

# Expression and possible role of *hPTTG1*/securin in cutaneous malignant melanoma

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Human pituitary tumour-transforming gene 1 or *hPTTG1* is a proto-oncogene that codes for securin, a protein involved in sister chromatid separation. Based on previous microarray data, we studied the expression of *hPTTG1*/securin in melanocytic lesions. In contrast to nevi and radial growth phase melanomas, securin was expressed by scattered cells in the vertical growth phase, suggesting a role in tumour progression. In a series of 29 nodular and 29 superficial spreading melanomas, matched for all histological prognostic parameters, securin expression was significantly correlated with the nodular subtype ( $P=0.018$ ) and not related to thickness. In other cancers, *hPTTG1* is involved in various oncogenic pathways, including induction of neovascularisation and aneuploidy, and inhibition of p53 activity. We found coexpression of securin with wild-type p53 in the same neoplastic cells in a minority of melanomas. Expression of securin was significantly correlated with the extent of aneuploidy but not with basic fibroblast growth factor immunoreactivity or microvessel density. DNA cytometry revealed that nuclei-overexpressing securin frequently showed tetraploidy or aneuploidy. Our data show that *hPTTG1* is frequently overexpressed in nodular melanoma, and suggest that *hPTTG1* may act as an oncogene in the vertical growth phase, either by inhibiting anaphase, thereby causing aneuploidy and genomic instability, or by modulating the function of p53, thereby impairing apoptosis.

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Pituitary tumour-transforming gene (*PTTG*) was originally cloned from a rat pituitary tumour<sup>1</sup> and the function of the human homologue *hPTTG1* (human pituitary tumour-transforming gene 1) was subsequently elucidated in foetal liver and testis.<sup>2,3</sup> *hPTTG1* is a proto-oncogene encoding for securin, a protein involved in several metabolic reactions, cell-cycle progression, appropriate cell division and chromosome stability; upon overexpression, securin is involved in malignant transformation and tumorigenesis.<sup>4</sup>

In normal tissues, securin expression is limited, in contrast to many human tumours, including pituitary adenomas,<sup>5</sup> lung and breast cancer,<sup>5–7</sup> colorectal cancer,<sup>8</sup> oesophageal cancer<sup>9</sup> and some lymphoid neoplasms.<sup>10</sup> The oncogenic mechanisms of *hPTTG1* are still barely known, but there is accumulating evidence that the oncoprotein activity of securin is exerted at different levels, that is, by induction of angiogenesis through basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF),<sup>11</sup> by prevention of separation of sister chromatids resulting in aneuploidy,<sup>12</sup> by activation of c-myc<sup>13</sup> and/or by specific interaction with p53, thereby blocking its transcriptional activity and inhibiting the ability of p53 to induce cell death.<sup>14</sup>

In accordance with its oncogenic activities, *hPTTG1* expression has been shown to have prognostic value. In tumours of the thyroid, securin is a

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prognostic marker for recurrence,<sup>15</sup> and in breast cancer, the extent of securin expression is associated with the presence of metastatic spread and lymph node invasion.<sup>6,16</sup> In cancer of the oesophagus and colorectum, high levels of *hPTTG1* expression correlate with tumour invasiveness.<sup>9</sup> Recently, *hPTTG1* has been identified as a key signature gene, with high expression predicting metastases in multiple tumour types.<sup>17</sup>

In a recent microarray study of primary malignant melanoma,<sup>18</sup> we found *hPTTG1* to be listed among the genes that were significantly negatively correlated with outcome since patients showing overexpression of *hPTTG1* had shortened distant metastasis-free survival (DMFS) at 4 years. This prompted us to investigate in detail the expression of securin in primary malignant melanoma, and to study the various pathways by which securin may function as an oncoprotein in melanoma. Our results show that securin is particularly overexpressed in the nodular subtype of malignant melanoma and that aneuploidy as well as interference with p53 appears to be the main oncogenic mechanisms.

## Materials and methods

### Immunohistochemistry

To study the pattern of securin expression in pigment cell lesions, we used tissue microarrays, comprising a total of 570 cores of 50  $\mu\text{m}$  width, taken from representative areas in 54 melanomas, 11 metastases and 14 nevi (with a mean of 7.1 cores per lesion). From the primary melanomas, only the vertical growth phase was sampled. The pertinent clinical and histological data are listed in Table 1. As the analysis of the oligonucleotide arrays suggested that overexpression of *hPTTG1/securin* occurred predominantly in the nodular subtype of melanoma, we studied securin expression in the vertical growth phase of 29 pairs of superficial spreading melanoma and nodular melanoma, matched for thickness, ulceration, number of mitotic figures, type of host response by tumour-infiltrating lymphocytes (ie brisk, nonbrisk or absent) and regression. In these 58 melanomas, all histological prognostic variables were assessed according to standard criteria. To assess the tumour progression phase in which securin immunoreactivity occurred, 10 additional cases of *in situ* malignant melanoma were also studied.

In all cases, endogenous peroxidase was inactivated, and heat-induced epitope retrieval was performed in Tris-ethylenediaminetetraacetic acid, pH 9.0. Since preliminary experiments on normal human testis showed that a mixture of mouse monoclonal and rabbit polyclonal anti-securin antibodies yielded better immunohistochemical results than either antibody alone (data not shown), all stainings were performed using a 1:1 mixture of

mouse monoclonal (clone DCS-280.2; Novocastra Labs. Ltd, Newcastle-upon-Tyne, UK) and rabbit polyclonal (antibody Z23.YU; InVitrogen, Carlsbad, CA, USA) anti-securin antibodies, both diluted 1:100 in phosphate-buffered saline. The second step consisted of a mixture of anti-mouse and anti-rabbit immunoglobulins, labelled with peroxidase-conjugated dextran polymers (Envision™, Dakocytomation, Haasrode, Belgium) and enzyme activity was detected using 3-amino-9-ethylcarbazole as substrate, revealing a brightly red reaction product that contrasted well with the brown melanin. The numbers of securin-positive melanoma cells in the tissue microarrays as well as in the 29 matched pairs of melanomas were counted in a maximum of five high power fields (HPF) in the areas of the vertical growth phase with the most abundant immunoreactivity.

