Androgen deprivation therapy regulation of ß1C integrin expression in prostate cancer

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Abstract. The ß1C integrin is an alternatively spliced variant of the ß1 integrin subfamily that at variance with its wild-type counterpart, i.e., the ß1A integrin, inhibits cell proliferation in prostate cancer cells. We have recently shown that transcriptional, translational and post-translational processes contribute to the selective loss of ß1C integrin during prostate malignant transformation. Here, we investigated whether androgen deprivation therapy (ADT) may affect ß1C mRNA expression in prostate cancer. Neoplastic prostates were obtained from patients undergoing radical prostatectomy who had received neoadjuvant ADT. The ß1C mRNA level was measured by Northern hybridization experiments and compared to normal prostates obtained from patients who underwent radical cystoprostatectomy for bladder cancer. Furthermore, the ß1C integrin gene transcriptional activity was measured by nuclear Run-on assays. We found an increase of ß1C mRNA expression (208±11%; p<0,01) in patients who received ADT in comparison to those who did not. Furthermore, we demonstrated an increase of gene transcriptional activity (360±10%; p<0,01) possibly partially or completely responsible for the regulation of the ß1C integrin mRNA levels. Short-term administration of ADT seems to interfere with ß1C integrin expression, suggesting the existence of androgen-mediated pathways involving ß1C. Precise characterization of the mechanisms that regulate the expression of this factor in cancer cells will provide further insight into the molecular mechanisms involved in tumor progression and possibly contribute to the identification of molecular targets for the development of new therapeutic strategies.

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Introduction

Prostate cancer is the most prevalent malignancy in the Western world and the second leading cause of male cancer death (1). Whereas recent studies have demonstrated that the expression of genes modulating cell adhesion, growth, cell cycle and apoptosis is altered or lost during progression from normal cell to invasive cancer, the etiology of prostate cancer remains largely obscure.

Adhesive contacts between cells and extracellular matrix (ECM) components play a crucial role in organ development, abnormal tissue growth, tumor progression and metastatic spread. Cell adhesion to the ECM is predominatly mediated by integrins, the most structurally and functionally diverse family of cell surface adhesion receptors that play a critical role in the acquisition and maintenance of neoplastic phenotype by preventing apoptosis and maintaining cell proliferation (2,3). Integrin expression profile dramatically changes upon the normal-to-neoplastic transition (4). These changes may reflect selective pressure imposed upon cancer cells to free themselves from adhesion to basement membranes and to up-regulate integrins that foster survival and proliferation (4).

Integrins are structurally organized into heterodimeric transmembrane complexes, variously assembled through a non-covalent association between α and ß subunit (5). Up to now 8 ß and 18 α subunits have been characterized, that associate to generate at least 24 different integrins characterized to date (3). Alternatively spliced forms of the α and ß integrin cytoplasmic domains have been identified adding further complexity to the regulatory pathways mediated by integrins (5). Five different ß1 integrins containing alternatively spliced cytoplasmic domains have been identified (ß1A, ß1B, ß1C, ß1C-2 and ß1D), all sharing a common N-terminal part up to the WDT 777 sequence, corresponding to the 3'-end of exon 6 in the ß1 gene (5-8).

The ß1C integrin is generated by the insertion of an unspliced sequence that causes a frame-shift in the 3'-end of the wild-type ß1 integrin subunit, coding for a unique 48-amino acid carboxyl-terminal sequence in the cytoplasmic domain (5).

In contrast with its wild-type counterpart ß1A (mostly referred to as ß1 only), ß1C expression inhibits cell prolife-

Table I. Clinicopathological features of 25 patients with prostate cancer.

Patients	Age (years)	Gleason grade	Hormonal therapy (months)	Hormonal therapy
N1	63	Control		
N2	80	Control		
N3	83	Control		
K1	73	9	-	-
K2	72	7	-	-
K3	70	8	-	-
K4	61	7	-	-
K5	61	9	-	-
K6	63	8	-	-
K7	70	7	-	-
K8	67	5	-	-
K9	72	8	-	-
K10	71	8	-	-
K11	64	6	-	-
K12	64	6	-	-
TK1	70	9	1	Goserelin + Bicalutamide
TK2	71	8	1	Goserelin + Bicalutamide
TK3	73	8	1	Goserelin + Bicalutamide
TK4	68	7	1	Goserelin + Bicalutamide
TK5	69	6	1	Goserelin + Bicalutamide
TK6	60	6	1	Goserelin + Bicalutamide
TK7	77	7	1	Goserelin + Bicalutamide
TK8	73	10	1	Goserelin + Bicalutamide
TK9	70	6	1	Goserelin + Bicalutamide
TK10	70	7	1	Goserelin + Bicalutamide

N, normal prostate; K, prostate cancer; TK, prostate cancer after 1-month of neoadjuvant ADT.

ration and causes growth arrest at the late G1 phase of the cell cycle (7,9) in prostate cancer epithelial cells (10), endothelial cells (11), fibroblasts (9) and CHO cells (7).

In vivo, ß1C is mostly expressed in non-proliferative and differentiated epithelium (12) whereas ß1A is ubiquitously expressed. At variance with ß1A, the ß1C protein is down-regulated in prostate adenocarcinoma (13), in endometrial proliferating disease (14), in some non-small cell lung carcinomas (15) and shows an inverse correlation with markers of cell proliferation in breast carcinoma (12).

