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# *RNASET2* as a Tumor Antagonizing Gene in a Melanoma Cancer Model

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The *RNASET2* gene, mapped in 6q27, was previously found to exert control of tumorigenesis in an ovarian cancer system. We present here results indicating a similar control in a melanoma cancer model. Thus, this gene is most likely involved in a common general pathway of tumorigenesis. Moreover, its antitumorigenic activity is manifested in vivo but not in vitro, suggesting that this gene belongs to the growing category of tumor antagonizing/malignancy suppressor genes. A possible role of *RNASET2* in the activation of a senescence program, whose responsible locus was mapped in the same chromosomal 6q27 region, seems to be inconsistent with our data.

Key words: Melanoma model; *RNASET2*; Antitumorigenic activity Tumor suppressor gene

## INTRODUCTION

In recent years, a solid body of epidemiological evidence has indicated that the peritelomeric region of human chromosome 6, namely 6q27, harbors one or more tumor suppressor genes involved in a wide range of tumor types (1–3). Despite intense efforts by several groups, including ours, no genes belonging to this class of tumor suppressors have been unequivocally isolated and characterized so far in this region.

Our group has recently isolated and partially characterized a gene from 6q27 called *RNASET2* (4). This gene is the first mammalian member of the widespread family of T2 RNAses to be described. Proteins belonging to the RNAse T2 family (EC 3.1.27.1) are present in all phila and are endorsed with disparate functions: for instance, in some microorganisms and plants T2 RNAses can mediate phosphate uptake by digesting extracellular RNAs (5), whereas in self-incompatible plants, specific T2 RNAses prevent self-fertilization by inhibiting the growth of the pollen tube (6). In still other species, T2 RNAses have been described to provide host protection against pathogens or to trigger cellular senescence (7,8). Although involved in apparently different biological processes, the most basic and relevant features of RNAseT2 proteins are their localization in the extracellular milieu (where RNAs are conventionally thought to be unavailable) and their ability to control cell growth. Therefore, a functional link between these two features has been envisioned, suggesting that RNAses belonging to this family, besides intracellular RNA digestion, are endorsed with novel biological functions that are performed outside the cell.

Among these biological functions, the well-described control of cellular growth mediated by T2 RNAses is particularly attracting from the oncological point of view. Indeed, *RNASET2* was found by our group to be hypoexpressed or absent at the transcript level in several primary ovarian tumors and ovarian cell lines (9). Moreover, we reported a clear inhibition of tumor growth following inoculation in nude mice of *RNASET2*-overexpressing cells from the Hey2Met3 ovarian cancer cell line (10). Despite such a strong effect in vivo, the *RNASET2*-transfected cells did not show any change when cancer-related parameters were assayed in vitro. The behavior of *RNASET2* is thus reminiscent of that described for the so-called "tumor-antagonizing" or "malignancy suppressor genes," which are characterized by an asymmetric oncosuppressive activity (namely, the ability to suppress tumorigenicity in vivo without affecting growth in vitro) (11–13), and whose occurrence in the human genome is estimated to be more frequent than predicted, although very few examples are available to date.

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Besides ovarian cancer, malignant melanoma is also associated with chromosomal rearrangements in the 6q27 region. We therefore wondered whether *RNASET2* could also behave as a tumor-suppressor gene for this highly aggressive neoplasia. Indeed, the gene product encoded by the *RNASET2* orthologous gene from *Aspergillus niger* (dubbed ACTIBIND) was recently reported to effectively inhibit growth, angiogenesis, and metastasis in a human melanoma model system, thus providing further support to our working hypothesis (14).

We present here experimental evidence in a melanoma model of an in vivo tumor-inhibitory effect of the human *RNASET2* gene that is not associated with alterations of the in vitro growth pattern, as previously observed in an ovarian cancer model. These results support the notion that *RNASET2* behaves in vivo as a tumor-antagonizing or malignancy suppressor gene involved in the pathogenesis of two independent cancer types (both associated with chromosome alterations in the 6q27 region) by means of a similar molecular mechanism.