Semiserical sections of 44 out of 58 melanomas were immunohistochemically stained for beta-catenin (clone 14; BD Transduction Labs., Franklin Lakes, NJ, USA), bFGF (polyclonal rabbit antibody sc79; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and p53 (clone DO-7; Dakocytomation). To study the coexpression of securin and p53, a sequential double staining was performed in 10 cases with large numbers of securin-expressing cells using peroxidase-conjugated and alkaline phosphatase-conjugated Envision™ reagents in the first and second staining; substrates for enzyme activity were amino-ethylcarbazole and BCIP, respectively, yielding contrasting red and blue reaction products. Immunohistochemical controls, which were consistently negative, consisted of omission of primary antibody and use of chromogen alone; in the double staining experiments, dye swapping was carried out by a reversal of the sequence of primary antibodies.

To assess the relationship between securin expression and angiogenesis, serial sections of the tissue microarrays were stained for securin and for CD31 (clone JC7OA; Dakocytomation) in order to label microvessels. Then, the total number of securin-immunoreactive cells as well as the microvessel density was assessed over the whole surface of each core according to the standard criteria.<sup>19</sup>

To study the relationship between securin expression and aneuploidy, the number of immunoreactive melanoma cells was counted in the vertical growth phase of 27 melanocytic tumours (seven primary melanomas, 20 metastases) that had been karyotyped previously.

### DNA Cytometry

To study the relationship between securin expression and DNA ploidy, a single 8- $\mu\text{m}$ -thick section of a melanoma that had an abnormal karyotype was stained for securin using the alkaline phosphatase-conjugated Envision™ method without counterstaining. This slide was then stained by the Feulgen

**Table 1** Pertinent clinical and histological features of the 54 primary cutaneous melanomas included in the tissue microarray

Case no.	Sex	Age	Status	Type	T	Thickness (mm)	Ulceration	Regression	Mitotic rate	TILS	Mean securin
1	f	78	AwRD	1	2A	1.34	-	+	2	2	24
2	f	65	DOD	3	3A	3.74	-	-	2	1	0
3	f	54	AwMD	2	4B	5.44	+	-	2	1	3.2
4	f	57	DOD	1	1B	0.98	+	-	2	2	7
5	f	75	AwRD	2	4B	7.58	+	-	2	1	63
6	f	70	DOC	1	1A	0.82	-	+	1	1	10
7	f	66	NED	1	3A	3.32	-	-	1	1	14
8	f	67	DOD	3	4A	6.40	-	-	2	1	10
9	m	72	DOD	2	3B	3.52	+	-	2	1	13.3
10	f	89	DOD	3	4B	7.04	+	-	2	0	14
11	f	77	DOC	1	3A	2.52	-	-	2	0	0
12	f	40	DOD	2	4B	10.88	+	-	2	0	35.5
13	f	76	DOD	2	4B	14.56	+	-	2	0	1.2
14	f	54	NED	2	3B	3.65	+	-	1	2	11.5
15	m	63	NED	1	3B	3.68	+	-	2	0	0
16	f	57	NED	1	1A	0.96	-	-	1	1	4
17	m	76	DOD	1	3B	2.21	+	-	1	1	0
18	f	74	NED	1	2A	1.59	-	+	2	2	0
19	f	92	NED	2	4B	4.70	+	-	2	1	0.8
20	f	93	NED	1	4B	6.72	+	-	2	0	2.2
21	f	65	DOD	1	2A	1.19	-	+	1	0	0
22	f	58	NED	1	2A	1.92	-	-	0	2	0
23	m	71	NED	2	4B	9.28	+	-	1	0	1
24	f	34	NED	1	1A	0.94	-	+	1	1	0
25	f	56	NED	2	3A	2.08	-	-	2	1	43.8
26	f	56	DOD	2	4B	8.16	+	-	2	0	12
27	f	37	DOD	1	2A	1.20	-	+	1	1	0
28	m	58	NED	1	3A	2.42	-	-	1	0	0
29	m	63	NED	4	4A	4.16	-	+	2	0	7.8
30	m	47	LOF	1	1A	0.66	-	-	0	0	0
31	m	38	DOD	1	4A	5.02	-	-	2	0	0.2
32	f	9	NED	4	4B	4.70	+	-	2	0	0
33	m	78	DOD	3	4B	14.18	+	-	2	0	4.8
34	m	74	AwRD	1	4A	5.18	-	-	1	1	0.4
35	f	68	DOD	1	3A	2.75	-	-	1	1	0.5
36	f	64	NED	2	4B	17.12	+	-	2	2	29.2
37	f	79	AwRD	2	4B	7.52	+	-	1	1	31.5
38	m	78	DOD	1	3B	3.04	+	-	1	2	16.6
39	f	1	DOD	2	4A	12.41	-	-	2	0	227
40	f	92	DOC	1	4B	6.81	+	-	1	2	39
41	f	47	NED	1	4A	4.61	-	+	1	2	0
42	m	63	DOC	1	4A	5.95	-	+	2	1	6
43	f	46	LOF	2	3A	2.08	-	-	1	2	0
44	m	33	NED	1	2A	1.54	-	-	1	1	16.3
45	f	55	DOD	3	3A	2.30	-	-	2	0	0
46	m	22	DOD	4	3A	3.36	-	-	2	1	17
47	f	64	NED	1	2A	1.44	-	+	1	2	0
48	f	72	DOD	2	4B	7.97	+	-	2	1	0
49	f	73	DOD	3	3B	3.36	+	-	2	1	0
50	m	85	DOD	4	4A	15.64	-	-	2	2	0
51	f	67	NED	1	3A	2.05	-	+	2	1	3
52	m	64	LOF	1	2A	1.40	-	+	1	1	3
53	f	53	NED	1	2A	1.02	-	-	1	0	2.6
54	f	56	DOD	2	4B	18.56	+	-	2	0	0

