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Characterization of candidate gene copy number alterations in the 11q13 region along with *BRAF* and *NRAS* mutations in human melanoma

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Amplification of the 11q13 chromosomal region is a common event in primary melanomas. Several candidate genes are localized at this sequence; however, their role in melanoma has not been clearly defined. The aim of this study was to develop an accurate method for determining the amplification pattern of six candidate genes that map to this amplicon core and to elucidate the possible relationship between *BRAF*, *NRAS* mutations and *CCND1* copy number alterations, all of which are key components of the MAP kinase pathway. Characterization of gene copy numbers was performed by quantitative PCR and, as an alternative method, fluorescence *in situ* hybridization was used to define the *CCND1* amplification pattern at the single cell level. Samples with amplified *CCND1* (32%) were further analyzed for copy number alterations for the *TAOS1*, *FGF3*, *FGF19*, *FGF4* and *EMS1* genes. Coamplification of the *CCND1* and *TAOS1* was present in 15% of tumors and was more frequent in ulcerated lesions ($P = 0.017$). Furthermore, 56% of primary melanomas had either *BRAF* or *NRAS* mutations, but these two mutations were not present in any of the lesions analyzed. Of these cases, 34% also had *CCND1* amplification. There was a significant relationship between *NRAS* activating mutations and UV exposure ($P = 0.005$). We did not find correlations between *CCND1* gene amplification status and any of the patients' clinicopathological parameters. However, *CCND1* amplification simultaneously with either *BRAF* or *NRAS* activation mutations was observed mainly in primary tumors with ulcerated surfaces ($P = 0.028$). We assume that coamplification of these candidate genes in the 11q13 region or *CCND1* gene alterations along with either *BRAF* or *NRAS* mutations might be more important for prognosis than the presence of these alterations alone.
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Malignant melanoma is the most aggressive form of skin cancer and has a multifactorial etiology. It is assumed that the disease progresses toward aggressive metastatic disease via the accumulation of nonrandom multiple genetic aberrations.[1]

Earlier, using comparative genomic hybridization (CGH) analysis, we and others found frequent amplification of the 11q13–q21 chromosome band in primary melanomas.[2–4] The Cyclin D1 (*CCND1*) gene has been widely considered to be a target gene in the region because its overexpression is commonly observed in several human cancers, including breast, head and neck, and bladder cancers.[5–7] Beside the *CCND1*, several other oncogenes and/or

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cancer-related genes reside within this amplicon core, such as *TAOS1*, *FGF3*, *FGF4*, *FGF19* and *EMS1*.[8–13]

CCND1 is the regulator subunit of cyclin-dependent kinase 4 (*CDK4*) and cyclin-dependent kinase 6 (*CDK6*). These enzymes are responsible for phosphorylating the retinoblastoma (Rb) protein and thus promoting cell entry into mitosis. Transcription of *CCND1* is stimulated by the MAP kinase pathway, and this is the connection point between the RAS-MAPK and p16-CCND1/CDK4-Rb pathways.[14,15] Elevated *CCND1* gene copies were found in a large series of acral melanoma subtypes, occasional amplification was described in lentigo maligna melanomas and superficial spreading melanomas (SSM), and only sporadic amplification in the nodular melanoma subtype (NM).[16,17]

The contribution of other genes in the 11q13 amplicon to several cancers has been investigated.[8–13] The *TAOS1* gene, which is located approximately 12 kb distal to *CCND1*, was found to be a novel marker for advanced esophageal squamous cell carcinoma and was overexpressed only if its gene amplification was present in the oral squamous cell carcinoma cell lines.[9,13] Amplification of the *EMS1* gene through the overexpression of cortactin protein has been shown to contribute to the invasive potential of tumor cells.[11,18] This protein is described as an actin-associated scaffolding protein that regulates the formation of actin-based structures closely associated with cell motility.[19] Overexpression of *EMS1* causes enhanced cell proliferation, predicts early recurrence and reduced survival in squamous cell carcinoma of the head and neck and in esophageal adenocarcinoma.[8,20,21] *CCND1* and *EMS1* are also thought to be likely to play pathogenic roles in the 11q13 amplicon in bladder tumors.[22] Coamplification of *CCND1, EMS1*, *FGF3* and *FGF4* genes was significantly associated with increased tumor stage and grade in a large series of urinary bladder cancer.[12] However, the possible role of these candidate genes within the 11q13 amplicon has not yet been investigated in primary melanoma.

Activating mutations in codon 600 of *BRAF* and in codon 61 of *NRAS* are, so far, the most common single mutations detected in human cutaneous melanoma.[23,24] Drugs targeting this pathway are, therefore, the most attractive clinical agents for the disease.[25] A subset of *BRAF* mutant melanomas are sensitive to these agents, examples of which are SB590885 (GlaxoSmithKline, Collegeville, PA, USA) and PLX-4032/PLX-4720 (Plexxikon, Berkley, CA, USA).[26–28] Identifying concomitant genetic alterations may help predict the response or resistance to *BRAF* and *NRAS* inhibitors.

One of the objectives of our study was to investigate gene copy number alterations in the 11q13 amplicon in primary and metastatic melanomas by performing Q-PCR analysis. First we focused on the detection of *CCND1* copy number alterations. All samples with amplified *CCND1* were further examined for copy number alteration of *TAOS1*, *FGF3*, *FGF4*, *FGF19* and *EMS1*. Although it is well documented that there is a high prevalence of *BRAF* (V600) and *NRAS* (Q61) mutations in melanoma, there are a few studies with discordant findings. These studies examined *CCND1* copy number alterations in conjunction with *BRAF* and *NRAS* mutational status of the disease. Another aim of our study was to elucidate the possible relationship between these oncogenes and their combined incidence and clinicopathological parameters. Furthermore, we compared *CCND1* copy number alterations as classified by real-time quantitative PCR (Q-PCR) with those classified by interphase fluorescence *in situ* hybridization (FISH) at a single cell level.