## MATERIALS AND METHODS

### *Cell Culture and Transfection*

All human melanoma cell lines were maintained in DMEM medium with 10% fetal bovine serum (FBS). The SK-MEL 28 cell line (a kind gift of Prof. Vanna Bianchi-Scarrà, Department of Clinical and Experimental Oncology, University of Genova, Italy) was transfected with Lipofectamine 2000 (Invitrogen). Single stably transfected clones were isolated following selection with G418 at 500 μg/ml. Primary cultures of melanocytes (Gentaur) were grown in HAM-F10 medium (Sigma) supplemented with Melanomax (Gentaur) and 10% FBS.

### *Quantitative RT-PCR*

Total RNA was extracted from primary melanocytes and melanoma cell lines with the TRI reagent (Sigma) and reverse transcribed with random examers using the High capacity cDNA RT kit (Applied Biosystem). Primers were designed with the Primer express program (Applied Biosystem). Real-time RT-PCR was performed on ABI PRISM 7000 with Power SYBR green PCR Master Mix (Applied Biosystems) following the manufacturer's instruction. The sequence of the primer pairs used for *RNASET2* amplification was as follows: forward: 5′-CG TAATTCACTCGTTTCCCAATC-3′ and reverse: 5′-CC CATGCTTTTCCCACTCAT-3′.

The reactions were performed in triplicate in a 25-μl volume containing target cDNA, 40 nM primers, 12.5 μl of Power Sybr Green PCR master mix (Applied Biosystem), and water to the final volume. Following a polymerase activation step at 95°C for 10 min, samples were denaturated at 95°C for 15 s and annealed/extended at 60°C for 1 min, for 40 cycles. Fluorescent signals generated during PCR amplification were monitored and analyzed with ABI Prism 7000 SDS software (Applied Biosystems). Comparison of the amount of each gene transcript among different samples was made using β-actin as reference. The amount of target RNA, normalized to the endogenous reference gene, was calculated by means of the difference-in-threshold-cycle parameter ($\Delta C_t$).

### *Proliferation Assay and Animal Experiments*

In vitro cell growth curves were assembled by plating 4000 cells in a 24-well plate and counting viable cells daily (in triplicate) on a Burker chamber for 7 days. To test for tumorigenicity in nude mice, cells were grown, trypsinized at 70% confluence, harvested, and resuspended in 0.5 ml of culture medium. Cells ($3 \times 10^6$) were then inoculated subcutaneously into 6-week-old nude mice. Tumor growth was monitored every 2 days for 1.5 months by measuring tumor size with a caliper.

### *Northern Analysis*

Total RNA was extracted from primary cultures of melanocytes with the TRI reagent (Sigma). RNA was subsequently cleaned up from melanin according to Lagonigro et al. (15) before being loaded (12 μg per lane) into 1.2% agarose formaldehyde-denaturing gel and blotted onto nylon membrane (Amersham) overnight, following standard procedures. Filters were prehybridized for 1–2 h and hybridized overnight at 68°C in Perfect-Hyb Plus Hybridization buffer (Sigma) with $^{32}$P-radiolabeled probes. After hybridization, filters were washed once with 2× SSC, 0.1% SDS 5 min at room temperature, twice with 0.5× SSC, 0.1% SDS 20 min at 68°C, and twice with 0.1× SDS, 0.1% SDS 20 min at 68°C.

### *Statistical Analysis*

The in vitro and in vivo data were analyzed with the Student's *t*-test and the Mann-Whitney *U*-test, respectively.

## RESULTS

To evaluate the potential oncosuppressive role of the *RNASET2* gene in the context of malignant melanoma, a panel of eight melanoma cell lines was screened for *RNASET2* expression levels by real-time RT-PCR (Fig. 1). With respect to normal melanocytes, four out of eight of these cell lines showed decreased levels of *RNASET2* mRNA. This is reminiscent of what we observed previously in another cancer model system, namely ovarian