Sex: f = female; m = male. Status: AwRD = alive with regional disease; AwMD = alive with metastatic disease; DOD = dead of disease; DOC = dead of other cause; NED = no evidence of disease; LOF = lost for follow-up. Type: histogenetic type; 1 = superficial spreading type; 2 = nodular type; 3 = acrolentiginous type; 4 = other types. T = tumour stage. Mitotic rate: 0 = no mitoses; 1 = <6/mm<sup>2</sup>; 2 = >6/mm<sup>2</sup>; TILS = tumour-infiltrating lymphocytes; 0 = no TILS; 1 = nonbrisk infiltrate of TILS; 2 = brisk infiltrate of TILS. Mean PTTG: mean number of cells with nuclear or cytoplasmic PTTG-staining, counted in the different cores of each melanoma.

method. Briefly, the slide was placed in 5 N HCl at 27°C for 30 min, washed 3 × 1–5 min in distilled water, stained with fresh Schiff reagent for 45 min, washed in running tap water for 15 min and washed for 1 min in aqua dest. From the same melanoma

sample, three 50-μm-thick sections were cut and processed for standard DNA flow cytometry as well as DNA image cytometry. For the last method, a cytospin specimen was prepared and stained by the Feulgen method as described above.

### Image Acquisition and Analysis of Three-Dimensional (3-D) DNA Content

Image stacks were acquired with a confocal laser scanning microscope (Leica TCS SP, Leica Microsystems, Heidelberg, Germany) fitted with  $\times 20/0.70$  NA HC Plan Apo objective. With a zoom factor of 2, final magnification was achieved of  $\times 40$ . In each field of vision, stacks of approximately 40 two-dimensional digital images ( $1024 \times 1024$  pixels) were obtained, depending on the effective thickness of the tissue sections. The bottom and top of the stack were identified interactively as the slices where only a few (cut) nuclei remained, after which image acquisition started with the lowest slice. Resolution at the specimen level was  $0.24 \times 0.24 \times 0.37 \mu\text{m}^3$ . To obtain measurements for at least 300 nuclei as previously set,<sup>20</sup> 24 fields of vision were imaged. The image stacks were analysed off-line using software developed at the Dept. Pathology, Free University of Amsterdam, Amsterdam, The Netherlands.<sup>21</sup> The DNA content of all individual nuclei was depicted in a DNA histogram (50 bins, scaling to 10c). The coefficient of variation of the diploid peak in a histogram was obtained with the MultiCycle software program (Phoenix Flow Systems, San Diego, CA, USA). It finds the best fit of several curves through the data points, including the Gaussian G0/1 and G2/M phase components. The coefficient of variation is based upon this mathematical model of the DNA content distribution.

### Mutational Analysis of TP53

Twenty 10- $\mu\text{m}$ -thick paraffin sections from 34 out of the 44 melanomas, in which p53 expression was analysed immunohistochemically, were used for DNA extraction. After deparaffination, genomic DNA was extracted using the QIAamp DNA Mini kit, according to the manufacturer's recommendations (Qiagen Benelux, Venlo, The Netherlands). For amplification of TP53 exon 7, the primer sets p53ex7s (AAGGCGCACTGGCCTCATCTTGG) and p53ex7as (AGGGGTCAGCGCAAGCAGAGG) were used, and for TP53 exon 8, the primer sets p53ex8s (ACAAGGTTGGTTGGAGTAGATGG) and p53ex8as (ACAAAGAGGCAAGGAAAGGTGATGG) were used. The PCR reactions were performed under the following conditions (FastStart High Fidelity PCR System, Roche): one denaturation step for 2 min at 95°C, 45 cycles melting at 94°C for 1 min, annealing at 66°C for the TP53 exon 7 and at 61°C for the TP53 exon 8 for 30 s and extension at 72°C for 2 min followed by a final elongation step for 10 min at 72°C (GeneAmp PCR System 2700, Applied Biosystems). ExoSapIt (GE Healthcare – Bio-Sciences) was added to the PCR reactions and incubated for 15 min at 37°C to digest the remaining primers. The reaction was stopped by incubation for 15 min at 80°C. The amplicons were forward and reverse sequenced using a cycle sequencing kit (Amersham Bio-

sciences, DYEnamic™ dye terminator kit) with the primers p53ex7s and p53ex7as for TP53 exon 7 and the primers p53ex8s and p53ex8as for TP53 exon 8. The sequencing reactions were desalted, denatured and run on an automated capillary DNA sequencing system (Amersham Biosciences, MegaBACE 500). The sequencing results were computer assembled and compared to the DNA sequence (Informax, VectorNTI) from the *Homo sapiens p53* gene (ID HSP53G, AC X54156).

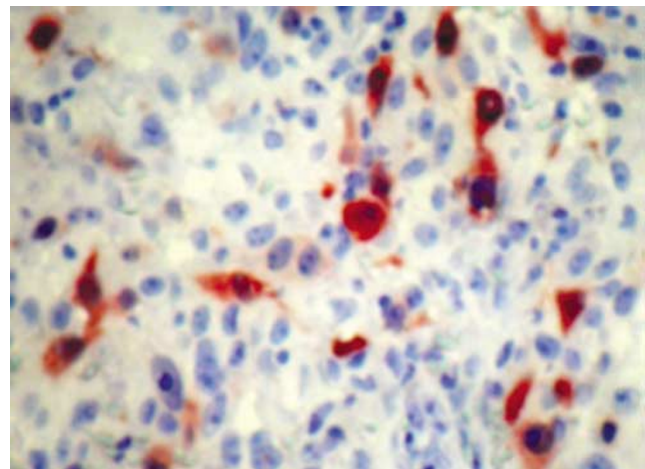
### Statistical Methods

These included paired and unpaired *t*-tests;  $R^2$ -values were computed for linear regression analyses. All *P*-values were two-sided and a significance level of 0.05 was applied.