We have demonstrated that both ß1C and ß1A mRNA expression is down-regulated in neoplastic prostate specimens, whereas only ß1C protein levels are reduced or even lost (13). Conversely, the protein expression of the ß1A integrin does not change, suggesting that transcriptional and/or post-transcriptional mechanisms specifically regulate the expression of ß1 integrin variants in prostate cancer (13). Moreover, to investigate the molecular mechanisms involved in the regulation of ß1C integrin expression, we measured the transcriptional activity of the ß1 integrin gene in normal and neoplastic prostate tissue, thus providing evidence that the transcriptional activity of the ß1 integrin gene was reduced, accounting for the down-regulation of ß1C mRNA expression in prostate adenocarcinoma (16).

We have also shown that the ß1C translation rate is decreased in cancer cells, in agreement with the decrease in mRNA levels, whereas the ß1A translation rate increases more than 2-fold, despite the reduction in mRNA levels (17). We found that both the ß1C and ß1A proteins are more rapidly degraded in cancer than in normal cells and that intermediates and/or rates of ß1C and ß1A protein maturation differ in cancer versus normal cells. Finally, transcriptional, translational and post-translational processes, contribute to the selective loss of ß1C integrin, a very efficient inhibitor of cell proliferation, in prostate malignant transformation.

In this study we measured mRNA expression of ß1C integrin, by Northern blot analysis and transcriptional activity by nuclear run-on assay, in prostate cancer with and without androgen deprivation therapy (ADT), using non-malignant prostates as controls.

Materials and methods

Human prostate tissue collection and procurement. This study was performed using 25 prostate specimens (Table I) obtained from patients hospitalized at the Department of Emergency and Organ Transplantation-Urology and Kidney Transplantation Unit of the University of Bari (Italy), in the

years 1997-2002. Informed consent was obtained from all patients. We identified three groups of patients. The first group included three patients (N1-N3; median age at surgery, 75±9 years) with a normal prostate who underwent radical cystoprostatectomy for bladder carcinoma not involving the prostate.

The second group included 12 patients (K1-K12; median age at surgery, 67±4 years) with prostate cancer, who underwent radical prostatectomy.

The third group included 10 patients (TK1-TK10; median age at surgery, 70±4 years) affected by prostate cancer who had undergone 1-month of neoadjuvant ADT, consisting of the association of a Luteinizing Hormone Releasing Hormone analog (goserelin depot, 3.6 mg/q 28 days) and a non-steroidal anti-androgen (bicalutamide, 50 mg/day). The median pre-operative PSA level was 13.6 ng/ml (range, 6.1-18.6 ng/ml) and 9.8 ng/ml (range, 2.2-16.3 ng/ml) for patients in groups K and TK, respectively.

Protocol-based tissue sampling techniques are used to procure tissue as soon as possible after removal from the patient (<20 min from time of removal at surgery to tissue freezing) while adequately preserving the specimen for pathological characterization (assessment of pathological stage, margin status and grade).

Soon after removal, the prostate was placed on a cutting table and a single incision was made through the capsule. Then the capsule is stripped from the gland. This procedure permits procurement of tissue from the gland for banking while allowing for diagnostic evaluation of the entire prostate capsule tissue for surgical margin status and stage. A sample was taken and cut in two parts. This procedure generated two mirror image halves. One half was snap-frozen, and cryopreserved in liquid nitrogen for RNA extraction and immunoblot analysis. The remaining tissue samples were fixed in 10% neutral-buffered formalin for 12-24 h, embedded in paraffin, and stained with hematoxylin and eosin (H&E) for histological evaluation. Identification of tumor areas in the frozen tissue was achieved by matching this tissue with H&E stained sections.

H&E stained sections were reviewed, and the tumor grade, according to the Gleason criteria (18), was estimated for each tumor sample: Gleason score <7 n=6 and ≥7 n=16.

A sample of human normal liver, obtained during cholecystectomy, was used to generate mRNA used as a negative control in the Northern hybridization experiments.

