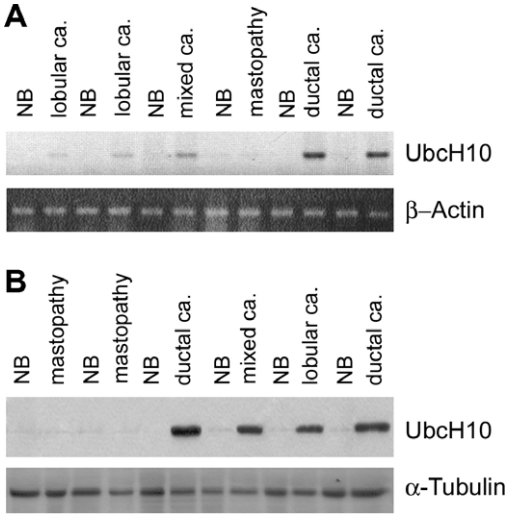

Fig. 2 – UbcH10 expression in human breast fresh tumour samples. (A) RT-PCR analysis of UbcH10 expression in human breast tumour samples versus their normal counterparts. β-Actin expression shows the same amount of RNAs used. NB, normal breast tissue; (B) Western blot analysis of UbcH10 protein expression 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

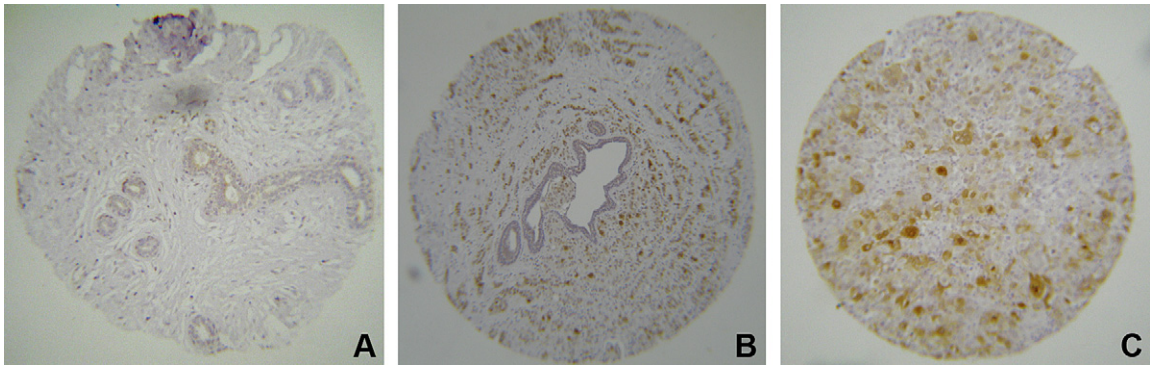

Fig. 3 – Immunostaining pattern of UbcH10 expression in breast cancer. UbcH10 expression in benign (A) (original magnification, 25×) and malignant (B, C) (original magnification, 25×) breast tissues. While benign tissue is lacking UbcH10 expression (A), ductal cancer (C) displays high levels of protein 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.

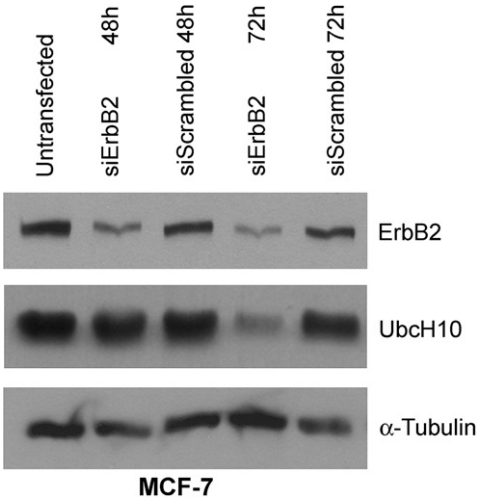

Fig. 4 – UbcH10 expression is dependent on ErbB2 expression. Inhibition of ErbB2 protein expression by siRNA in MCF7 cell line evaluated by Western blot analysis shows, at 72 h after siRNA transfection, a decrease of the UbcH10 expression. Cells transfected with a scrambled duplex (siScrambled) and untransfected 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