## Results

### Expression of *hPTTG1* in Pigment Cell Lesions

Out of 54 evaluable primary melanomas in the tissue microarrays, 47 (87%) contained neoplastic cells that expressed nuclear and/or cytoplasmic securin. In 26 of these, the number of immunoreactive cells was rather small (ie below 10%), whereas in 8/52 cases, more than 50% of the neoplastic cells expressed securin. In the metastatic melanomas, only one out of 10 cases lacked securin expression; in the remaining metastases, five showed low numbers of immunoreactive cells (ie below 10%), and in three cases, more than 50% of the tumour cells expressed securin. Remarkably, immunoreactivity in both primary and metastatic melanomas occurred in scattered tumour cells and was predominantly found in large, highly atypical melanoma cells with bizarre or multiple nuclei (Figure 1). In 10 cases of melanoma *in situ*, rare securin-positive cells were found in two cases. Out of 11



**Figure 1** Securin is expressed in scattered, highly atypical and pleomorphic melanoma cells. Three-step Envision technique.

evaluable nevi, six contained rare (ie below 1%) securin-expressing cells.

A previous supervised analysis of the differences in gene expression between 49 superficial spreading melanomas and 15 nodular melanomas had revealed

that PTTG was the most significantly differentially expressed gene (unpublished results). This was confirmed in the present study by counting the number of immunoreactive cells in the 54 melanomas in the tissue microarray sections, revealing

**Table 2** Expression of securin and p53 in 29 pairs of nodular (NM) and superficial spreading melanomas (SSM), matched for thickness (column 3), ulceration (column 4), mitotic index (column 5; 1 = <1/mm<sup>2</sup>; 2 = between 1 and 6/mm<sup>2</sup>; 3 = >6/mm<sup>2</sup>), tumour-infiltrating lymphocytes (TILS, column 6; 0 = absent; 1 = nonbrisk; 2 = brisk) and regression (column 7)

Pair	Type MM	Thickness	Ulcer	Mitoses	TILS	Regression	Mean securin	Mean p53
1	NM	1.1	+	3	0	–	55.2	ND
	SSM	1.2	+	1	1	–	4.8	ND
2	NM	1.3	+	2	1	–	47.4	111.7
	SSM	1.2	+	2	1	–	3.6	8.4
3	NM	1.3	+	1	1	–	71.2	47
	SSM	1.3	+	2	1	+	18.2	4.2
4	NM	1.3	–	3	1	+	48.4	ND
	SSM	1.3	–	2	1	–	28.8	ND
5	NM	1.5	–	3	1	–	23.2	47.4
	SSM	1.5	–	2	0	–	21.2	29.3
6	NM	1.7	–	1	0	–	69.6	5.8
	SSM	1.7	–	1	1	–	11	4.7
7	NM	1.8	–	3	1	–	7	7
	SSM	1.8	–	3	1	–	64.2	202.5
8	NM	1.9	–	3	0	–	76	179
	SSM	1.9	–	1	1	–	17.6	87.6
9	NM	1.9	+	1	1	–	7	10.8
	SSM	1.9	+	3	1	–	70.2	53.5
10	NM	2.1	–	3	0	–	33.6	34.8
	SSM	2.1	–	2	0	–	29.2	11.8
11	NM	2.3	+	3	0	–	81	ND
	SSM	2.3	–	2	0	–	19	ND
12	NM	2.6	+	1	0	–	29	ND
	SSM	2.7	+	2	1	–	9	ND
13	NM	2.7	–	3	1	–	74.2	95.6
	SSM	2.7	–	1	0	–	19	27.8
14	NM	2.9	–	3	0	–	17.4	17.4
	SSM	2.8	–	1	1	+	6	0.8
15	NM	3.1	+	2	1	–	21.6	ND
	SSM	3.1	+	3	0	–	49.4	ND
16	NM	3.1	–	3	1	–	27.8	25
	SSM	3.1	–	2	1	+	33.8	68.4
17	NM	3.2	+	2	1	+	55.8	90
	SSM	3.2	+	2	1	–	60.2	55
18	NM	3.3	–	1	2	–	21.2	26.3
	SSM	3.3	–	1	1	–	11	41.5
19	NM	3.3	+	3	1	–	19.8	32.8
	SSM	3.3	+	3	1	–	48.4	46
20	NM	3.4	+	3	1	–	25	4.8
	SSM	3.6	+	2	0	–	18.2	28.8
21	NM	3.7	+	2	0	–	64.4	33
	SSM	3.7	+	3	0	–	19.8	25.2
22	NM	3.8	+	2	1	–	9.2	16.3
	SSM	3.8	+	3	1	–	36.4	50.4
23	NM	4	+	3	0	–	69.4	0.8
	SSM	4	+	3	1	–	121.4	72.3
24	NM	4	+	3	0	–	81	73.8
	SSM	4	+	3	0	–	32.6	5
25	NM	4.2	+	3	1	–	95.4	186
	SSM	4.3	+	1	1	–	29.2	2
26	NM	4.9	+	3	0	–	87.6	73.6
	SSM	5	–	2	1	–	16.4	42.2
27	NM	5.7	–	3	0	–	74.6	ND
	SSM	5.9	–	3	2	+	4.4	ND
28	NM	7.1	+	3	0	–	59.2	65.4
	SSM	6.8	+	3	0	–	40	48.8
29	NM	11.5	–	2	1	–	34.8	ND
	SSM	12	–	3	1	–	3	ND

Securin and p53-expressing cells were counted in five representative high power fields. ND = not done.

significant ( $P=0.02$ ) differences between nodular (average number of immunoreactive cells per core section = 31.5) and superficial spreading melanomas (average number = 5.5) (Table 1). However, since the nodular melanomas in the tissue microarray series had greater mean thickness than superficial spreading melanomas, we studied securin expression in a separate series of 29 pairs of nodular and superficial spreading melanomas, matched for all histological prognostic markers including tumour thickness (Table 2). Paired *t*-tests revealed significant ( $P=0.018$ ) differences in securin expression between nodular and superficial spreading melanomas (mean number of immunoreactive cells in five HPF: nodular melanoma = 48.1; superficial spreading melanoma = 29.2), whereas there was no correlation between securin expression and tumour thickness.

