Nuclear mRNA retention and aberrant doppel protein expression in human astrocytic tumor cells

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Abstract. Doppel (Dpl) is a paralogue of the mammalian Prion (PrP) protein. It is abundant in testis and, unlike PrP, it is expressed at low levels in the adult central nervous system (CNS). Besides, Dpl overexpression correlates with some prion-disease pathological features, such as ataxia and death of cerebellar neurons. Recently, ectopic expression of doppel was found in two different tumor types, specifically in glial and haematological cancers. In this study the doppel gene (*PRND*) mRNA and protein expression in PRT-HU2 and IPDDC-A2 astrocytoma-derived cell lines was investigated. Northern blot analysis revealed two equally abundant *PRND* mRNA isoforms, while real-time PCR, on nuclear and cytoplasmic RNA fractions, and cRNA *in situ* hybridization, on astrocytoma cells and bioptical specimens, showed a nuclear retention of *PRND* transcripts. Western blot analysis showed that the amount of protein expressed is low compared to the level of mRNA. Moreover deglycosylation studies indicated that Dpl undergoes unusual glycosylation processes. Immunohistochemistry experiments demonstrated that Dpl was mainly localised in the cytoplasm of the astrocytic tumor cells, and that it failed to be GPI-anchored to the cell membrane. This unusual cellular localization was also confirmed through EGFP-Dpl expression in astrocytomas; on the contrary, HeLa cells exhibited the expected Dpl membrane localization. Our findings suggest an aberrant doppel gene expression pattern, characterized by a substantial nuclear retention of the transcript, an altered post-translational modification of the protein and an unusual cytoplasmic localization.

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Introduction

In 1999, the first prion-gene paralogue, doppel (*PRND*), was described in rodents (1,2). Subsequently, the doppel gene was confirmed in humans (3), cattle, sheep (4), and goats (5). Comparative gene expression analysis was reported on different patterns of temporal and spatial expression among prion and doppel. Thus, whereas prion protein (PrP) is widely expressed, showing the highest expression profiles within the CNS (6), doppel shows barely detectable levels in most tissue (4,7). Doppel protein (Dpl) was found highly expressed only in adult and fetal testis (8), and different groups recently proposed an involvement in male gametogenesis (9-11). Based on the structural similarities between PrP (12) and Dpl (13), a role of doppel in the development of prion neurodegenerative diseases was hypothesised (14). Specifically, when ectopically expressed in some $Prnp^{0/0}$ transgenic lines, Dpl causes Purkinje cell death and ataxia (reviewed in ref. 15). However, Dpl-induced neurodegeneration can be rescued by the introduction of a *Prnp* transgene (16), suggesting the possibility that the protein interacts or competes with PrP in a sort of molecular antagonism-model (17). Further studies were performed to demonstrate a direct involvement of doppel in prion-diseases in humans (7) as well as in animals (18,19). From these analyses, doppel seems not to be associated with the diseases, neither if one considers the gene variability and expression, nor if one examines the role of the protein at the pathological lesions. Consequently, the expression of the doppel gene has been investigated in other pathological contexts. Ferrer and collaborators (20) have recently reported a selective Dpl immunoreactivity in dystrophic neuritis in Alzheimer's disease patients. In tumors, the first evidence of a possible implication of Dpl has been recently produced by our group, reporting an aberrant *PRND* expression in the astrocytic and leukaemic specimens with different malignancy grades (21,22).

Astrocytomas are fairly common tumors of neuroectodermal origin that typically show a high degree of malignancy (23). Specific pathological features of astrocytomas comprise a high degree of neoplastic cell proliferation and invasion within the brain peritumoral areas and, in addition, prominent angiogenesis in the neoplastic tissue. Many genes have been

identified and found to be associated with astrocytic tumor progression, however their functional involvement has not been completely clarified (24). Many of these genes are involved in signal transduction processes or in cell cycle arrest pathways (25). Several differential expression markers have been identified specifically for astrocytomas, such as the *TP53* (26) and the *EGFR* genes (27); furthermore, the majority of these tumors exhibit differences in astrocytic developmental markers such as GFAP and S-100B proteins (28). Recent studies that used genomic high-throughput technologies such as DNA microarrays provided more comprehensive molecular profiling of human astrocytic tumors (29-31). These studies indicated that astrocytomas over-expressed various gene families related to cell adhesion, motility, invasion, and angiogenesis. However, only a fraction of the alterations in the pattern of mRNA expression has been confirmed at the protein level and the functional interplay of these genes in inducing various malignant phenotypes remains elusive (32).