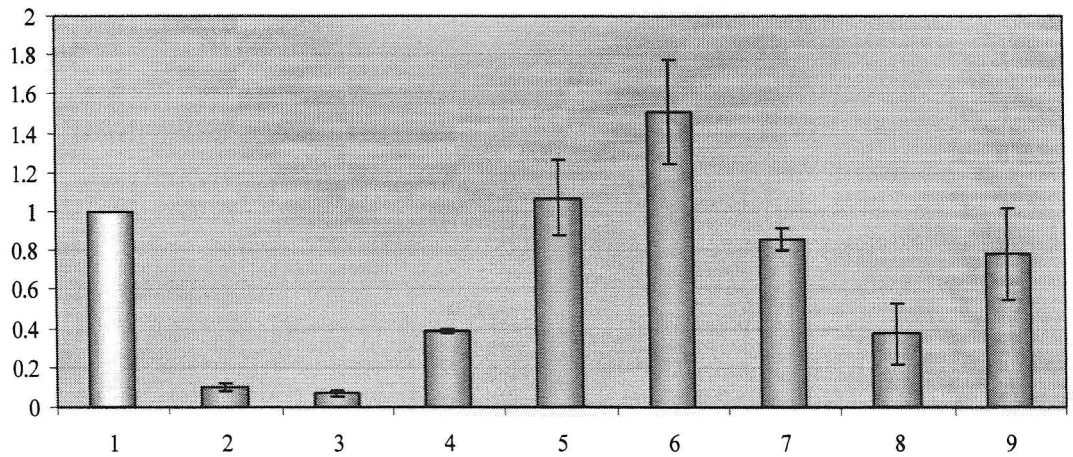


**Figure 1.** Quantitative real-time RT-PCR for *RNASET2* expression levels in a panel of melanoma cell lines. The PCR assay was performed as described in Materials and Methods. *RNASET2* expression was compared to that observed in presenescent normal melanocytes following normalization with the β-actin reference gene. The figure shows expression data from three independent experiments. 1: normal melanocytes; 2: 9923 P°; 3: 9923 M; 4: SK MEL 28; 5: BV; 6: 20842 P; 7: 20842 M2; 8: mel. SP; 9: mel. PAP.

tumors (9). Therefore, a melanoma cell line with low *RNASET2* mRNA transcript levels (SK MEL 28) was chosen for further characterization. Although some cell lines displayed an even lower *RNASET2* expression level when compared to the SK-MEL 28, they were discarded for further analysis due to their poor replication rate in vitro and malignancy potential in vivo (data not shown). The SK-MEL 28 cell line was therefore stably transfected with either the full-length *RNASET2* cDNA cloned into the pcDNA3 vector or the empty plasmid vector, and in vitro cell growth rates were subsequently measured for transfected clones whose *RNASET2* expression levels were investigated by Northern analysis. As depicted in Figure 2a, when *RNASET2* cDNA-transfected clones were compared with control clones, no statistically significant differences could be established. Furthermore, a clonogenic assay performed with both control and *RNASET2*-transfected cells did not show any difference between the two experimental groups (data not shown). To determine the possible effect of *RNASET2* expression on tumor growth in vivo, the transfected clones were then injected SC into the flank of nude mice. Consistent with our previous results in an ovarian cancer model, mice bearing control clones transfected with the empty plasmid showed a statistically significant increase in tumor sizes when compared to mice bearing *RNASET2*-transfected clones, beginning at the fourth week after injection (Fig. 2b). Thus, *RNASET2* was shown to suppress tumor growth in vivo in this melanoma-derived cell line.

Early genetic studies have suggested that the peritelomeric portion of chromosome 6, namely 6q27, could harbor a tumor-suppressor gene involved in the senescence program (16). Because *RNASET2* has been mapped in the same region, we tested whether this gene could be involved in postreplicative senescence by monitoring *RNASET2* mRNA levels in primary melanocytes at different population doublings, starting from 11 PDs up to 37 PDs when replicative senescence occurred. As shown in Figure 3, *RNASET2* mRNA levels in primary melanocytes were invariant from early to late passages (the same result was observed for primary fibroblasts; data not shown), thus ruling out replicative senescence as the biological mechanism by which *RNASET2* carries out its oncosuppressive activity in this cellular model.