Materials and methods

Tumor Samples and DNA Isolation

Tumors were collected in the Department of Dermatology, Medical and Health Science Center, University of Debrecen, Hungary. The study was approved by the Regional and Institutional Ethics Committee, Medical and Health Science Center, University of Debrecen, and conducted according to regulations. Written informed consent was always obtained from the patients. Tumor diagnosis was carried out on formalin-fixed paraffin-embedded tissue sections using hematoxylin and eosin staining. Melanoma tumor staging was determined according to the new TNM staging system.[29] The distinction between tumor groups arising from chronically sun-exposed site or intermittently sun-exposed site was based on the presence or absence of solar elastosis of the dermis surrounding the melanomas.

Clinicopathological data from the 68 primary tumors are summarized in Table 1. DNA from peripheral blood cells of healthy individuals and DNA from a melanoma cell line (M24) were also included into the analysis. Tumor DNA was extracted using the G-spin Genomic DNA Extraction Kit following the instructions of the manufacturer (Macherey-Nagel, Düren, Germany). NucleoSpin Extract II kit (Macherey-Nagel) was used to purify DNA if needed. Only high quality DNA template was accepted, with an absorbance ratio range of 1.8–1.9 (260–280 nm) or 1.7–2.5 (260–230 nm), as measured by the NanoDrop 1000 instrument (NanoDrop Technologies, Wilmington, De, USA). Control DNA was extracted from peripheral blood cells using the Nucleo Spin Blood mini kit according to the manufacturer's instructions (Macherey-Nagel). All DNA samples were stored at -20°C.

Table 1 Associations of *CCND1* amplification, *CCND1/TAOS1* coamplification, *BRAF* and *NRAS* mutation with clinicopathological parameters of patients

	CCND1[a]; n/*total (%)*	P	*CCND1/TAOS1*[b]; n/*total (%)*	P	*BRAF*+[c]; n/*total (%)*	P	*NRAS*+[d]; n/*total (%)*	P
All tumor	22/68 (32)	—	10/68 (15)	—	25/68 (37)	—	13/68 (19)	—
Tumor type								
NM	10/26 (39)	0.433	4/26 (15)	1.000	9/26 (35)	0.802	7/26 (27)	0.220
SSM	12/42 (29)		6/42 (14)		16/42 (38)		6/42 (14)	
Gender								
Male	13/33 (39)	0.302	5/33 (15)	1.000	13/33 (39)	0.802	6/33 (18)	1.000
Female	9/35 (26)		5/35 (14)		12/35 (34)		7/35 (20)	
Age								
20–50	6/18 (33)	1.000	4/18 (22)	0.437	5/18 (28)	0.407	1/18 (6)	0.160
>50	16/50 (32)		6/50 (12)		20/50 (40)		12/50 (24)	
Breslow thickness[e] *(mm)*								
<2.00	8/27 (30)	0.430[f]	3/27 (11)	0.079[f]	7/27 (26)	0.801[f]	5/27 (19)	1.000[f]
2.01–4.00	3/13 (23)		0/13 (0)		7/13 (54)		3/13 (23)	
>4.00	11/28 (39)		7/28 (25)		11/28 (39)		5/28 (18)	
Clark's level								
I, II, III	8/31 (26)	0.313	2/31 (7)	0.097	11/31 (36)	1.000	4/31 (13)	0.354
IV, V	14/37 (38)		8/37 (22)		14/37 (38)		9/37 (24)	
Ulceration								
Absent	7/31 (23)	0.129	1/31 (3)	0.017	9/31 (29)	0.313	5/31 (16)	0.758
Present	15/37 (41)		9/37 (24)		16/37 (43)		8/37 (22)	
Anatomic site								
Intermittently sun-exposed site	17/53 (32)	1.000	7/53 (13)	0.680	21/53 (40)	0.545	6/53 (11)	0.005
Chronically sun-exposed sites	5/15 (33)		3/15 (20)		4/15 (27)		7/15 (47)	
Metastasis formation[g]								
Nonmetastatic	7/25 (28)	0.406	2/25 (8)	0.273	8/25 (32)	1.000	4/25 (16)	0.350
Metastatic	13/32 (41)		7/32 (22)		11/32 (34)		9/32 (28)	

NM, nodular melanoma; SSM, superficial spreading melanoma.
[a]Number of tumors with *CCND1* amplification.
[b]Number of tumors with *CCND1/TAOS1* coamplification.
[c]Number of tumors with *BRAF* codon 600 mutation.
[d]Number of tumors with *NRAS* codon 61 mutation.
[e]Thickness categories based on the current melanoma staging system.
[f]<4.00 *versus* >4.00 mm.
[g]Only patients with at least 3 years of follow-up were included.

FISH Analysis of *CCND1*

FISH was performed on tumor imprint preparations using the LSI Cyclin D1 (11q13) SpectrumOrange/CEP 11 SpectrumGreen Probe (Vysis, Downers Grove, USA) as described previously.[30] This is a mixture of two probes: the *CCND1* probe is approximately 300 kb long, containing the *CCND1* gene, and the second probe is specific for the D11Z1 alpha satellite centromeric repeat of chromosome 11. *CCND1* copy number alterations were determined with both FISH and Q-PCR methods in 35 melanoma samples.

Primer Design

The primers were designed for the genes *GNS*, *UBE2E1*, *FGF3*, *FGF4*, *FGF19*, *EMS1* and *TAOS1* using Primer Express 2.0.0 software (Applied Biosystems, Foster City, USA) and Primer3 (Whitehead Institute, Cambridge, USA; http://biotools.idtdna.com/primerquest/; Table 2). To avoid the secondary structures, we used the web-based MFOLD version 3.2 software (http://www.bioinfo.rpi.edu/applications/mfold/).[31] The sequences of the primers for the *CCND1* gene were downloaded from the RTPrimerDB database (http://medgen.ugent.be/rtprimerdb/).[32] Primers were purchased from Biocenter Kft. (Szeged, Hungary).