In this study we investigated the features of *PRND* over-expression in astrocytoma, with a fine characterization of its complex transcriptional and translation patterns. The results showed a peculiar nuclear retention of the messenger and an ectopic expression of the protein in the cytoplasm of the tumor astrocytic cells.

Materials and methods

Cell cultures and astrocytoma tissue sections. Human astrocytoma cell lines PRT-HU2 (33), provided by Dr Carlo Zibera (Fondazione Maugeri, Pavia) and IPDDC-A2 (ECACC, Dorset, UK), were cultivated in RPMI-1640 and D-MEM media, respectively, supplemented with 10% FBS, 100 units/ml penicillin, 0.1 μg/ml streptomycin and 1% L-Glutamine (Invitrogen, Carlsbad, CA). The human HeLa cell line (ATCC, Rockville, MD) was maintained with D-MEM medium supplemented with 10% FBS, 100 units/ml penicillin, 0.1 μg/ml streptomycin and 1% L-Glutamine. Glioblastoma multiforme tumor tissues, classified according to the WHO guidelines (34), were 9 surgical resection specimens, obtained after informed consent of the patients and stored at -80˚C. Human testis tissue was obtained after autoptic resection and immediately stored at -80˚C.

Northern blot analysis. Total RNA (50 μg) from PRT-HU2 and IPDDC-A2 cell lines, of the Glioblastoma multiforme specimens, extracted with the Trizol reagent (Invitrogen), and from a human healthy brain total RNA (Clontech, Palo Alto, CA), were run on a 1.0% formaldehyde-agarose gel, blotted to a nylon membrane and hybridized with 100 ng of a ^{32}P-dCTP random-labeled probe (Amersham Biosciences, Freiburg, Germany). *PRND* (NM_012409) and *GAPD* (AY340484) probes, respectively of 531 and 458 nucleotides, corresponding to the entire *PRND* coding region and to a portion of the *GAPD* transcript, were previously amplified (32 cycles of 94˚C x 15 sec, 62˚C x 30 sec, 72˚C x 45 sec) from commercial human genomic DNA (Clontech), using the following primers: *PRND* forward 5'-ATG AGG AAG CAC CTG AGC TGG TG-3', reverse 5'-TTA TTT CAC CCT GAG CCA GAT CAA A-3', *GAPD* forward 5'-GGC CAA TGA TAA CCT TAT AAG AGG-3', reverse 5'-CAT GAC GAA CAT GGG GGC AT-3'. Probes were gel purified, spectrophotometrically quantified and sequenced. Post hybridization washes were performed at high stringency (0.1 x SSC, 0.1% SDS at 55˚C).

cRNA in situ hybridization. Sense and antisense *PRND* cRNA probes were produced by a 'run off' T7-based transcription protocol. For sense *PRND* probe, the forward primer 5'-ACT GTC TGC ATG CTG CTC TTC-3' and the T7 promoter-tailed reverse primer 5'-TAA TAC GAC TCA CTA TAG GGG ATC AAA GCC AGA AGG CAG AG-3' were specifically employed to amplify a portion of the *PRND* coding region from human genomic DNA. Differently, the forward 5'-TAA TAC GAC TCA CTA TAG GGA CTG TCT GCA TGC TGC TCT TC-3' and the reverse 5'-GAT CAA AGC CAG AAG GCA GAG-3' primers were adopted to generate the antisense probe. Purified PCR (10 μg) products were *in vitro* transcribed using NTP-DIG-(11)-UTP labelling mixture and 40 units of T7 RNA Polymerase (Roche, Indianapolis, IN) for 2 h at 37˚C. Labelled cRNAs were then ethanol-precipitated and redissolved in 100 μl DEPC-water. Probes were then stored at -20˚C until use. For *in situ* detection on astrocytoma-derived cells or Glioblastoma multiforme 4-μm tissue sections, the Innogenex ISH kit (Biogenex, San Ramon, CA) were employed according to the manufacturer's specifications, in the presence of 10 pg of the *PRND* sense or antisense probes, and using fast red as chromogen.

RNA isolation from astrocytoma cell lines. Isolation of cytoplasmic and nuclear RNA was performed from 5x10^7 PRT-HU2, IPDDC-A2 and HeLa cells grown at confluence with the RNeasy midi kit (Qiagen, Hilden, Germany). The RNA content and the OD ratios were accurately measured using a spectrophotometer (Biophotometer, Eppendorf, Hamburg, Germany) with different dilutions and replicas.