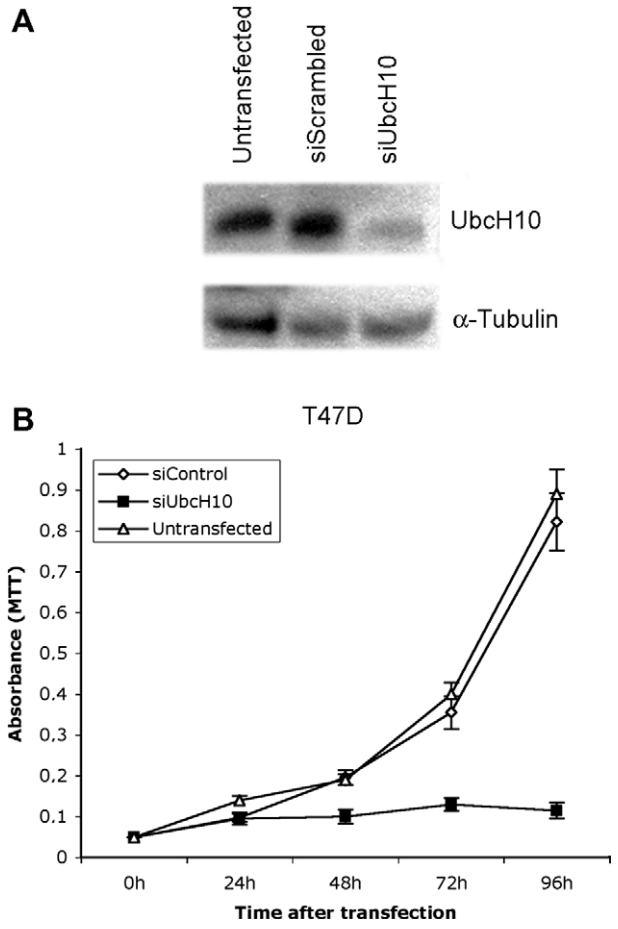

Fig. 5 – The block of UbcH10 protein synthesis by RNA interference inhibits the proliferation of breast carcinoma cells. (A) Inhibition of UbcH10 protein expression by siRNA in T47D cell line evaluated by Western blot analysis. At 48 h after siRNA transfection, total cell lysates were prepared and normalised 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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REFERENCES

1. Vogelstein B, Kinzler KW. Has the breast cancer gene been found? *Cell* 1994;**79**(1):1–3.
2. Michiels S, Koscielny S, Hill C. Interpretation of microarray data in cancer. *Br J Cancer* 2007;**96**(8):1155–8.
3. Pallante P, Berlingieri MT, Troncone G, et al. UbcH10 overexpression may represent a marker of anaplastic thyroid carcinomas. *Br J Cancer* 2005;**93**(4):464–71.
4. Hershko A, Ciechanover A. The ubiquitin system. *Annu Rev Biochem* 1998;**67**:425–79.
5. Joazeiro CA, Weissman AM. RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 2000;**102**(5):549–52.
6. Berlingieri MT, Pallante P, Guida M, et al. UbcH10 expression may be a useful tool in the prognosis of ovarian carcinomas. *Oncogene* 2007;**26**(14):2136–40.
7. Porter DA, Krop IE, Nasser S, et al. SAGE (serial analysis of gene expression) view of breast tumor progression. *Cancer Res* 2001;**61**(15):5697–702.
8. Tavassoli FA, Devilee P. *World health organization classification of tumours. Pathology and genetics of tumours of the breast and female genital organs*. Lyon: IARC Press; 2003.
9. Demichelis F, Sboner A, Barbareschi M, Dell'Anna R. TMABoost: an integrated system for comprehensive management of tissue microarray data. *IEEE Trans Inf Technol Biomed* 2006;**10**(1):19–27.
10. Liu X, Minin V, Huang Y, Seligson DB, Horvath S. Statistical methods for analyzing tissue microarray data. *J Biopharm Stat* 2004;**14**(3):671–85.
11. Development Core Team R. A language and environment for statistical computing. R foundation for statistical computing. Vienna, Austria, ISBN 3-900051-07-0, 2005.
12. Wagner KW, Sapinoso LM, El-Rifai W, et al. Overexpression, genomic amplification and therapeutic potential of inhibiting the UbcH10 ubiquitin conjugase in human carcinomas of diverse anatomic origin. *Oncogene* 2004;**23**(39):6621–9.
13. Welcsh PL, King MC. BRCA1 and BRCA2 and the genetics of breast and ovarian cancer. *Hum Mol Genet* 2001;**10**(7): 705–13.

14. Brierley JD, OÕSullivan B, Gospodarowicz M. Cancer staging, 6th edition of the UICC/AJCC TNM classification. *Oncology Rounds* 2003.
15. De Leeuw WJ, Berx G, Vos CB, et al. Simultaneous loss of E-cadherin and catenins in invasive lobular breast cancer and lobular carcinoma in situ. *J Pathol* 1997;**183**(4):404–11.
16. Korkola JE, DeVries S, Fridlyand J, et al. Differentiation of lobular versus ductal breast carcinomas by expression microarray analysis. *Cancer Res* 2003;**63**(21):7167–75.
17. Shackney SE, Silverman JF. Molecular evolutionary patterns in breast cancer. *Adv Anat Pathol* 2003;**10**(5): 278–90.
18. Cleator S, Ashworth A. Molecular profiling of breast cancer: clinical implications. *Br J Cancer* 2004;**90**(6):1120–4.
19. Burger AM, Gao Y, Amemiya Y. A novel RING-type ubiquitin ligase breast cancer-associated gene 2 correlates with outcome in invasive breast cancer. *Cancer Res* 2005;**65**(22):10401–12.