Real-Time Quantitative PCR Analysis

Quantification of gene copy number was performed on 68 primary melanoma tissues and 6 melanoma

Table 2 Sequences of oligonucleotides used for this study

Gene	*Forward primer (5′→3′)*	*Forward primer (5′→3′)*
GNS	TCCAACTTTGAGCCCTTCTT	CGTTCCATGGATGTTGAAGT
UBE2E1	GGTGGGAAGTATTGCCACTCA	GTGAAACCCCAATTTATGTAGCGTAT
CCND1	GCTCCTGGTGAACAAGCTCAA	TTGGAGAGGAAGTGTTCAATGAAA
TAOS1	TGCACGCACCTGTTTAAATTTTC	TTGGAGAGGAAGTGTTCAATGAAA
FGF3	GGGAACGCGAGTCCCTTTA	CCTTTTGTTGGCGAACCGT
FGF19	CGGATCTCCTCCTCGAAAGC	CCACTGTGGATTGCTCAGAGC
FGF4	CAACGCCTACGAGTCCTACA	AGGAAGTGGGTGACCTTCAT
EMS1	CAAGCTGAGGGAGAATGTCTT	TTGTTCCACACCAAATTTCC
BRAF V600	CTCTTCATAATGCTTGCTCTGATAGG	TAGTAACTCAGCAG-CATCTCAGG
NRAS Q61	CACCCCCAGGATTCTTACAGA	GATGGCAAATACACAGAGGAAGC
Fluorescence probes	*Sensor (5′→Fluoroscein-3′)*	*Anchor (5′-LCRed640→Phosphate-3′)*
BRAF V600	AGCTACAGTGAAATCTCGATGGAG	GGTCCCATCAGTTTGAACAGTTGTCTGGA
NRAS Q61	ATACAGCTGGACAAGAAGAG	AGTGCCATGAGAGACCAATACATGAGGA

Underlined sequences correspond to *BRAF* codon 600 and *NRAS* codon 61, respectively.

metastases using Q-PCR. We aimed to quantify the relative amounts of the target genes, all located in the 11q13 amplicon core, using two reference genes for normalization, *GNS* (12q14.3) and *UBE2E1* (3p24.2). These reference genes have not yet been reported as having genetic abnormalities in melanomas analyzed by array CGH. To screen the copy number changes, two reference genes are needed to produce a robust, reliable and accurate quantification.[33–36] Reactions were carried out using an ABI Prism 7000 sequence detector (Applied Biosystems).

The amplification mixtures (25 μl) contained 1 μl template DNA ($\sim$10 ng/μl), 12.5 μl Power SYBR-Green PCR Master Mix (Applied Biosystems) and 100 nM of each primer for *TAOS1*, *FGF3*, *FGF19*, *UBE2E1* and *FGF4,* 200 nM of the primer for *CCND1* and 300 nM of the primers for *EMS1* and *GNS*. The reactions were performed under the following conditions: 10 min of polymerase activation at 95°C then 40 cycles at 95°C for 15 s and 60°C for 1 min. Assay for each gene included: (1) no template control (in duplicate), (2) 10 ng of calibrator DNA (Applied Biosystems; in triplicate) and (3) approximately 10 ng of tumor DNA (in triplicate). A melting curve analysis was run after the amplification was completed and consisted of a 20 min slow ramp from 60 to 92°C using an ABI PRISM 7000 Sequence Detection System (Applied Biosystems). The derivative melting curves showed single melting peaks, which confirmed the high Q-PCR specificity (absence of primer dimers and other nonspecific products).

Quantification and Data Analysis

Quantification was performed using the Pfaffl method.[37] This requires the efficiency to be known, which is determined from standard curves. To evaluate more accurately the PCR reaction efficiency, five-point standard curves from three different fourfold dilutions (ranging from 80.0 to 0.3 ng; in triplicate) were set up using melanoma cell line DNA (M24) for the different target genes and control DNA for the reference genes. To calculate the PCR amplification efficiency ($E = 10^{-1/\text{slope}} - 1$) we calculated the slope value from the three separated standard curves for the target genes and reference genes as well. Only standard curves R^2 value >0.99 were accepted (Table 3). Instead of interpolating unknown samples from a standard curve, we calculated the relative copy number (Ratio) solely based on the observed C_T values (equation (1))

$$\text{Ratio} = \frac{(1+E_{\text{target gene}})^{-\Delta C_{\text{T target gene}}}}{\sqrt{(1+E_{\text{reference gene1}})^{-\Delta C_{\text{T reference gene1}}} \times (1+E_{\text{reference gene2}})^{-\Delta C_{\text{T reference gene2}}}}} \quad (1)$$

$E_{\text{target gene}}$ is the efficiency of the PCR reaction for the target gene, $E_{\text{reference gene}}$ is the efficiency of the PCR reaction for the reference gene, $\Delta C_{\text{T target gene}}$ is the difference in threshold cycle value between the test sample and calibrator sample for the target gene, $\Delta C_{\text{T reference gene}}$ is the difference in threshold cycle value between test sample and calibrator sample for reference gene.