### *hPTTG1* and Angiogenesis in Melanoma

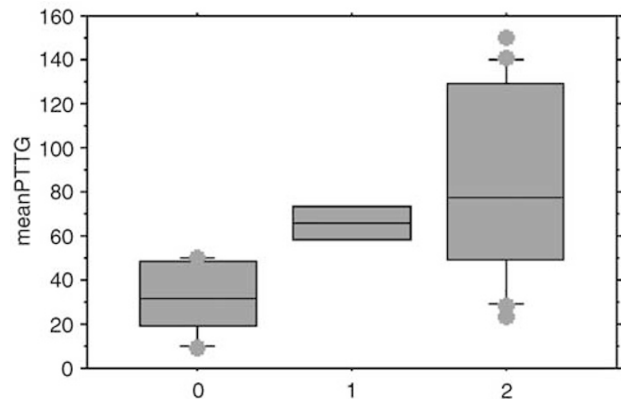
Ten malignant melanomas stained on semiserial sections for securin and bFGF showed no spatial relationship in immunoreactivity for either antibody. In addition, no correlation was found between the number of CD31-immunoreactive microvessels and the number of securin-expressing cells.

### *hPTTG1* and Beta-Catenin Expression in Melanoma

Zhou *et al*<sup>22</sup> detected cytoplasmic accumulation of beta-catenin in oesophageal carcinomas that over-expressed *PTTG1* and suggested that overexpression of *PTTG1* in these tumours was likely due to the activation of beta-catenin/*WNT* signalling. However, in 10 melanomas overexpressing securin, the patterns of expression of beta-catenin varied considerably (ie membranous staining in 6/10; nuclear staining in 2/10; nuclear and cytoplasmic staining in 2/10).

### *hPTTG1* and Aneuploidy in Melanoma

Twenty-seven karyotyped melanomas were used. Based on their karyotype, the 27 lesions could be divided into three groups, that is, a group of six lesions (four primary melanomas, two metastases) with entirely normal karyotype, a group of five cases (one primary and four metastases) with few structural abnormalities and a group of 16 lesions (two primary melanomas, 14 metastases) with highly abnormal karyotype. Comparison of the mean number of securin-expressing melanoma cells in these groups revealed significant differences between the melanomas with normal karyotype, on the one hand, and melanomas with few numerical abnormalities ( $P=0.01$ ) or melanomas with highly abnormal karyotype ( $P=0.006$ ), on the other (Figure 2).



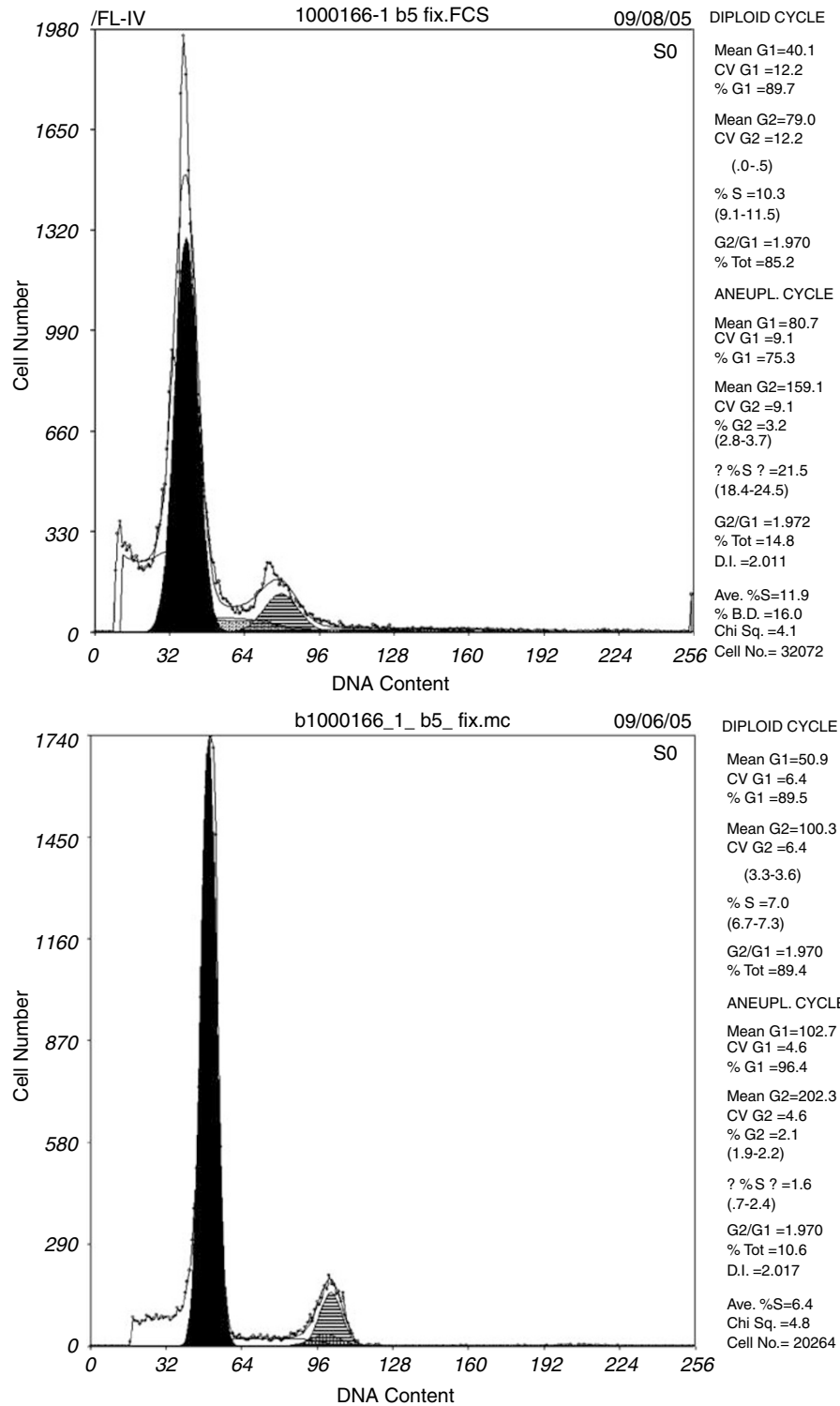
**Figure 2** Box plots of the number of securin-positive cells in melanomas with normal karyotype (0), mild karyotypic abnormalities (1) and severe aneuploidy (2). The grey box shows the limits of the middle half of the data (the black line inside the box represents the median). Whiskers are drawn to the nearest value not beyond a standard span (1.5 interquartile range) from the quartiles. Extreme points are highlighted by dots. Significant differences were found between groups 0 and 1 ( $P=0.01$ ), and between groups 0 and 2 ( $P=0.006$ ).