Real-time expression analysis. Nuclear and cytoplasmic RNA (2 μg) from PRT-HU2 and IPDDC-A2 astrocytoma-derived and HeLa cells reverse-transcribed using the High Capacity cDNA Archive kit (Applera, Foster City, CA) according to the supplier's specifications. *PRND* and *GAPD* primers were designed with Primer Express software (version 3.1, Applera) with identical annealing temperatures. Primers were designed on adjacent exons of the genes in order to avoid DNA-derived amplicons, that amplified fragments of 460 and 458 bp, respectively for *PRND* and *GAPD*. *PRND* primers were 5'-CTC CAG AGA GAG CCA AGG TT-3' and 5'-ATG CTT GAG GGA GCA GAG CT-3', *GAPD* primers were 5'-GGC CAA TGA TAA CCT TAT AAG AGG-3' and 5'-CAT GAC GAA CAT GGG GGC AT-3'. Real-time PCR was performed on a MJ Opticon II model (MJ Research, Waltham, MA) in three replicas for each of the investigated samples. Samples were subjected to the following thermal profile: 95˚C x 15 min, followed by 40 amplification cycles of 94˚C x 15 sec, 65˚C x 25 sec, 72˚C x 45 sec. For each sample, amplifications were performed in 20-μl volumes containing 1X DyNAmo SYBR-Green qPCR mix (Finnzymes, Espoo, Finland), 0.5 μM of each primer pair and 10 ng of nuclear or cytoplasmic cDNA. Negative controls were no-template control and no-retrotranscribed RNA mixtures.

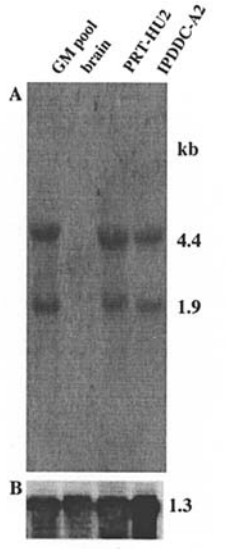

Figure 1. Expression profile of the human PRND gene. Northern blot analysis was carried out with a PRND-specific probe (A). The lanes contain RNAs of human astrocytoma cell lines (PRT-HU2 and IPDDC-A2), of a pool of Glioblastoma multiforme specimens and of normal adult brain. A human GAPD probe acted as a control for the amount of RNA loaded and for integrity (B).

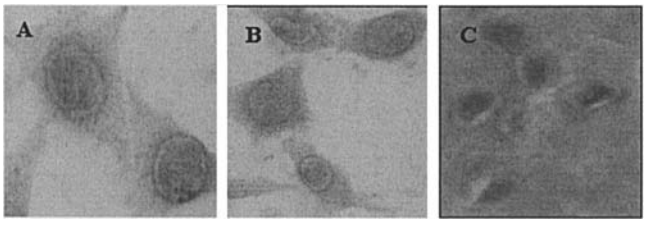

Figure 2. Cellular localization of PRND mRNA in astrocytoma-derived cell lines and bioptical resections. PRT-HU2 (A) and IPDDC-A2 cells (B) and Glioblastoma multiforme tissue (C) were hybridised with a PRND digoxigenin-labelled antisense cRNA probe. Light optical magnification of X1,000.

Biochemical studies. PRT-HU2 and IPDDC-A2 cells (~10^8) were grown to confluence, washed twice with buffer A (50 mM sodium phosphate pH 6.5, 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride) and collected with a cell scraper. After centrifugation, cells were resuspended in buffer A containing 730 mM sucrose, and disrupted by sonication. To separate the cytosolic from the microsomial fraction, the cell-free extract obtained after 10-min centrifugation at 1000 x g, was spun for 1 h at 100,000 x g. The microsomial fraction was then solubilised at room temperature in buffer A containing 2% Triton X-100 and centrifuged as before. To prepare microsomial fraction of testis Dpl, autoptic sample (1 g) was mechanically lysed using a potter in 20 ml of buffer A containing 730 mM sucrose, and treated as above. The total protein concentration was determined according to Lowry *et al* (35). Aliquots of the purified proteins (2 μg and 10 μg for testis and astrocytoma cells, respectively) were subjected to 12% SDS-PAGE followed by Western blot analysis. Western blot analysis was performed with purified antisera from rabbit immunised with recombinant human Dpl. The blots were finally revealed by chemiluminescence ECL system (Amersham).