The equation was applied to calculate the standard error (s.e.) of the relative copy number of the normalized target gene, as described Hoebeeck *et al*.[35] To determine whether the copy numbers of the investigated genes were significantly different from those of the controls, we determined a tolerance interval (TI) for the relative gene copy number, using the mean standard deviation (s.d.) of the ΔC_T values for the target and reference genes in 12 healthy individuals according to the equation $\text{TI} = 2 \pm 2 \times \text{s.d.}\Delta C_T$, as described elsewhere.[38] The TI range was 1.28–2.72 for *CCND1*, 1.42–2.58 for

Table 3 The calculated PCR amplification efficiencies

Gene	R^2 *value*	*Linear regression equation*	*95% confidence intervals, slope*	*Mean efficiency* ($E = 10^{-1/slope} - 1$)
GNS	0.9977	$Y = -3.38 \times X + 27.08$	−3.51 to −3.25	0.98
UBE2E1	0.9974	$Y = -3.47 \times X + 30.10$	−3.61 to −3.33	0.94
CCND1	0.9960	$Y = -3.52 \times X + 27.94$	−3.79 to −3.25	0.92
TAOS1	0.9970	$Y = -3.79 \times X + 28.96$	−3.95 to −3.64	0.84
FGF3	0.9966	$Y = -3.49 \times X + 28.74$	−3.65 to −3.32	0.94
FGF19	0.9977	$Y = -3.92 \times X + 28.90$	−4.08 to −3.77	0.80
FGF4	0.9978	$Y = -3.69 \times X + 28.56$	−3.83 to −3.55	0.80
EMS1	0.9971	$Y = -3.99 \times X + 28.34$	−4.39 to −3.60	0.78

TAOS1, 1.18–2.82 for *FGF3*, 1.10–2.90 for *FGF4*, 1.13–2.87 for *FGF19* and 1.04–2.96 for *EMS1.* If the calculated copy number ± s.e. exceeded the calculated upper limit, the gene(s) in the tumors were considered to be amplified.

Detection of *BRAF* and *NRAS* Mutations

Analysis of mutations in the *BRAF* codon 600 and in the *NRAS* codon 61 was performed on LightCycler real time PCR System (Roche Diagnostics, GmbH, Mannheim, Germany) by melting curve analysis using fluorescent probes. All primers and probes were purchased from TIB Molbiol (Berlin, Germany; Table 2). Amplification was performed in glass capillaries using 50 ng of sample DNA in a 10 μl volume containing 1 μl 10 × LightCycler FastStart DNA Master HybProbe (Roche Diagnostics), 0.8 μl 25 mM $MgCl_2$, 0.75 μl DMSO, 1 μl (5 μM) forward and reverse primer and 1 μl (2 μM) of the anchor and sensor hybridization probe.

The reaction was performed under the following condition: initial denaturation at 95°C for 10 min and 45 cycles of amplification consisting of denaturation at 95°C for 0 s, annealing at 52°C for 10 s and amplification at 72°C for 20 s. Melting curve analysis was done as follows: PCR products were denatured for 1 min at 95°C then cooled down to 40°C for 1 min and warmed up to 72°C (ramping at 0.1°C/s) with continuous fluorescence detection before by a final cooling step at 40°C for 30 s. The accuracy of the method was confirmed by direct sequencing (BIOMI Kft., Gödöllő, Hungary) of PCR products that showed deviation from the wild-type (WT) genomic DNA melting peak (Figure 1).

Statistical Analysis

Statistical tests were performed using STATA version 9.0 (StataCorp LP, TX, USA). Categorical variables were compared by Fisher's exact test and continuous variables by the Mann–Whitney *U*-test. Correlations between the results of Q-PCR and FISH analysis were defined by calculating the Spearman's rank correlation (r_s). Results were considered significant if $P < 0.05$.

Results

Tumor Samples

A total of 74 fresh or frozen tissue samples were obtained from 70 patients (34 men and 36 women), of which 68 were primary melanomas and 6 were melanoma metastases, including 4 primary tumor-metastasis pairs. The number of patients with at least 3 years of follow-up was 57. If the follow-up period was less than 3 years, clinical data for metastasis formation were not included in Table 1, which summarizes the clinicopathological data.

Confirmation of Reference Genes Suitable for Normalization

To check the validity of the genes used as reference genes (*GNS*/*UBE2E1*), we determined the copy number ratio of the genes in 14 normal control DNA samples and 74 tumor samples. The ratio for normal and tumor DNA were 0.92 (s.d. = 0.072) and 0.96 (s.d. = 0.205), respectively. The copy number ratios of the two reference genes in the normal and tumor DNA were similar ($P = 0.7236$, Mann–Whitney test).

Q-PCR Intra- and Interassay Variability

To determine the intra- and interassay variability of the Q-PCR assay, the mean coefficient of variance (CV) and mean standard deviation (s.d.) were determined for each gene, calculated from samples run in triplicate using the transformation of C_T to the linear 2^{-C_T} value. Overall, the intraassay variability was 8% (s.d. = 0.11) and the interassay variability was 25% (s.d. = 0.38; Table 4). Samples were retested if the CV was higher than 20%.

CCND1 Amplification in Melanoma Samples Detected by Q-PCR

A total of 68 primary and 6 metastatic lesions were tested to identify *CCND1* amplification by Q-PCR relative to the two reference genes (*GNS* and *UBE2E1*). Using this technique, we found *CCDN1* amplification in 22 primary melanomas (32%) and 1 metastasis