### *hPTTG1* and 3-D DNA Content

Both DNA flow cytometry and DNA image cytometry of the cytospin specimen showed a DNA tetraploid histogram (percentage of nuclei in G2/M larger than 10%) as shown in Figure 3. The 3-D DNA content measurements on 24 fields (representing an area of 1.5 mm<sup>2</sup>) yielded 2076 intact nuclei. In total, 76 nuclei (4% of all nuclei analysed) were securin positive. Since the image analysis software only measures DNA content of single nuclei, the complex multiple nuclei within a single cell as shown in Figure 1 were measured as single nuclei. Since double staining has been performed (securin staining did not interfere with Feulgen fluorescence), all intact nuclei that were clearly positive for securin were labelled as such in the DNA histogram (Figure 4). Securin-positive nuclei have DNA contents that partly are in the G0/G1 and G2/M range and partly beyond G2/M. Of the 76 securin-positive cells, 49 (64%) fall outside the G0/G1 or G2/M phase of a normal cell cycle. These cells in the G0/G1 and G2/M range are either DNA aneuploid or belong to the S phase of the normal cell cycle; the nuclei beyond G2/M are aneuploid.

### *hPTTG1* and p53 in Melanoma

Of the 58 melanomas previously studied for securin expression, 44 cases were stained for p53 and the number of immunoreactive cells was counted in the same way as had been done for securin. In addition, DNA was amplified from 34 melanomas and studied for mutations in codons 7 and 8 of *TP53*. For all melanomas, the number of neoplastic cells with



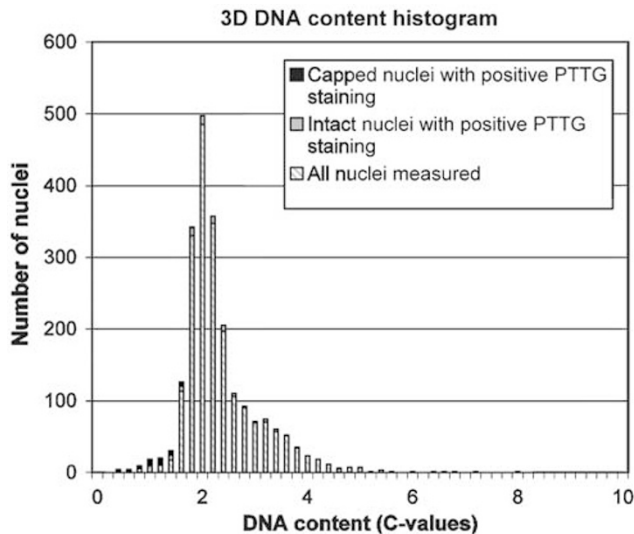
**Figure 3** DNA flow cytometry (top) and image cytometry (bottom) histograms from a melanoma. The first peak is defined as the diploid cell population and the second peak represents the tetraploid cell population.

nuclear p53 accumulation correlated well with the number of securin-positive cells ( $R^2 = 0.34$ ;  $P < 0.001$ ); this remained significant when only NM ( $R^2 = 0.39$ ;  $P = 0.001$ ) or SSM ( $R^2 = 0.25$ ;  $P = 0.01$ ) were considered. Mutational analysis of 34 out of

these 44 melanomas revealed no mutations in codons 7 and 8 of *TP53*.

In 10 melanomas, sequential double staining for securin and p53 was performed. In each of these cases, the majority of securin-positive neoplastic

cells lacked nuclear p53, and the majority of scattered melanoma cells with nuclear accumulation of p53 apparently lacked securin overexpression (Figure 5a). Dye-swapping experiments showed the same results (Figure 5b). In four cases however, scattered melanoma cells were found to exhibit a purple colour; computer-assisted elimination of either red or blue colours revealed coexpression of cytoplasmic securin and nuclear p53 in the same melanoma cells (Figure 6).

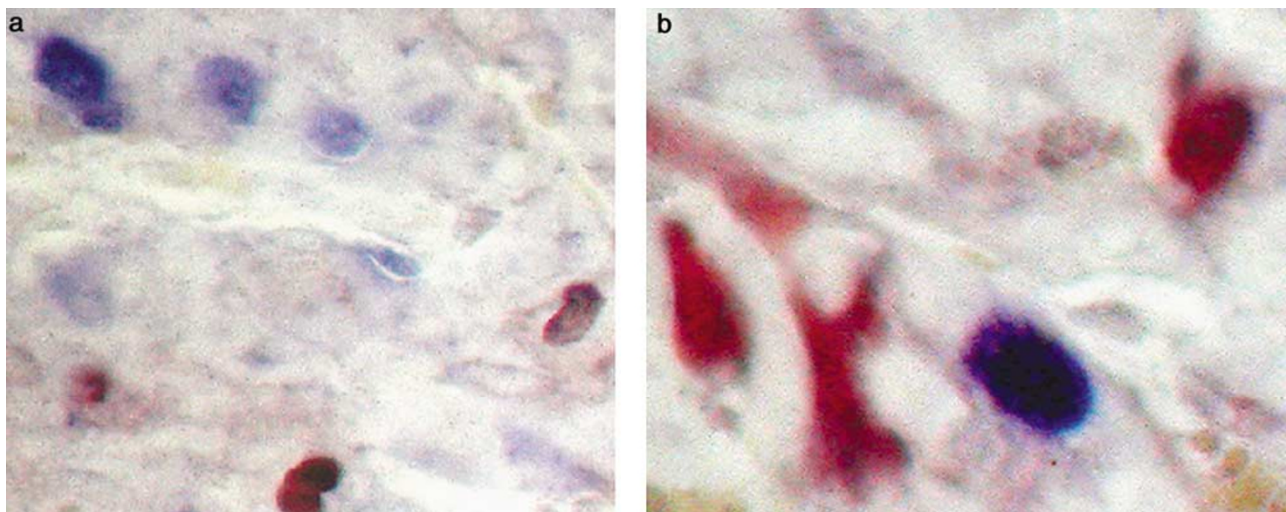


**Figure 4** 3-D DNA content histogram based on 2076 nuclei measured. On top of this histogram, the nuclei that belong to PTTG/securin expressing cells are shown (76 complete nuclei and 37 capped nuclei). The DNA diploid peak has a coefficient of variation of 8.5%. Intact nuclei that show positive staining for PTTG are spread over the histogram, within the diploid (G0/G1 phase) as well as the tetraploid (G2/M) as the S phase or aneuploid region.