## DISCUSSION

In this report, we present further evidence of a tumor-suppressive role for the *RNASET2* gene in a melanoma cancer system. Coupled with our previous results in an ovarian cancer system and the recently described oncosuppressive role of ACTIBIND (the gene product of the *RNASET2* ortholog from *A. niger*) in a human melanoma model, these data suggest that *RNASET2* controls tumorigenicity in at least two different cancer systems. We have also investigated whether this secreted and functionally active RNAse (17) could be responsible for the activation of a senescence program in primary melanocytes and fibroblasts, but no alterations in *RNASET2* mRNA levels could be detected as cells were passaged from early to late duplication stages. These preliminary data seem therefore to exclude this gene as being responsible for such programs in these cellular models. However, in both cancer types the most salient feature of *RNASET2* appears to be a consistent control of tumor-

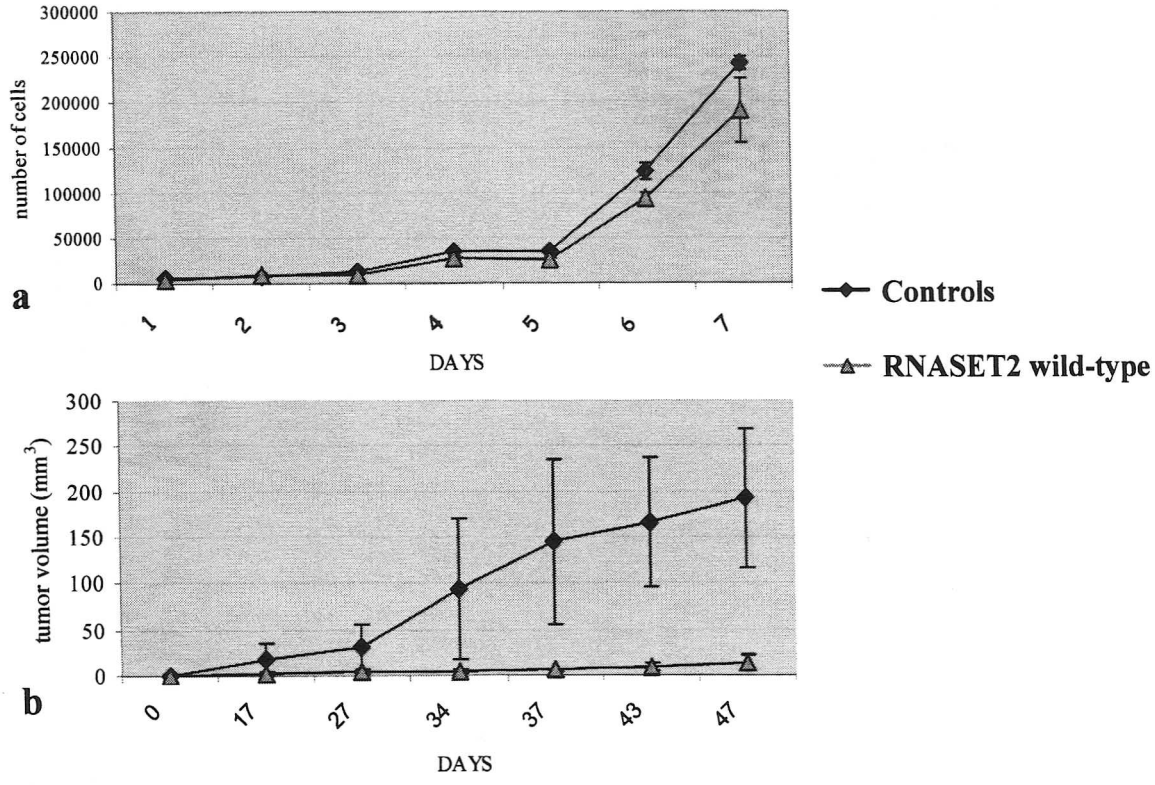


**Figure 2.** (a) In vitro growth of *RNASET2*-expressing SK MEL 28 clones and controls. The growth curves were assembled from three independent clones for each transfected construct. (b) In vivo tumorigenic test in nude mice with transfected SK MEL 28 clones. Two *RNASET2*-transfected and two control clones were used for this experiment. Each clone was inoculated into five independent animals.

igenesis in vivo, associated with a lack of growth control in vitro.

Genes endowed with an asymmetric suppression of tumorigenesis (i.e., controlling tumor growth in vivo but not in vitro) have gained increased attention over the last few years (11–13). For example, the group of Klein has carried out a pioneering work on this category of genes, and to date five of them (LF, LIMD1, HYAL1, HYAL2, and VHL) have been isolated and characterized from the 3p chromosome (13). Very recently another gene, called DEC1 and mapped to 9q32-q34, was found to belong to the same category (18). Other notable examples of genes showing asymmetric tumor suppression are those postulated by the early studies carried out in the 1970s and 1980s by the groups of Harris, Stanbridge, and Kline (12), which after more than two decades are still unidentified. Interestingly, these genes do not correspond to any of the tumor-suppressor genes identified by the widely employed assay of LOH or by comparable molecular cytogenetic approaches.