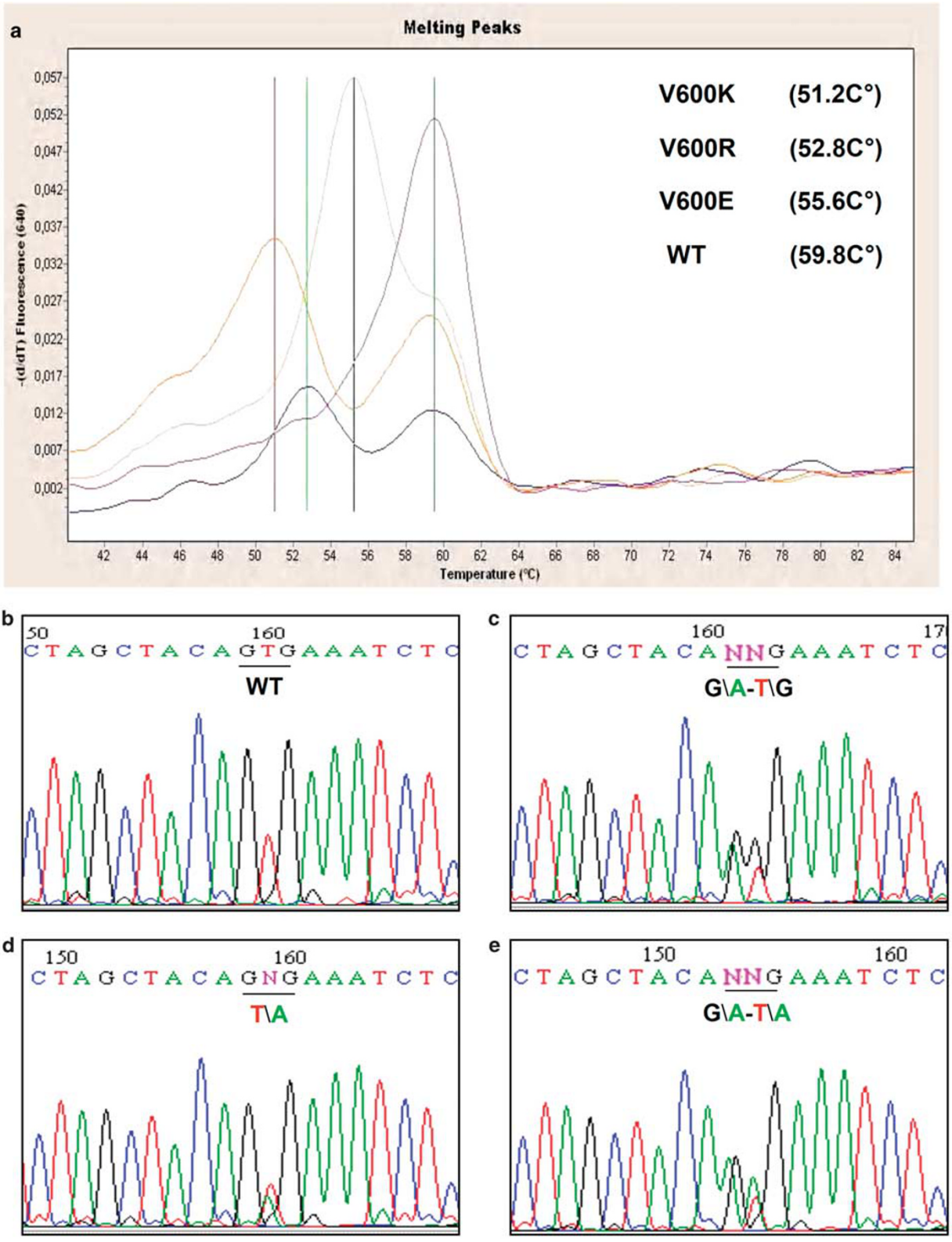


Figure 1 LightCycler melting curves and sequence traces of different *BRAF* types. (**a**) Typical results of melting curve analysis, (**b**) *BRAF* WT sequence (codon 600 is underlined) (**c**) V600R mutation (GT1798-99AG double base pair substitution), (**d**) V600E mutation (T1799A base pair substitution), (**e**) V600K mutation (GT1798-99AA double base pair substitution).

(Table 1). We were able to compare the *CCND1* gene amplification pattern in the primary and its metastatic tumor in four cases: the *CCND1* gene was amplified in all the primary lesions (case numbers 5, 22, 8 and 13; Table 5) but we could detect *CCND1* amplification only one metastasis (data not shown).

Table 4 Coefficient of variance (CV) and standard deviation (s.d.) for triplicate readings for intra- and interassay variability using 2^{-C_T} values

	A: Intraassay variability			*B: Interassay variability*		
	Mean s.d. and CV from triplicate readings, in the same run			*Mean s.d. and CV from triplicate readings, across separate PCR runs*		
Genes	*Mean s.d.*	*Mean CV (%)*	*Samples measured in triplicate*	*Mean s.d.*	*Mean CV (%)*	*Samples measured in triplicate over 2 runs*
GNS	0.13	9	79	0.47	30	7
UBE2E1	0.11	8	81	0.46	30	6
CCND1	0.12	8	73	0.21	15	6
TAOS1	0.08	6	25	0.56	36	3
FGF3	0.09	5	27	0.33	23	5
FGF19	0.13	9	23	0.4	27	3
FGF4	0.14	10	26	0.18	12	4
EMS1	0.11	8	29	0.4	27	4
	Overall s.d.	*Overall CV (%)*	*Total*	*Overall s.d.*	*Overall CV (%)*	*Total*
	0.11	8	363	0.38	25	38

Table 5 Coamplification of *TAOS1*, *FGF3*, *FGF19*, *FGF4* and *EMS1* with *CCND1* detected by Q-PCR and *BRAF* V600, *NRAS* Q61 mutations in primary melanomas