Isoelectric focusing was carried out on 7-cm Immobiline DryStrips (pH 3-10) with a Multiphor II system (Amersham). The DryStrips were rehydrated overnight at room temperature in 8 M urea, 2% CHAPS, 2% IPG buffer 3-10, 100 μl of Dpl solution dialyzed against water (100 μg of protein). Focusing was performed at 20˚C according to the following gradient voltage program: 0-200 V, 1 min; 200-3,500 V, 2 h; 3,500 V, 2 h. After focusing, the strips were equilibrated in 10 ml of isoelectric focusing equilibration buffer (50 mM Tris-HCl, pH 8.8, 8 M urea, 2% SDS, 30% glycerol, and 0.002% bromophenol blue) and layered on top of a vertical 12% SDS-polyacrylamide gel for the second dimension separation. Western blot analysis and Dpl detection was performed as previously described.

Chemical deglycosilation was performed by trifluoromethanesulfonic acid (TFMS) according to Edge *et al* (36). Briefly, 1 mg of liophilized protein was dissolved in 300 μl of anisole TFMS 1:2, and, after bubbling nitrogen for 10 sec, the solution was stirred at 25˚C for 3 h. To recover the deglycosilated proteins, a 50-fold excess of diethyl ether containing 10% n-hexane (v/v) solution was added and the mixture was freezed at -80˚C for 1 h. The precipitated proteins were collected by centrifugation, resuspended in ice-cold 95% ethanol, centrifuged, liophilized, resuspended in Laemmli buffer and subjected to SDS-PAGE and Western blot analysis.

Enzymatic Dpl deglycosilation was carried out using PNGase F or/and neuroaminidase (New England Bioloabs, Ispwich, MA). Briefly, Dpl was heat-denaturated for 10 min in the presence of 2.5% SDS and 140 mM β-mercaptoethanol. 10% (v/v) of NP-40 was added and the mixture was incubated overnight at 37˚C in the presence of 1,000 U of PNGase F or/and 100 U of neuraminidase. The reactions were stopped with trichloroacetic acid at a final concentration of 25% (w/v). The precipitated proteins were collected by centrifugation and washed twice with acetone. Proteins were then resuspended in Laemmli buffer and subjected to SDS-PAGE and Western blot analysis.

Immunohistochemistry. PRT-HU2, and IPDDC-A2 cells were fixed with ethanol 70% at 4˚C for 10 min. Indirect immunoperoxidase staining of cultured cells was performed using the 'Cell and tissue staining kit AP-BCIP/NBT' (R&D System, Minneapolis, MN) with the 4-nitroblue tetrazolium chloride as chromogen. The human G-20 anti-Dpl primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was incubated (at a 1:1,000 dilution) at room temperature for 30 min. Samples were counterstained with hematoxylin for 45 sec. Negative control staining reactions were performed using the secondary antibody alone.

Dpl-EGFP plasmid construction and transfection. The pEGFP-N1 plasmid (Clontech) was mutagenized by PCR using the primers 5'-CCG GAA TTC CCA CCA TGG TGA GCA AGG GCG AG-3' and 5'-GTC GCG GCC GCT TTC TTG TAC AGC TCG TCC ATG-3', to remove the stop codon at the end of *EGFP*. The pEGFP-N1/Dpl(1-176) construct was produced amplifying the *PRND* amino terminal signal peptide