RNA extraction and Northern blot analysis. Frozen tissue samples were pulverized and cellular RNA was extracted using the guanidinium isothiocyanate-cesium chloride procedure as previously described (13). Total RNA (25 μg) isolated from the tissues was electrophoresed through 1% denaturing agarose gel containing 660 mmol/l formaldehyde, and transferred to a nylon membrane (Hybond N^+; Amersham, Milan, Italy), and Northern blot analysis of ß1C and ß1 integrin mRNA was performed as previously described (13). The filters were subsequently prehybridized overnight at 42˚C with a buffer consisting of 50% formamide, 5X Denhardt's solution (1% Ficoll 400, 1% polyvinylpyrrolidone, 1% bovine serum albumin), 5X sodium chloride/sodium phosphate/ethylenediaminetetraacetic acid (SSPE) (3 mol/l NaCl, 200 mmol/l $Na_2H_2PO_4$, pH 7.0, 19 mmol/l ethylenediaminetetraacetic acid), 0.5% sodium dodecyl sulphate (SDS) and 100 μg/ml of sonicated salmon sperm DNA. The filters were then hybridized for 20 h at 42˚C by adding $3x10^6$ cpm of [^{32}P]-labeled probe/ml to the prehybridization solution. The filters were washed once with 2X SSPE, 0.1% SDS for 10 min at room temperature, then with 1X SSPE, 0.1% SDS at 42˚C, followed by several washes in 0.1X SSPE, 0.1% SDS, at 65˚C and finally exposed at -80˚C overnight or longer to Kodak X-Omat AR 5 film (Kodak, Rochester, NY, USA). Radiolabeled probes were generated using the Megaprime DNA labeling kit (Amersham), 100 μCi of [α-^{32}P]-dCTP (3000 Ci/mmol, Amersham) and 25 ng of double-stranded either 116-bp fragment specific for the ß1C integrin or a full-length human ß1 cDNA (13). The specific 116-bp ß1C fragment (nucleotides 2435-2550) (13) was generated by polymerase chain reaction using the pBS-ß1C plasmid as template and the resulting fragment was subcloned in the pBluescript vector. mRNA levels were normalized using ribosomal 28S RNA, a constitutively expressed gene (13). For this purpose, blots were stripped in 0.1% boiling SDS and reprobed with the radiolabeled [^{32}P]-28S cDNA probe. Quantitative analysis was performed by densitometric scanning of the autoradiographs using a Bio-Rad GS-700 densitometer (Bio-Rad, Richmond, CA, USA); multiple exposures of the same Northern blots in a linear range were performed.

Nuclear run-on transcription assays. Isolation of nuclei and transcriptional assays were performed as previously described (16). In the nuclear Run-on transcription assay aliquots of 100 μl of frozen nuclei were added to 100 U placental RNase inhibitor (Promega), 50 nmoles each of rATP, rCTP and rGTP (Promega), 100 μCi of [α-^{32}P]UTP (3000 Ci/mmole; NEN Life Science Products, Boston, MA, USA) and incubated for 30 min at 30˚C with periodic mixing. Transcription was terminated by lysing the nuclei with 0.3 M NaCl. DNA was digested by adding 12 μl of a 1 mg/ml solution of RQ1 DNase (Promega) followed by a 15 min incubation at 30˚C. The reactions were extracted twice with phenol/chloroform/isoamyl alcohol (25:24:1) and once with chloroform/isoamyl alcohol (24:1). The aqueous solution was adjusted to 0.3 M sodium acetate and the RNA precipitated twice in 3 volume of absolute ethyl alcohol for 30 min at -80˚C. Nylon N^+ membranes (Amersham), containing 10 μg of cDNA fragments previously bound by slot blot, were prehybridized overnight at 42˚C with a buffer consisting of 50% formamide, 5X Denhardt's solution (0.1% Ficoll 400, 0.1% polyvinylpyrrolidone, 0.1% bovine serum albumin), 5X sodium chloride/sodium phosphate/ethylenediaminetetraacetic acid (SSPE) (0.75 M NaCl, 40 mM $Na_2H_2PO_4$, pH 7.0, 5 mM ethylenediaminetetraacetic acid), 0.5% sodium dodecyl sulfate (SDS) and 100 μg/ml of sonicated salmon sperm DNA. The filters were then hybridized for 48 h at 42˚C with $3.0x10^6$ cpm/ml [^{32}P]-labeled RNA obtained in the run-on transcription assay.

The following cDNA fragments were used in the nuclear Run-on analysis: the 116-bp specific ß1C fragment (nucleotides 2435-2550) isolated by Eco-RI digestion from the pBluescript-ß1C plasmid (13), the 2.6 kb full-length ß1 fragment, isolated by Eco-RI digestion from the pBluescript-