The behavior of the *RNASET2* gene in a melanoma cancer system that we describe here further suggests that this gene should be ranked as a novel member of this growing class of "tumor-antagonizing genes." Indeed, as we already reported in an ovarian cancer model system (9), *RNASET2* was shown to suppress the tumorigenic potential of a melanoma cell line in vivo without affecting the growth rate in vitro. These results are particularly intriguing in light of our previous observation that *RNASET2* is usually secreted in the extracellular milieu (17). Thus, an *RNASET2*-mediated paracrine signaling pathway may be envisioned, by which *RNASET2* secreted by a particular cell type (i.e., an epithelial cell) can trigger a biological response in a different cell type (i.e., component of the tissue stroma) that ultimately evoke an antiproliferative signal. Such a response would probably not be observed in a "one cell-type only" model system, such as in vitro culture of *RNASET2*-expressing cells. As far as the plausible biological response evoked by this gene is concerned, at least three different mechanisms can be postulated, according to the putative functional features attributed by Klein to tumor antagonizing genes (12): 1) inhibition of angiogenesis; 2) stimulation of cellular differentiation; and 3) control of tissue architecture. Significantly, the relative role of these three complex events in the suppression of tumori-

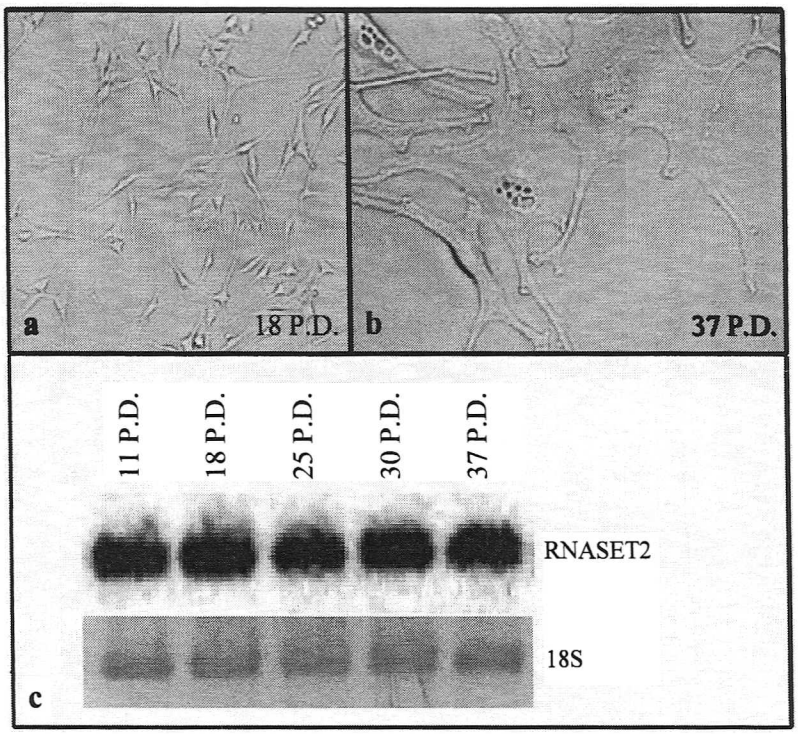


**Figure 3.** Photomicrograph of primary cultures of human melanocytes. (a) Melanocytes at 18 PD (10× magnification). (b) Senescent melanocytes at 37 PD (40× magnification). (c) Northern blot for *RNASET2* expression in primary cultures of melanocytes at different population doublings.

genicity should be easily addressed by using in vivo tumorigenic assays in nude mice as an experimental model.

Thus, our current efforts are devoted to dissect in vivo the molecular signaling pathway(s) by which *RNASET2* is acting. We reckon that, besides improvements in the theoretical aspects aimed at widening the operational definition of tumor-suppressor genes, the in-depth characterization of the *RNASET2* gene could provide useful information that can be exploited in a more clinically oriented avenue. In particular, therapeutic potentials are most appealing in the light of the recent assessment of some RNases in phase III trials and the recently described antitumorigenic properties of ACTIBIND (14).

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