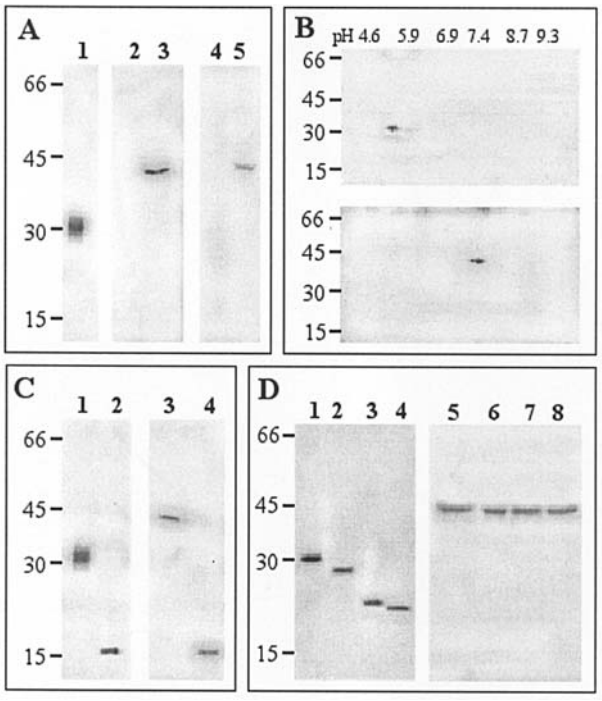

Figure 3. Biochemical characterization of Dpl in astrocytoma cells. A, Western blot analysis of Dpl in microsomial healthy human testis (lane 1), PRT-HU2 (lane 2) and IPDDC-A2 (lane 4) cell fractions, compared to the cytosolic fractions of the astrocytoma cells (lanes 3,5). B, Dpl two-dimensional electrophoresis of PRT-HU2 cells and normal human testis. C, chemical deglycosilation by TFMS treatment of Dpl: testis extract (lanes 1,2), and PRT-HU2 cytosolic cell fraction before (lane 3) and after (lane 4) treatment. D, enzymatic deglycosilation of Dpl with PNGase F in testis and in PRT-HU2 cells, respectively, before (lanes 1,5) and after treatment (lanes 2,6), treated with neuroaminidase (lanes 3,7) and with both enzymes (lanes 4,8). Two-dimensional, chemical and enzymatic deglycosilation analyses in IPDDC-A2 cells gave identical results, compared to the PRT-HU2 cells (data not shown). Molecular mass is indicated in kilodaltons.

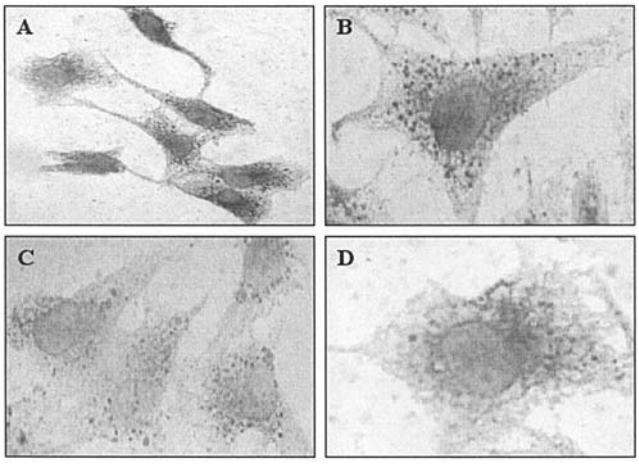

Figure 4. Immunohistochemical analysis of Dpl expression in human astrocytoma cell lines. Dpl cytoplasmic staining of PRT-HU2 (A,B) and IPDDC-A2 cells (C,D). Light optical magnification of X700 (A), X800 (C) and X1,000 (B,D).

coding region (from 1 to 25 Dpl residues) with the primers 5'-CCG CTC GAG CGG ATG AGG AAG CAC CTC AGC TG-3' and 5'-CCG AAT TCC GCC CCT CTC CAA CCA AAA C-3'. This *PRND* portion was then cloned into the pEGFP-N1 mutagenized plasmid, 5' of *EGFP*, in the XhoI and EcoRI restriction sites. The remaining *PRND* peptide coding region (26-176 Dpl residues) was amplified using the primers 5'-ATT TGC GGC CGC TGC AGG ACT TCG GGT CAC-3' and 5'-TGC GGC CGC TTA TTT CAC CAT GAG CCA GAT CA-3'. The product was then cloned downstream of *EGFP* in the modified pEGFP-N1 plasmid, using the NotI restriction site, thus obtaining pEGFP-N1/Dpl(1-176). Transfections were transiently performed in HeLa and IPDDC-A2 cells at 95% confluence into 22-mm glass bottom dishes using the Lipofectamine-2000 reagent (Invitrogen), with a DNA (mg)/reagent (ml) ratio of 2:4 and 3:8, respectively. Cells were then incubated for 24 h at 37˚C and 5% CO_2. Images were acquired on a Leica TCS SPII microscopy equipped with the confocal inverted microscopy system Leica DM IRBE, using a 63X, NA 1.32 oil immersion objective. EGFP was excited at the wavelength of 488 nm with a filtered argon laser.

Results

Characterization of PRND mRNA in astrocytoma cell lines. As previously reported, doppel resulted to be overexpressed in different tumor cells such as those of astrocytic and leukaemic lineages (21,22).