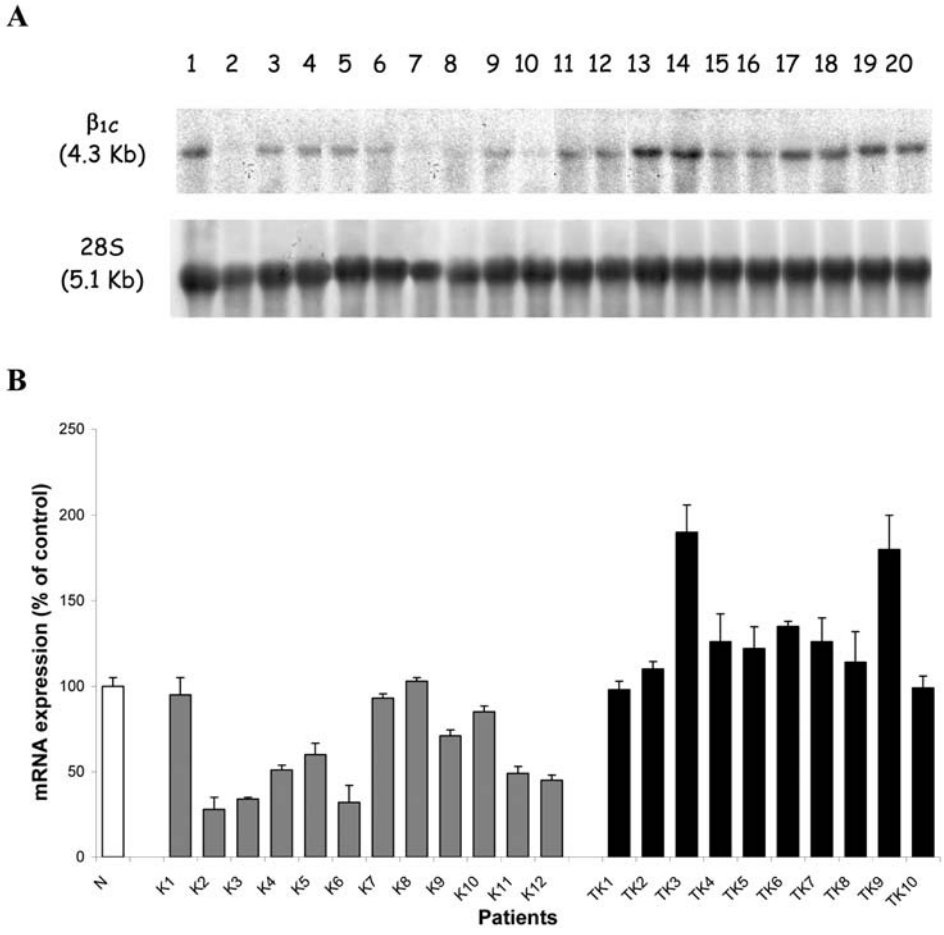

Figure 1. ß1C mRNA expression. (A) Total RNA was isolated from 3 normal, 12 neoplastic prostate specimens and 10 neoplastic tissue obtained from patients treated with 1-month of hormonal therapy. Total RNA (25 μg) were used for each sample. Lane 1, RNA from HL60 cells was used as positive control. Lane 2, RNA from human liver was used as negative control. Lanes 3-5, RNA isolated from 3 patients with normal prostate tissues. Lanes 6-12, RNA isolated from 7 patients with neoplastic prostate tissues. Lanes 13-20, RNA isolated from 7 patients with neoplastic prostate tissues who had undergone 1-month of hormonal therapy. To normalize the amount of total RNA loaded for each sample, the blot was stripped and rehybridized using a 28S rRNA probe. (B) The average of ß1C mRNA expression levels in 3 normal prostate tissue specimens (N) was set at 100. ß1C mRNA levels in neoplastic tissues not hormonally treated (K1-K12) and in neoplastic tissue with 1-month of hormonal therapy (TK1-TK10) were calculated as percentage of N. Mean values ± SEM from at least three different experiments are shown.

full-length ß1C plasmid (13), and the 1.3 kb 28S fragment isolated by *Bam*H1 digestion from the p28S plasmid (13).

After hybridization, the membranes were washed once in 2X SSPE, 0.1% SDS for 10 min at 42˚C, twice in 1X SSPE, 0.1% SDS for 10 min at 42˚C, twice in 0.5X SSPE, 0.1% SDS for 10 min at 42˚C, once in 0.1X SSPE, 0.1% SDS for 10 min at 50˚C, and then exposed to Kodak X-OMAT AR 5 film.

Autoradiographs of the RNA-DNA hybrids obtained after 7 days exposure at -80˚C were analysed using a GS-700 Imaging densitometer (Bio-Rad). All values were standardized according to the 28S rRNA signal used as internal standard. The average of ß1C transcriptional activity in normal prostate biopsies derived from three patients was set at 100 (arbitrary units). ß1C transcriptional activity in neoplastic tissues was calculated as percentage of the transcriptional activity of the normal prostate.

HL60 cells. Human leukemia HL60 cells were grown in RPMI-1640 (Gibco, Life Technologies, Milan, Italy), with 50 mg/ml gentamycin, 2 mmol/l glutamine, and 15% inactivated fetal calf serum, at 37˚C in presence of 5% CO_2. Total RNA from differentiated cells was prepared 24 h after incubation with 160 nmol/l TPA (or PMA phorbol-12-myristate-13-acetate; Sigma) as previously described (13) and used as positive control in the Northern hybridization experiments.

Statistical analysis. Data are reported as the mean ± SEM. Statistical analysis was performed using the ANOVA followed by Duncan post-hoc test. All experiments were repeated at least three times.

Results

Effect of the hormonal therapy on the expression of ß1C integrin. Steady-state level of ß1C mRNA in prostate tissue was evaluated by Northern blot analysis. A 4.3-kb band was detected in all samples corresponding to the ß1C mRNA length since the probe used is specific for exon C, only found in the ß1C variant (Fig. 1A).

To normalize the differences due to mRNA loading and transfer, the same blots were dehybridized and re-hybridized