	Copy number ± s.e.							
Cases	CCND1	TAOS1	FGF3	FGF19	FGF4	EMS1	BRAF+ *V600*	NRAS+ *Q61*
1	13.4 ± 0.60	10.0 ± 0.71	5.00 ± 0.61	7.4 ± 0.65	7.9 ± 0.62	5.8 ± 0.48	V600E	WT
2	5.4 ± 0.31	3.7 ± 0.31	4.5 ± 0.22	5.1 ± 0.32	4.1 ± 0.25	4.4 ± 0.34	WT	Q61R
3	4.9 ± 0.31	3.6 ± 0.23	3.7 ± 0.26	3.9 ± 0.26	3.3 ± 0.31	4.6 ± 0.29	V600E	WT
4	4.4 ± 0.34	4.4 ± 0.27	3.3 ± 0.38	7.4 ± 0.65	3.7 ± 0.26	5.2 ± 0.32	WT	WT
5	11.7 ± 0.34	7.0 ± 0.44	3.8 ± 0.23	4.9 ± 0.36	4.0 ± 0.44	No Amp	WT	WT
6	5.2 ± 0.72	5.0 ± 0.71	4.5 ± 0.28	4.6 ± 0.19	4.2 ± 0.31	No Amp	WT	WT
7	3.2 ± 0.10	3.0 ± 0.22	3.3 ± 0.13	3.8 ± 0.22	No Amp	3.8 ± 0.13	WT	WT
8	3.7 ± 0.19	2.8 ± 0.16	3.3 ± 0.26	No Amp	No Amp	No Amp	V600E	WT
9	4.2 ± 0.28	2.8 ± 0.12	No Amp	No Amp	No Amp	3.8 ± 0.13	V600E	WT
10	3.5 ± 0.48	3.2 ± 0.15	No Amp	No Amp	No Amp	3.0 ± 0.06	WT	WT
11	9.6 ± 0.74	No Amp	No Amp	No Amp	No Amp	No Amp	WT	WT
12	4.6 ± 0.10	No Amp	No Amp	No Amp	No Amp	No Amp	WT	WT
13	3.8 ± 0.24	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K
14	3.5 ± 0.15	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K
15	3.4 ± 0.22	No Amp	No Amp	No Amp	No Amp	No Amp	WT	WT
16	3.1 ± 0.30	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K
17	3.1 ± 0.12	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K
18	3.1 ± 0.14	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K
19	3.0 ± 0.19	No Amp	No Amp	No Amp	No Amp	No Amp	V600E	WT
20	3.0 ± 0.16	No Amp	No Amp	No Amp	No Amp	No Amp	V600R	WT
21	2.9 ± 0.09	No Amp	No Amp	No Amp	No Amp	No Amp	WT	WT
22	2.9 ± 0.12	No Amp	No Amp	No Amp	No Amp	No Amp	WT	Q61K

No Amp, amplification was not detected; WT, wild type *BRAF* or *NRAS* genotype.

Comparing Gene Copy Number for *CCND1*: Q-PCR *versus* FISH

When we compared the *CCND1* gene copy number alterations obtained by Q-PCR with those detected by FISH, we found a good agreement between the two methods (Figure 2). The average *CCND1* copy number varied from 1.9 to 15.0 by FISH and from 1.8 to 13.4 by Q-PCR. We were able to detect amplification by Q-PCR in 8 out of 10 cases in which the frequency of amplified cells was more than 30% (copy number ≥ 5/cell) and in all cases in which the gene/centromere ratio was above 2 (Table 6). We did not observe amplification by Q-PCR if the disomic

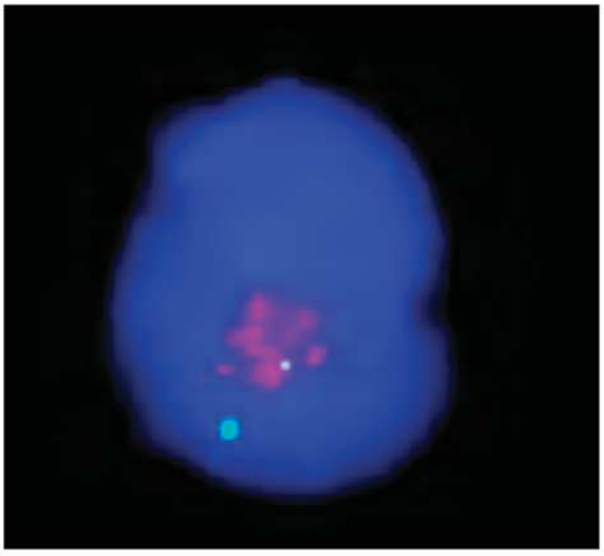
cell population was above >50% (data not shown). The Spearman's rank correlation coefficient showed strong correlations between FISH and Q-PCR results ($r=0.6$, $P<0.001$). In sample 7, a high-level amplification was seen by FISH, but the average copy number of the *CCND1* gene was only 3.2 by Q-PCR. The reason for this discrepancy may be that the touch preparation and the DNA were from slightly different parts of the tumor or a high copy number heterogeneity existed within the sample.

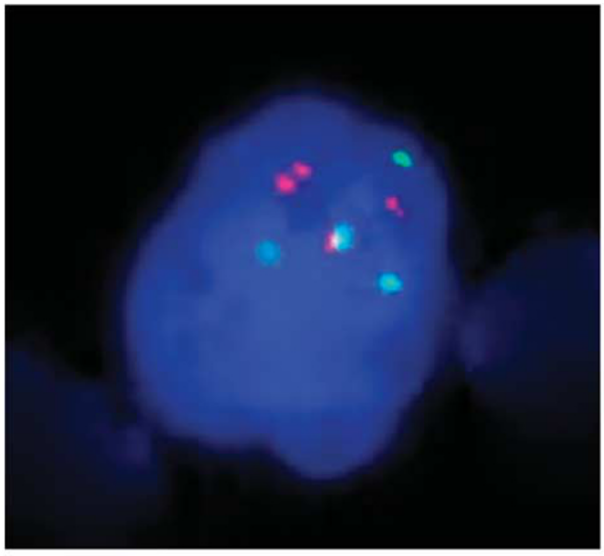
Evaluation of *TAOS1*, *FGF3*, *FGF4*, *FGF19* and *EMS1* Gene Copy Numbers by Q-PCR

Samples with *CCND1* amplification (22 primary melanomas) were further characterized for the *TAOS1*, *FGF3*, *FGF4*, *FGF19* and *EMS1* gene copy number alterations by Q-PCR. Among these 22 samples, 18% (4/22) showed amplification for all of these additional five genes (Table 5). In 46% of tumors (10/22) *CCND1* was coamplified with *TAOS1*; 36% (8/22) of *CCND1*-amplified tumors also displayed amplification of *FGF3*. Coamplification of *CCND1* and *EMS1* was observed in 7 lesions (32%). The nine primary melanoma samples which exhibited higher *CCND1* gene copy numbers (range 4.2–13.4) tended to display *TAOS1* (7/9), *FGF3* (6/9), *FGF19* (6/9), *FGF4* (6/9) and *EMS1* (5/9) amplifications as well (see details in Table 5).