To investigate and clarify the doppel expression in human astrocytomas, different molecular and cellular approaches were employed. Initially, in Northern blot analysis, a nucleotide probe complementary to the *PRND* coding region was hybridised to a panel of human total RNAs, representative of the PRT-HU2 and IPDDC-A2 astrocytoma-derived cell lines, to a pool of RNAs from Glioblastoma multiforme specimens and to a normal adult brain, used as a negative control. Northern blot analysis revealed two main bands of 4.4 and 1.9 kb, equally represented within each sample (Fig. 1). However, the bands showed different intensities between the investigated RNAs, i.e. absence of signals in normal brain and with the highest expression in the Glioblastoma multiforme pool and in the PRT-HU2 cells. Different RACE (Rapid Amplification of cDNA ends)-based approaches such as the SMART and the Creator technologies (Clontech), as well as the Takara LA PCR kit (Takara), using combination of several oligo-dT primers of different length (15-35 nucleotides) were employed in order to characterize the observed transcripts. None of these attempts were successful. To directly visualise doppel mRNA in the astrocytoma cells, a probe corresponding to a portion of the *PRND* coding region was *in vitro* T7-transcribed and used in cRNA *in situ* hybridisation experiments, on PRT-HU2 and IPDDC-A2 cells (Fig. 2A and B), as well as on Glioblastoma multiforme bioptical resections (Fig. 2C). These experiments showed that doppel transcripts formed discrete signals localised mostly within the nuclei of the cells, highlighting foci of accumulation of the transcripts. In addition, less abundant granular doppel hybridisation signals were apparent in correspondence of the perinuclear envelope and, although less abundant, within the cytoplasm of the cells. Similar evidence came from the analysis of bioptical sections of the Glioblastoma multiforme specimens. Negative controls, using doppel sense-probes, failed to reveal hybridisation signals (data not shown). In order to confirm these findings and to compare the amount of

Table I. *PRND* and *GAPD* expression in nuclear and cytoplasmic RNA fractions of PRT-HU2, IPDDC-A2 and HeLa cells.

	C_t *(PRND)*			C_t *(GAPD)*		
Cell line	Nuclear	Cytoplasmic	RQ	Nuclear	Cytoplasmic	RQ
PRT-HU2	32.4±0.3	36.7±0.4	45.3	35.6±0.2	29.5±0.4	0.3
IPDDC-A2	32.9±0.1	35.2±0.3	11.3	34.2±0.3	28.4±0.2	0.4
HeLa	36.1±0.3	34.9±0.3	1	32.2±0.2	27.9±0.1	1

C_t are the amplification threshold cycles. RQ (relative quantity) refers to the nuclear/cytoplasmic PRND or GAPD relative quantitation, given by $2e^{-\triangle\triangle C_t}$ algorithm, as previously described (47), normalised to the HeLa endogenous reference. A validation test for the $2e\text{-}\triangle\triangle C_t$ algorithm was applied as previously described (21).

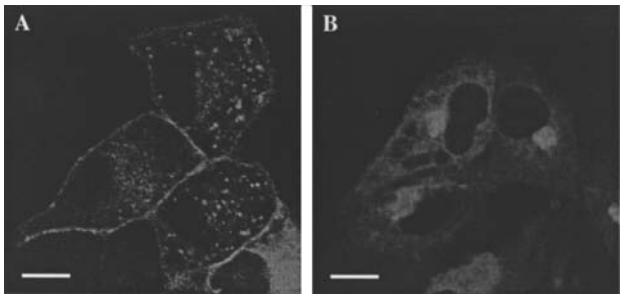

Figure 5. Confocal microscopy immunofluorescence staining of Dpl in human tumor cell lines. HeLa (A) and astrocytoma IPDDC-A2 human cells (B) were transfected with the pEGFP-N1/Dpl (1-176) expression vector and visualized with a confocal microscope. In HeLa, the construct stains the membrane edges of the cells. In IPDDC-A2, the Dpl signals are diffusely distributed in the cell cytoplasm. Scale bars, 10 μm.

gene-specific transcripts in the two cellular compartments, nuclear and cytoplasmic RNA fractions were isolated from growing astrocytoma and HeLa cells, spectrophotometrically quantified and subjected to real-time PCR amplifications. Specific primers were designed on adjacent exons of the *PRND* and *GAPD* genes and adopted in real-time PCR. As shown in Table I, the comparison of the PCR threshold cycles (C_t) and the *PRND* and *GAPD* nuclear/cytoplasmic relative quantities in the astrocytoma-derived cell lines, demonstrated that the two genes exhibited opposite patterns of expression. In fact *PRND* amplification signals were found mostly in the nuclear RNA fraction of PRT-HU2 and IPDDC-A2 cells, whereas they were mainly cytoplasmic in HeLa cells, used as control. Also, *GAPD* signals were as expected, i.e. cytoplasmic, both in the astrocytic tumor cells and in the HeLa control cells.