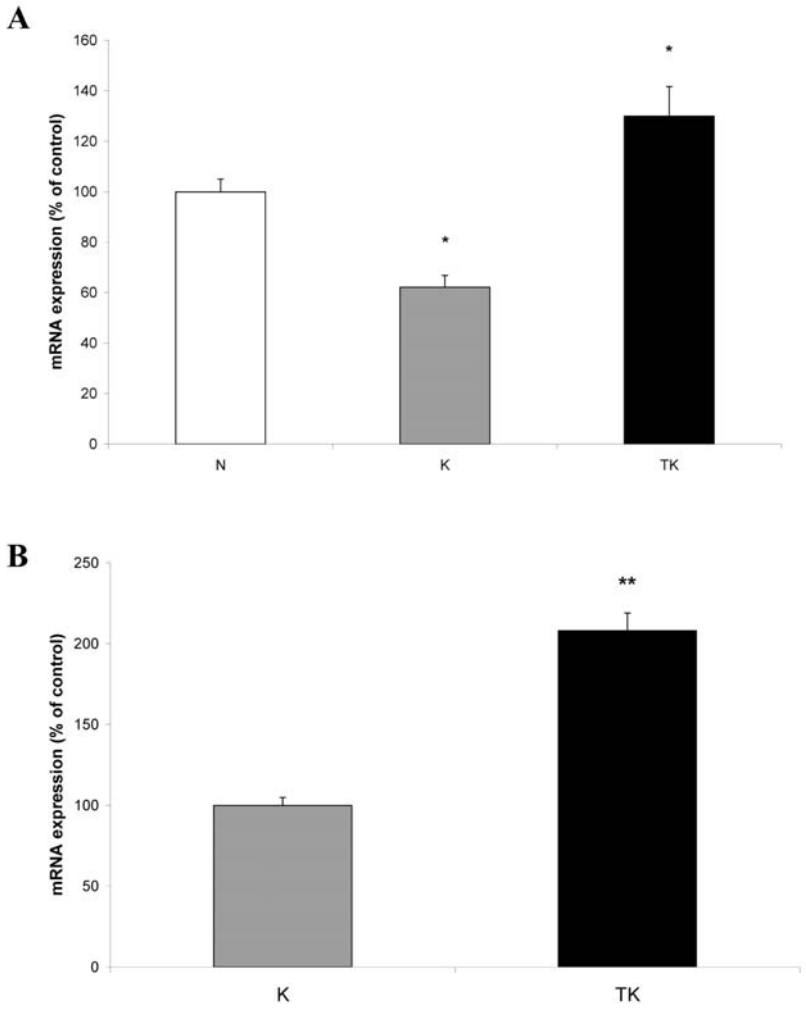

Figure 2. (A) Median average of percentile ß1C expression in neoplastic prostate tissues. The ß1C mRNA level in 3 samples of normal prostate tissues (N) was set at 100, and the mRNA levels in the other tissues were calculated as a percentage of N. K, neoplastic tissues isolated from 12 patients without hormonal therapy; TK, neoplastic tissue isolated from 10 patients treated with 1-month of hormonal therapy. Error bars indicate SEM. *P<0.05. (B) Median average of percentile ß1C expression in neoplastic prostate tissues. The average ß1C mRNA levels in 12 samples of neoplastic tissue isolated from patients without hormonal therapy (K) was set at 100, and the mRNA levels in neoplastic tissue isolated from 10 patients treated with 1-month of hormonal therapy (TK) was calculated as a percentage of K. Error bars indicate SEM. **P<0.01.

with a human 28S cDNA probe. The ratio between the 4.3-kb long ß1C mRNA band and the 28S rRNA band was calculated for each sample to take into account differences in RNA loading. The average of ß1C mRNA expression levels in normal prostate control derived from three patients was set at 100 (arbitrary units). ß1C mRNA levels in neoplastic prostate were calculated as percentage of normal prostate mRNA levels hybridized on the same filter. For each specimen, the mean value (±SEM) of results obtained in at least three experiments was calculated.

Semi-quantitative analysis of Northern hybridization (Fig. 1B) showed variations in the expression of ß1C mRNA in the neoplastic tissue isolated from patients who received neoadjuvant hormonal therapy (TK1-TK10) or not (K1-K12), compared to the mRNA from normal tissue specimens (N) used as normal control.

Increased ß1C steady-state mRNA levels were detected in 90% of group TK specimens when compared with normal prostate samples (N). ß1C mRNA expression levels in TK group ranged between 190% (±16%) and 98% (±5%) of the normal control (N) (Fig. 1B). On the contrary, decreased ß1C steady-state mRNA levels were detected in 80% of group K specimens ranging from 28% (±7%) to 103% (±2%) of the levels found in normal prostate tissues used as control (Fig. 1B).

Northern blot analysis showed a statistically significant ($p<0.05$) increase (130±12%) of ß1C mRNA levels in TK group in comparison with normal tissues (N) (Fig. 2A). Moreover, our results showed a statistically significant ($p<0.05$) decrease (62±5%) of ß1C mRNA level in K group compared with normal prostate tissues (N) (Fig. 2A), and these results are in accordance with our previously reported data (13).

If we compare the average of the ß1C percentile expression in TK group with K group, Northern blot analysis showed a statistically significant ($p<0.01$) increase (208±11%) of ß1C mRNA levels (Fig. 2B). On the contrary, there were not significant variations in the level of ß1C integrin expression measured in the patients after 3- and 6-months of neoadjuvant hormonal therapy in comparison to the K group (data not shown).

Table II. ß1C and ß1 mRNA expression in neoplastic prostate specimens and in neoplastic prostate specimens with hormonal therapy.