## Discussion

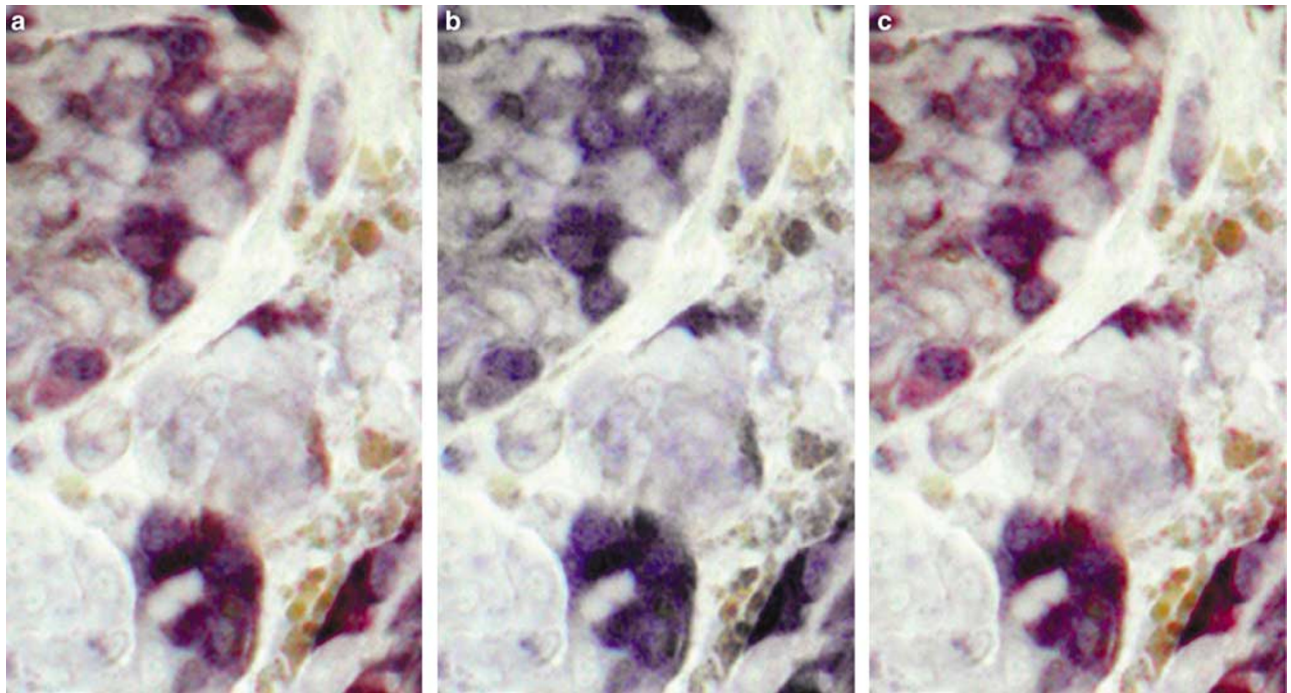
Using immunohistochemistry, we have shown that the vast majority of primary cutaneous melanomas show overexpression of securin in scattered neoplastic cells. Since immunoreactivity was rare in the early, radial growth phase in contrast to the vertical growth phase, our data indicate that securin is involved in tumour progression, rather than in the development and early growth of melanoma. Since *hPTTG1* is a proto-oncogene by virtue of its transforming capacity and ability to induce tumours in nude mice,<sup>23,24</sup> our results not only add melanoma to the list of neoplasms in which *hPTTG1* overexpression is observed, but also add *hPTTG1* to the list of oncogenes that may play a role in progression of melanoma.

As shown by our microarray and immunohistochemical data, overexpression of securin was particularly observed in the nodular subtype of melanoma. Nodular melanomas differ from superficial spreading melanomas in various aspects, for example, relation to sun exposure, anatomical distribution and histological and immunohistochemical features. On morphology, nodular melanomas show an invasive, vertical growth from the beginning in contrast to superficial spreading melanomas that initially spreads in the epidermis in the form of a radial growth phase. On immunohistochemistry, the overexpression of VEGF<sup>25</sup> in nodular melanoma has been inferred to explain the poorer prognosis of this subtype. Genetically, nodular melanomas carry more frequently extra *CMYC* copies<sup>26</sup> and are characterised by deletions in 1p36 and aberrations of chromosome 10.<sup>27</sup> Finally, nodular melanoma has been claimed to exhibit more prominent aneuploidy.<sup>28</sup> Here, we show that both clinicopathological subtypes of melanoma also differ in the expression of securin.



**Figure 5** Double staining for p53 and securin. Nuclear p53 (in red in a; in blue in b) and nuclear/cytoplasmic securin (in blue in a; in red in b) were found in distinct, nonoverlapping cells.





**Figure 6** Double staining for p53 (blue) and securin (red) reveals purple, double-stained cells (a); when red (b) or blue (c) is removed, several melanoma cells can be seen to coexpress nuclear p53 (blue in b) and cytoplasmic securin (red in c).