BRAF and *NRAS* Mutation Frequencies

BRAF mutations at codon 600 were found in 25 out of 68 primary melanomas (37%) and in 2 out of 6 metastases. Eighteen (27%) primary and two metastatic lesions had *BRAF* exon 15, codon V600E mutations. This mutation is a single-base T→A transition (T1799A), resulting in a valine to glutamine change and causing substitution with a negatively charged residue. Seven primary tumors (10%) with V600 mutations were double base pair tandem substitutions; five (7%) had a V600K (GT1798-99AA) mutation, causing a valine to lysine change in the amino acid; two (3%) had a V600R tandem mutation





	Signals/cell by FISH (%)				Average gene copy number	
Tumor sample	2	3 - 4	≥ 5	Gene /centromere ratio	FISH	Q-PCR
(a) tumor # 1	0	0	100	7.5	15.0	13.4
(b) tumor # 42	79	17	0	1.0	2.3	2.2

Figure 2 Comparison of copy number alterations of the *CCND1* gene detected by FISH and Q-PCR analysis. *CCND1* gene specific probe was labeled with Spectrum Orange (appearing as red signals) and chromosome 11 centromeric probe was labeled with Spectrum Green (appearing as green signals), cell nuclei were labeled with blue fluorescent dye (DAPI). (**a**) Image represents a high-level amplification for the *CCND1* gene whereas (**b**) image shows tetrasomy for both centromeric and gene regions.

Table 6 Comparison of copy number alterations of the *CCND1* gene obtained by FISH and Q-PCR analysis

	Signals/cell by FISH (%)				*Average copy number*	
Tumor sample	*2*	*3–4*	*≥5*	*Ratio (gene/centromere)*	*FISH*	*Q-PCR*
1	0	0	100	7.5	15.0	13.4
7	0	0	100	6.9	15.0	3.2
5	5	39	56	2.4	9.1	11.7
2	8	36	56	3.4	8.7	5.4
11	28	39	33	2.3	5.9	9.6
3	18	49	31	1.5	5.3	4.9
6	13	30	54	2.4	5.0	5.2
10	8	49	43	1.2	5.0	3.5
24	8	46	47	1.6	6.8	No amp
25	31	35	32	0.8	4.1	No amp

No Amp, amplification was not detected.

(GT1798-99AG), causing a valine to arginine change, both of which mutations resulted in a substitution of valine for a positively charged amino acid (Figure 1).

NRAS mutations at codon 61 were found in 13 out of 68 primary melanomas (19%) and in 2 out of 6 metastases. The two most frequently observed mutations were a CAA to AAA transversion, which occurred in seven primary lesions (10%) and two metastases (resulting in a Q61K change) and a CAA to CGA transition, which occurred in five primary tumors (7%; resulting in a Q61R change). A CAA to TTA tandem mutation was observed in one lesion (2%; resulting in a Q61L change). Furthermore, 56% of primary melanomas and 3 metastases had either *BRAF* or *NRAS* mutations, but both mutations were never simultaneously present. *CCND1* was amplified in 34% of *BRAF* or *NRAS* mutated primary melanoma samples (Table 5).

In four patients, both primary and metastatic tumors were analyzed. Mutations in the primary tumors (one carrying a V600E mutation, two harboring a Q61K mutation) also occurred in the corresponding metastatic lesions (data not shown).

Correlation of Gene Alterations with Clinicopathological Parameters

We did not find correlations between *CCND1* gene amplification status and any of the patients' clinicopathological parameters.

Tumors with *CCND1* and *TAOS1* coamplification were classified with higher Clark's level (IV–V). Ulcerated tumors had a statistically significant association with *CCND1* and *TAOS1* coamplification ($P=0.017$; Table 1). In addition, coamplification of *CCND1* with *TAOS1* ($P<0.001$), *FGF3* ($P=0.001$), *FGF19* ($P<0.001$), *FGF4* ($P=0.002$) and *EMS1* ($P=0.004$) genes, respectively, were more frequently found in thick (≥9 mm Breslow thickness) melanomas. Younger age at diagnosis was significantly associated with coamplification of these five oncogenes (*CCND1*, *TAOS1*, *FGF3*, *FGF19* and *FGF4*; $P=0.026$, Mann–Whitney test; data not shown).

NRAS codon 61 mutations were significantly more frequent in tumors originating from chronically sun-exposed sites ($P=0.005$; Table 1). Other clinical parameters did not show significant association with the prevalence of *BRAF* or *NRAS* mutations. However, an increased *CCND1* gene copy number in conjunction with either *BRAF* or *NRAS* activation mutations was significantly more common in primary tumors with ulcerated surfaces ($P=0.028$; data not shown).

Discussion

We have elaborated a relative Q-PCR assay to assess the copy number profile of candidate genes located within the 11q13 chromosomal segment in primary malignant melanomas. This Q-PCR assay resulted in accurate measurement of DNA copy number alterations and can be used as an independent, sensitive method in parallel with other techniques such as Southern blot and FISH. The advantages of the technique are low cost and a requirement for only a very small amount of DNA, meaning that early-stage lesions of small size can be also analyzed. In addition, this PCR assay can be designed and validated for any loci with a known sequence in the human genome within a short period of time.