	ß1C (% of expression ± SEM)	ß1 (% of expression ± SEM)
N	100±5	100±3
K	62±5	67±7
TK	130±12	144±10

In addition, using the full-length ß1 cDNA probe, that hybridizes with all ß1 variants (19), we analyzed ß1 mRNA levels in all the above-described prostate tissues (Table II). The results showed reduced ß1 mRNA levels (67±7%) in all the prostate tissues isolated from K patients compared with the normal prostate tissues (N). Among the TK group, the results showed a statistically significant ($p<0.01$) increase in ß1 mRNA levels (144±10%), thus indicating that an altered splicing mechanisms is not responsible for the modification of the ß1C mRNA steady-state levels occurring in prostate cancer (13).

Transcriptional regulation of the ß1C integrin gene expression. To investigate the molecular mechanism underlying the increase of ß1C mRNA levels, occurring in prostate cancer during hormonal therapy, we compared the transcriptional activity of the ß1C integrin gene in neoplastic tissues isolated from patients treated for 1-month with ADT (TK), versus neoplastic tissues isolated from patients who did not receive hormonal therapy (K) by nuclear Run-on experiments.

The autoradiographic signals for one representative neoplastic prostate specimen (K5) and one representative neoplastic prostate specimen after 1-month of ADT (TK6) obtained in a typical run-on experiment, are shown in Fig. 3A.

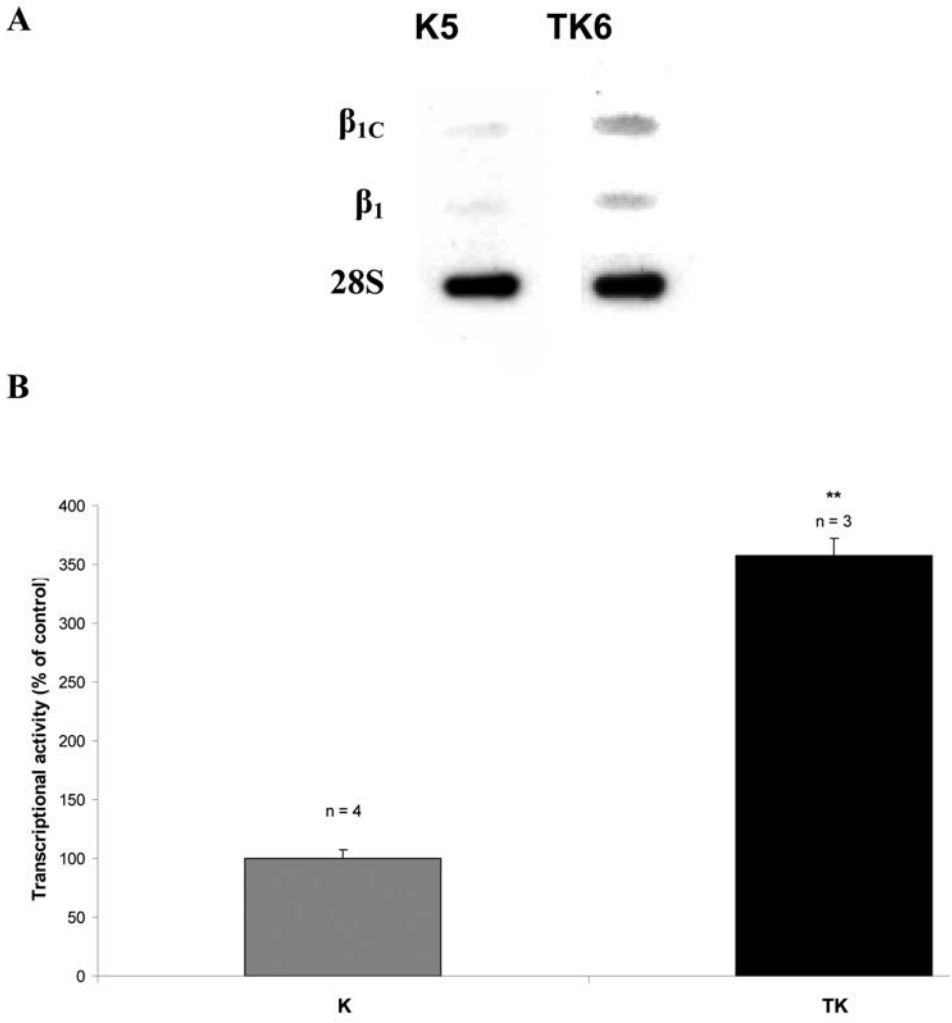

Figure 3. Transcriptional regulation of the ß1C integrin gene in prostate cancer. Nuclear run-on transcriptional analysis of nuclei isolated from 4 neoplastic human prostate specimens (K3, K5, K7, K10) and 3 neoplastic human prostate specimens after 1-month of hormonal therapy (TK3, TK6, TK9) was performed. Equal amounts of [^{32}P]-labeled nuclear transcripts were hybridized to filters on which denatured cDNA (5 μg) for ß1C integrin, full length ß1 integrin and 28S rRNA had been immobilized. (A) The autoradiography for 1 representative neoplastic prostate (K5) and 1 representative neoplastic prostate after 1-month of hormonal therapy (TK6) is shown. (B) Values for ß1C mRNA were normalized to the internal standard 28S rRNA, for each specimen, and transcriptional activity in 3 neoplastic tissues after 1-month of hormonal therapy (TK) was calculated as percentage of the average of the transcriptional activity in 4 neoplastic human prostate specimens (K), set at 100. Mean values ± SEM from two different experiments are shown. Error bars indicate SEM. $^{**}P<0.01$.