Analysis of our previous oligonucleotide microarray data revealed that *hPTTG1* overexpression in the vertical growth phase of malignant melanoma was significantly associated with shortened 4-year DMFS.<sup>18</sup> These data are in line with studies on breast, colorectal and thyroid carcinomas in which overexpression of *PTTG* is associated with early recurrence, or metastatic spread and thus with a poor prognosis.<sup>8,15,16</sup>

In normal cells, the securin protein is expressed in a cell-cycle-dependent manner and regulates sister chromatid separation to opposite poles of the cell during anaphase.<sup>4</sup> Following cytoplasmic to nuclear translocation, it peaks in the G2/M phase and subsequently is ubiquitinated and degraded in the proteasome. The process of sister chromatid segregation is tightly regulated and ensures that a complete set of chromosomes is transmitted from one generation to another. It is triggered by a conserved cysteine proteinase called separase, which is responsible for the cleavage of Scc1, which forms the bridge between the sister chromatids. During most of the cell cycle, separase is inactive due to its binding to securin. At the metaphase-to-anaphase transition, securin is degraded by a multi-subunit protein ligase, the anaphase-promoting complex (APC) or cyclosome (APC/C) that is bound to accessory factors Cdc20 and Cdh1.<sup>4</sup>

Overexpression of securin may be caused by several mechanisms. First, genomic aberrations in *hPTTG1* (promoter mutations, amplification) have as yet not been detected, or do not play a major role

in the enhanced transcription or insufficient degradation of securin.<sup>29</sup> Second, somatic mutations in one or more components of *APC* have been reported in breast<sup>30</sup> and colon cancer,<sup>31</sup> suggesting that the cause of securin overexpression in these cancers is due to insufficient degradation. Although the predominant cytoplasmic localisation of securin in melanomas as well as in other neoplasms<sup>5</sup> is in agreement with this hypothesis, mutations affecting *APC* genes (*APC3*, *APC6* and *APC8*) have not been found in a number of melanoma cell lines (A Puisieux, personal communication, Lyon, France).

Third, securin overexpression may be the result of enhanced transcription due to the activation of several signalling pathways, which are known to promote tumorigenesis in melanoma. In this respect, insulin-like growth factor I may be of possible interest. Chamaon *et al*<sup>32</sup> showed that insulin-like growth factor I and insulin as well as their downstream effectors, that is, the phosphoinositol-3 kinase and mitogen-activated protein kinase signalling pathways regulate *hPTTG1* transcription in both normal and neoplastic human cells. Insulin-like growth factor I is one of the most critical proteins required for the migration and growth of melanoma cells.<sup>33</sup> Its binding protein is upregulated in a dose-dependent manner in melanomas and its expression increases from common nevi to dysplastic nevi and primary and metastatic melanoma,<sup>34</sup> implying a role in the tumour progression of melanoma.<sup>35</sup> Clearly, further studies are necessary to investigate the role of insulin-like

growth factor I in the *hPTTG1* overexpression in melanoma.

Irrespective of its cause, overexpressed securin is capable of acting as an oncoprotein. Recent molecular studies have suggested different mechanisms by which *hPTTG1* overexpression promotes tumorigenesis. By inhibiting chromosome segregation and thus disruption of mitosis, *hPTTG1* has been shown to generate aneuploidy, a ubiquitous feature of human solid tumours that causes genetic instability and also promotes further aneuploidy.<sup>12,36</sup> Aneuploidy is a common feature of tumours overexpressing *PTTG*, and in breast cancer, the largest number of securin-positive cells was found in the tumours with the highest degree of pleomorphism.<sup>6,16</sup> We also found securin expression preferentially in pleomorphic melanoma cells with highly atypical or multiple nuclei. We studied the ploidy status in relation to *hPTTG1* overexpression using a series of melanomas that had been karyotyped, and observed a significantly higher number of securin-positive cells in aneuploid cases. Moreover, analysis of the DNA content in securin-positive nuclei in one case showed a substantial number of these to reside within the aneuploid subset, or to show tetraploidy, which has been shown to precede aneuploidy.<sup>37</sup>

In addition to aneuploidy, many studies have suggested an important role for *hPTTG1* in angiogenesis through the induction of bFGF.<sup>11</sup> Forced overexpression of *hPTTG1* in human embryonic kidney cells or NIH3T3 fibroblasts leads to an increased expression of angiogenic factors such as bFGF, VEGF and interleukin 8.<sup>4,11,38</sup> These factors may act as effectors for securin-driven angiogenesis since they contain a proline-rich SH3 domain that binds to the C-terminal double PXXP motif of securin, a putative SH3-interacting domain.<sup>39</sup> Ishikawa *et al*<sup>11</sup> showed that *hPTTG1* induces an angiogenic phenotype in both *in vitro* and *in vivo* angiogenesis models. Highly vascularised colorectal cancers express high levels of securin,<sup>8</sup> and in breast carcinoma, securin-mediated angiogenesis may contribute to an invasive breast tumour phenotype.<sup>6</sup> We could not confirm these results as we did not find a correlation between securin expression, on the one hand, and bFGF immunoreactivity or microvessel density, on the other. Therefore, our results suggest that mechanisms other than angiogenesis are operating in *hPTTG1*-overexpressing melanomas.

Using phage display screening, Bernal *et al*<sup>14</sup> identified interaction between securin and p53 *in vitro* and *in vivo*. In breast cancer cells, securin blocks the specific binding of p53 on DNA, thereby inhibiting its transcriptional activity and the ability to induce cell death. In a lung tumour cell line, overexpression of both *hPTTG1* and *TP53* resulted in the downregulation of p53-regulated apoptosis together with the downregulation of the p53-induced expression of downstream genes, including Bax, suggesting that in these cells, *hPTTG1* inhibits the function of p53 rather than its expression. In our

series of melanomas, nuclear accumulation of p53 was a common finding, although bidirectional DNA sequencing revealed no mutations in codons 7 and 8 of *TP53*. These findings are in line with those reported in the literature.<sup>40</sup> Our results showed that the number of securin- and p53-positive cells are significantly correlated, and that in some melanomas, overexpression of both proteins occurred in the same neoplastic cells. Although we have no firm *in vitro* data, we can speculate that, in analogy with other cancers, *hPTTG1* overexpression in melanoma modulates the transcriptional activity of p53, resulting in impairment of apoptosis. This oncogenic pathway of p53 inhibition may explain the common finding of nuclear accumulation of p53 without concomitant mutation in malignant melanoma.

In conclusion, we have reported the overexpression of *PTTG* in the vertical growth phase of malignant melanoma, and suggest a role of this oncogene in the progression of nodular melanoma, either through induction of aneuploidy or through modulation of the function of p53.

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