We had to consider a number of important parameters during the design of the Q-PCR assays for the quantification of the target genes within the 11q13 amplicon core. For normalization and to increase the reliability of the assay, we chose two reference genes (*GNS* on 12q14.3 and *UBE2E1* on 3p24.2), which are not thought to have copy number alterations in melanoma. The validity and reliability of both genes as reference genes were revealed through the detection of the copy number ratio in normal DNA and melanoma tumor samples as described earlier by De Preter *et al*.[33] The gene copy number ratio of the tumor samples is frequently calculated using the method described by Pfaffl

et al.[37] When using this method, the relative gene copy number ratio is calculated only from the Q-PCR efficiencies and the crossing-point deviation of an unknown sample *versus* a control sample; it needs no calibration curve, as control levels are included in the model. However, to evaluate the reproducibility of the Q-PCR assays we calculated the coefficient of variation (CV) using the transformation of C_T to the linear 2^{-C_T} value (calculating CV based on raw C_T values produces incorrect results because of its logarithmic nature[39]). Using our approach, the overall intra- and interassay variability of the experiments was 8 and 25%, respectively. These values are in good agreement with those reported by others.[40–42] The calculated low CVs were critical for the reliability of the designed Q-PCR assay and were the result of the accurate PCR reaction setup and the high quality of the DNA templates used during the entire experiment.

We were able to compare FISH and Q-PCR data for the *CCND1* gene in case of 35 primary melanomas and found good concordance. Q-PCR was sensitive enough to detect gene amplification if the copy number was ≥ 5 in more than 30% of tumor cells. The rare discrepancies between Q-PCR and FISH were probably resulted from higher normal cell contamination or could be explained by genetically heterogeneous tumor cell clones in the analyzed samples.[33]

In addition, we evaluated the *CCND1* gene copy number in all melanoma samples. Thirty-two percent of the primary lesions exhibited amplification. In contrast to a previous study,[16] but in confirmation of a recently published data,[17] we found that *CCND1* amplification was similarly frequent in SSM and NM. We did not observe any associations between *CCND1* gene alterations and clinicopathological parameters. Comparing the *CCND1* gene copy numbers in four primary and metastatic tumor pairs, we found gene amplification in all primary tumors but only one metastasis.

Recently it was found that *CCND1* is coamplified with other genes located within the 11q13 region in several malignancies, and it is likely that these coamplifications play a pathogenic role in those cancers.[12,13,22] However, no data are available for melanoma. To define the coamplification pattern of genes in the 11q13 amplicon, we designed Q-PCR assays for the *TAOS1*, *FGF3*, *FGF19*, *FGF4* and *EMS1* genes. The coamplification of *CCND1* with *TAOS1* was the most frequent event and had a significant association with the presence of ulceration ($P = 0.017$), a clinical feature that can predict poor prognosis. *TAOS1* has been described as a possibly important gene that might drive the 11q13 amplification in oral squamous cell carcinoma and associated with poor prognosis.[10,13] RNA interference method predicts that *TAOS1* would participate in cell-cycle control and regulate cell proliferation, similarly to *CCND1*.[10] We have shown that coamplifications of each of these genes with *CCND1* are characteristic for thick melanomas and are present in patients of younger age. Based on our data we assume that coamplification of these candidate genes can contribute to a more aggressive phenotype than *CCND1* amplification alone. It is also possible that the coamplifications are merely a result of genetic instability that increases during tumor progression but do not contribute directly to the phenotypic alterations.[43]

The *BRAF* V600E mutation frequency in our samples was 26%, which is in concordance with other studies[44,45] but the occurrence of this mutation was lower than it has been reported elsewhere.[4,46] We detected two other *BRAF* mutations that were distinct from the V600E mutation. In each case, subsequent sequencing revealed the presence of V600K or V600R tandem mutations with rates of 7 and 3%, respectively. The two most frequently observed *NRAS* mutations were Q61K and Q61R, with rates of 10 and 7%, respectively, and these were associated exclusively with tumors derived from chronically sun-exposed sites, which agrees with past studies.[46,47] Metastatic lesions harbored the same mutations as the primary tumor from which they originated, which supports the idea that these mutations are preserved throughout melanoma progression.[48]

A recent study analyzing 126 melanomas found that lesions with increased *CCND1* expression level had either mutation in *BRAF* or *NRAS* or increased copy number of the *CCND1* gene, without exception. The study reported a strong inverse correlation between *BRAF* mutations and increased *CCND1* copies. This suggests that an increased level of *CCND1* protein (the downstream component of the *RAS*/*BRAF*/*MAP* kinase pathway), as a result of either mutations in upstream genes or increased gene dosage, represents a crucial event driving melanoma progression.[4] Evaluating the relationship between increased *CCND1* gene dosage and the frequency of *BRAF* and *NRAS* mutations, we found that 34% of the primary melanomas harboring one of these activating mutations also had *CCND1* alterations. Among these tumors, melanomas with ulcerated surfaces were significantly more frequently present ($P = 0.028$), indicating the clinical relevance of this finding. This association is supported by a recent study in which *CCND1* was found to be amplified and overexpressed in 17% of *BRAF* V600E-mutated human metastatic melanomas and contributed to increased *BRAF* inhibitor resistance in melanomas.[49]

In conclusion, Q-PCR is a fast, reliable and accurate method for detecting amplification present in the 11q13 amplicon core in malignant melanomas. We found that neither the increased *CCND1* gene dosage nor the *BRAF* or the *NRAS* mutations alone contributed to more aggressive phenotype. However, we assume that coamplification of these candidate genes in the 11q13 region or carrying a *CCND1* alteration along with either the activating

BRAF or *NRAS* mutation may be more important for prognosis in subgroups of aggressive melanomas than the presence of these alterations alone.

Disclosure/conflict of interest

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