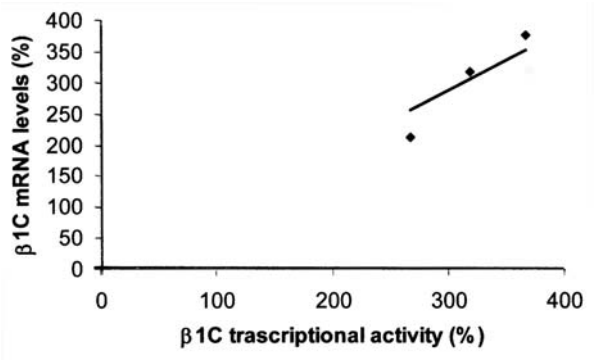

Figure 4. Regression analysis between ß1C transcriptional activity and ß1C mRNA expression in prostate cancer after 1-month of hormonal therapy. ß1C transcriptional activity was evaluated as described in Fig. 3, the average of ß1C transcriptional activity in 4 neoplastic prostate tissue specimens (K) was set at 100, ß1C transcriptional activity in 3 neoplastic prostate tissues after 1-month of hormonal therapy was calculated as the percentage of K. ß1C mRNA expression levels were evaluated by Northern blotting analysis as described in Fig. 1: the average of ß1C mRNA levels in 12 neoplastic prostate tissue specimens (K) was set at 100, ß1C mRNA levels in 3 neoplastic prostate tissues after 1-month of hormonal therapy were calculated as percentage of K. Regression analysis showed positive correlation between ß1C transcription rate and ß1C mRNA expression levels: r=0.90.

We measured an increase (360±10%) of the ß1C integrin transcriptional activity in TK tissues compared to the K tissues, which resulted statistically significant ($p<0.01$) (Fig. 3B).

This result suggest that the ß1C integrin gene transcriptional activity is possible responsible for the up-regulation of the ß1C integrin mRNA levels measured in patients who had undergone 1-month of neoadjuvant ADT.

The increase in ß1C transcriptional activity directly correlated with the up-regulation of ß1C mRNA steady-state expression in TK group ($r=0.90$; $p<0.001$) as shown in Fig. 4.

Clinical progression and ß1C mRNA expression. To investigate whether the effect of the neoadjuvant ADT on the ß1C mRNA expression is associated with the aggressiveness of prostate cancer, we made an attempt to correlate ß1C mRNA levels to the Gleason score.

As shown in Fig. 5, the ß1C mRNA levels measured in TK specimens showed a statistically significant ($p<0.05$) increased expression (259±10%) in comparison with patients without therapy (K) when Gleason score was <7. In Gleason score ≥7 prostate cancer, ß1C mRNA levels measured in TK specimens showed a lower increase of expression (186±10%) (Fig. 5).

In conclusion, even if these preliminary results need to be verified, we may suppose that ADT effect on the ß1C recovery decreases with advancing tumor since the increase of the ß1C mRNA levels is greater in tumors with Gleason <7.

Discussion

In this study we investigated whether ß1C mRNA expression was modulated by ADT. In a previous study we demonstrated no statistically significant differences in ß1C mRNA levels between patients who received 3- or 6-months of neoadjuvant ADT and patients who did not undergo hormonal treatment (13). In the only available case where 1-month neoadjuvant therapy had been administered, there was an increase in ß1C mRNA levels compared with those in patients that had not received hormonal therapy. Therefore, in the present study we investigated the effect of a short-term