Biochemical characterization of the doppel protein. The doppel protein expressed in PRT-HU2 and IPDDC-A2 astrocytoma-derived cell lines was subjected to a biochemical characterization. Western blot analysis performed on microsomial and cytosolic fractions (Fig. 3A), showed that Dpl from both cell lines was present in the cytoplasm, in a soluble form (lanes 3 and 5). Conversely, Dpl from testis, used as a positive control, was found as expected in the microsomial fraction (lane 1), as previously reported (10). Besides, a higher Dpl expression was detected in PRT-HU2, compared to IPDDC-A2 cells (lanes 3 and 5). Additionally, whereas Western blot analysis on testis Dpl showed always a hetero-disperse band of ~32 kDa, Dpl from astrocytoma cells presented one major band of molecular mass ~42 kDa. The pI value of Dpl from astrocytoma cells turned out to be shifted to a higher value compared to the testis Dpl protein (Fig. 3B, pI value 7.0 vs 5.3). After chemical deglycosylation by means of TMSF, both testis and astrocytoma Dpl proteins showed a band of ~15 kDa, suggesting similar polypeptide integrity (Fig. 3C, lanes 2 and 4). On the contrary, unlike testicular Dpl (Fig. 3D, lanes 1 and 2), astrocytoma Dpl was not deglycosylated by PNGase F (lanes 3 and 4); moreover, no sialic acid cleavage was obtained by means of neuraminidase, even after prolonged enzymatic treatment, either before or after addition of PNGase F (Fig. 3D, lanes 5-8).

Doppel protein cellular localization in astrocytoma cell lines. Immunohistochemistry studies were performed in order to confirm the aberrant cytoplasmic doppel protein expression in the astrocytoma cell lines. The results, reported in Fig. 4, confirmed that, similarly to the Western blot analysis, PRT-HU2 presented a higher cytoplasmic protein content (A,B), compared to the IPDDC-A2 cells (C,D). The cytoplasmic localization was also confirmed with confocal microscopy, following transfection of the IPDDC-A2 cells with the pEGFP-N1/Dpl (1-176), a Dpl-EGFP expression vector described in Materials and methods. Interestingly, as reported in Fig. 5, HeLa cells, used as a control, exhibited the expected membrane localization of the chimeric Dpl-EGFP protein (A), while the astrocytoma cells showed a diffuse cytoplasmic fluorescence (B).

Discussion

In this study, we explored the aberrant doppel gene expression in astrocytomas, describing a peculiar nuclear retention of the messenger in the nucleus, abnormal post-translational processes and an ectopic expression of the protein in the cell cytoplasm.

In a previous study we reported overexpression of the *PRND* gene in two different tumor lineages, of astrocytic (21) and haematological origin (22). The finding was interesting

because in the corresponding healthy tissue, i.e. brain and bone marrow, *PRND* expression was absent, in accordance with other studies (7,8). However, the high amount of doppel transcripts did not correlate to the expression levels of the corresponding protein. To get insights into the doppel expression pattern we investigated the generation of the transcript within the nucleus, its presence in the cytoplasm and, finally, the cellular localization of the corresponding protein. Our results suggest a nuclear *PRND* RNA retention as shown with two different approaches, cRNA *in situ* hybridisation and real-time PCR analysis of nuclear and cytoplasmic RNA fractions. In summary, the data indicate that only a minor fraction of *PRND* mRNA was exported outside the nucleus.

Nuclear mRNA retention and compartmentalisation is increasingly recognized as an important mechanism to regulate the activity of transcription-related proteins and to modulate cell growth and death (37). Export of nuclear mRNAs is constantly challenged by the opposing force of mRNA retention and decay (38). This balance ensures that only 'perfect' transcripts persist, and that non-functional and potentially deleterious transcripts are removed early in their biogenesis (39).