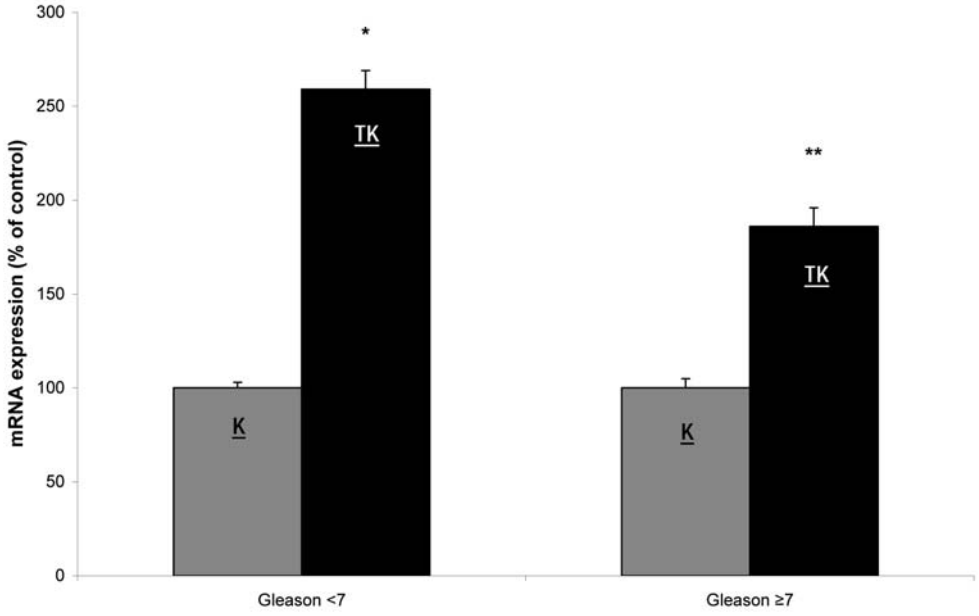

Figure 5. Association of ß1C mRNA expression with Gleason score. The average of ß1C mRNA expression levels in neoplastic tissues isolated from patients without hormonal therapy (K) was set at 100. ß1C mRNA levels in neoplastic tissues after 1-month of hormonal therapy (TK) were calculated as percentage of K. Mean values ± SEM from at least 3 different experiments are shown. The 22 patients, described in Table I, were divided into 2 groups. The first included 3 patients affected by prostate cancer without hormonal therapy and 3 patients affected by prostate cancer treated with 1-month of hormonal therapy, all with Gleason score <7. The second included 9 patients affected by prostate cancer and 7 patients affected by prostate cancer treated with 1-month of hormonal therapy, all with Gleason score ≥7. Error bars indicate SEM. $^{*}P<0.05$; $^{**}P<0.01$.

(1 month) ADT treatment on ß1C mRNA expression levels in a larger number of patients.

We show for the first time, a significant increase (130±12%) of ß1C mRNA levels in prostate adenocarcinoma after 1-month of neoadjuvant ADT compared with the normal prostate tissues. On the contrary, we confirmed a significant decrease (62±5%) of ß1C mRNA levels in neoplastic tissues (group K) compared with normal prostate tissue (group N) (13).

Moreover, we found that altered splicing mechanisms are unlikely to explain the increased of ß1C mRNA levels measured in hormonally treated prostate cancer, since all ß1 integrin mRNAs were up-regulated (13).

These results suggest that a short course of ADT specifically interfere with ß1C integrin expression, possibly indicating that androgen-mediated mechanisms act through pathways involving ß1C integrin. Adaptation and clonal selection models could explain these differences on ß1C integrin expression between a short course and a 3- or 6-month course of neoadjuvant ADT. In particular, according to the former hypothesis resistant clones could develop through adaptive transformation of the initial homogenous population in response to castration (20).

Whereas the mechanisms of hormonal regulation of integrin expression in human prostate cancer cells remains to be elucidated, Hughes *et al* demonstrated the suppression of integrin activation through modulation of affinity state by Ras/MAPK pathway (20). This pathway may be activated by the androgen receptor (21). Moreover, it has been shown that androgen receptor-mediated negative regulation of gene expression occurs mainly by physical interaction of the androgen receptor with Ets proteins, and most of the genes that are transcriptionally down-regulated by the androgen receptor have Ets binding sites in their promoter region (21). In this light, it is conceivable that interaction of the androgen receptor with ß1 integrin transcription factors might represent a pathway regulating this gene.

In-depth understanding of the molecular mechanism regulating the cross-talk between ß1C expression and ADT, needs additional more definitive experiments by using an *in vitro* cell system, thus allowing a closer examination of the ß1C integrin interaction with androgen responsive elements, or other alternative mechanisms such as an indirect modulation of a co-repressor or other post-translational changes. We also tried to evaluate the association between ß1C mRNA levels and the tumor aggressiveness.

Our preliminary results seems to indicate as a general trend that the hormonal therapy effects decrease with advancing tumor grade since the increase of the ß1C mRNA levels is greater in tumors with Gleason <7. Obviously it will be necessary to verify these preliminary results by using an *in vitro* cell system given that it is not possible to recruit a larger and more representative population as recent international guidelines do not recommend the use of neoadjuvant therapy. In fact a recent Cochrane review (22) analysed the role of neoadjuvant hormonal therapy and prostatectomy, showing that for patients undergoing prostatectomy, the addition of a neoadjuvant hormonal treatment did not improve overall survival, neither did it provide any significant advantage in disease-free survival over prostatectomy alone.

Finally, since it is conceivable that either a transcriptional or a post-transcriptional regulation of ß1C expression might be responsible for the increased mRNA levels, we measured the transcriptional activity of ß1C gene in the prostate tissue by run on analysis experiment, as previously set up in our laboratory (16,23). We show that the increased transcriptional activity of the ß1C integrin gene was correlated with the up-regulation of ß1C mRNA expression in prostate cancer patients treated with neoadjuvant ADT.

Since it is not possible at this point to exclude that post-transcriptional mechanisms could also be involved in the regulation of ß1C mRNA expression, further studies will be performed by using an *in vitro* cell culture system, on the mRNA half-life. These studies, aimed at highlighting the mechanisms involved in the selective regulation of ß1C integrin expression in prostate cancer, could provide new insights into the molecular control of ß1C expression and, therefore, prostate cancer cell proliferation.

In conclusion, our data show that short-term administration of ADT interferes with ß1C integrin expression, suggesting that androgen-mediated mechanisms may act through the ß1C pathways.

The precise characterization of the mechanisms regulating the expression of this adhesion factor in prostate cancer cells will provide further insight into the molecular mechanisms involved in tumor progression.

A better understanding of the biological mechanisms responsible for the uncontrolled growth of hormonally regulated cancer such as those of the prostate tissue, is critical not only to contribute to the understanding of the molecular mechanisms that support malignant cell transformation and progression, metastasis establishment and growth, but also to possibly contribute to devise novel effective diagnostic and/or prognostic molecular approaches.

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