The characterization of the doppel transcripts by Northern blot analysis showed the 4.4-kb isoform, previously found in normal human testis (8), as well as a novel and equally abundant alternative 1.9 kb transcript. This new transcript was confirmed to be a *PRND*-specific isoform through the hybridization with a different probe (data not shown). However, this transcript did not correspond in length to the 1.3-kb *PRND* 3' RACE deposited sequence (GeneBank acc.n. AF187844), isolated from human brain cerebral cortex. Despite numerous attempts designed to characterise the nucleotide composition of this alternative *PRND* transcript, performed by different RACE and retro-transcription approaches, these experiments failed. These negative results could be attributed to a particular hypo-adenylation of the doppel transcript, as well as to a *PRND*-specific deadenylase activity within the astrocytomas nucleus. To explain the observed *PRND* nuclear retention phenomenon, many different processes could be evocated such as unsuccessful capping, abortive RNAPII transcription, the improper packaging, as well as unspliced mRNAs.

The doppel protein in astrocytoma-derived cells exhibited some biochemical peculiarities that are different to those observed in the testis tissue. It turned out to be a soluble protein, endowed with a more complex and larger glycan moiety. Similarly to many glycoproteins described in different tumor cells (40), it probably undergoes unusual glycosylation events leading to an abnormal glycan pattern, different from those previously described in the human testis tissue (10). Thus, the inability of PNGase F to digest the Dpl of both cell lines could be due to a steric hindrance created by the altered glycan moiety or to the presence of an unusual fucose residue in proximity to the N-glycosylation sites. Moreover, the failure of the neuroaminidase in releasing sialic acid residues could be due to a probable absence of sialic acids in the oligosaccharide side chains of the doppel astrocytoma glycoprotein. This is in agreement with the higher pI-value determined by isoelectric focusing. It is worth mentioning that the unusual glycoforms found in astrocytoma-derived cell lines were also detected in astrocytoma brain homogenates (Chiarelli, personal communication), strengthening the suggestion that changes in glycosylation are often one of the hallmarks that accompany malignant transformations (41). The doppel protein isolated from astrocytoma-derived cell lines exhibited a cytoplasmic cellular localization, different from that expected for GPI-anchored membrane signal, as revealed in immunohistochemical staining of the Sertoli cells performed on sections of normal and pathological testes from human (10,11), mouse (42) and cattle (43). Besides, the reported Dpl-EGFP expression comparison experiments in astrocytoma and in HeLa cells demonstrated that a different tumor cell environment could justify *per se* the membrane or cytoplasmic sorting of the Dpl protein. Similarly to the prion protein, doppel is a glycosylphosphatidylinositol-anchored protein as biochemically revealed in mouse neuroblastoma N2a cells (42). GPI-proteins contribute to the overall organization of membrane bound proteins and are important in apical protein positioning. These proteins play also a critical role in a variety of receptor-mediated signal transduction pathways (44). Membrane GPI-anchoring failure of Dpl could also be explained by mutations at the GPI attachment site, leading to incomplete or erroneous GPI synthesis. However, the sequencing of the *PRND* coding regions in both astrocytoma-derived cell lines and specimens, failed to reveal nucleotide variations (Comincini, personal communication). As an alternative explanation, membrane glycosylphosphatidylinositol deficiency or downregulation that has been observed in different neoplasia, might lead to possible mechanisms of loss of antigenicity and immune escape (41). Despite these important pathological implications, no extensive data are available in the literature about GPI-anchoring defects within astrocytic tumor cells.

The reasons for doppel mRNA overexpression and the cytoplasmic accumulation of the protein in astrocytic tumor cells are unknown. Unfortunately, very little is known, at present, about the physiological functions of Dpl; even less information is available about its possible pathological role. Different studies have demonstrated that Dpl exacerbates oxidative damage *Prnp*-null mice cell lines (45). In addition, the up-regulation of astrocyte-specific genes in PrP-deficient mice ectopically expressing Dpl, has suggested that Dpl is actively involved in glial cell activation in the brain (46). Recently, however, Ferrer and collaborators described a Dpl accumulation in abnormal neurites of senile plaques, probably originated from impaired transport and subcellular localization or by an anomalous turnover of the protein (20). Similarly, tumor astrocytes exhibited a Dpl accumulation within the cells, that seems associated with the WHO astrocytoma malignancy grades (Chiarelli and Valentini, unpublished data). In order to reveal a possible contribution of the doppel protein to some of the most relevant tumor cell peculiarities, i.e. differences in proliferation, adhesion, apoptosis, migration, Dpl silencing and overexpressing experiments are in progress.

In conclusion, although doppel-tumor research is still in its infancy, the reported molecular and cellular evidence, combined with the above suggested functional experiments, could therefore demonstrate if doppel protein is involved in

some molecular steps of the astrocytic tumor progression and if it could be considered a potential therapeutic molecular target.

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