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Prognostic relevance of the expressions of CAV1 and TES genes on 7q31 in melanoma

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1. ABSTRACT

The 7q31 locus contains several genes affected in cancer progression. Although evidences exist regarding its impact on tumorigenesis, the role of genetic alterations and the expressions of locus-related genes are still controversial. Our study aimed to define the 7q31 copy number alterations in primary melanomas, primary-metastatic tumor pairs and cell lines. Data were correlated with clinical-pathological parameters. Genetic data show that 7q31 copy number distribution was heterogeneous in both primary and metastatic tumors. Extra copies were highly accompanied by chromosome 7 polisomy, and significantly increased in primary lesions with poor prognosis. Additionally, we determined the mRNA and protein levels of the locus-related CAV1 and TES genes. TES mRNA level was associated with metastatic location. CAV1 mRNA and protein levels were significantly higher in thicker tumors, however, lack of protein was also observed in a subpopulation of thin lesions. Expressions of CAV1 and TES were not associated with 7q31 alterations. In conclusion, 7q31 amplification can predict unfavorable outcome. Alterations of TES mRNA level may predict the location of metastasis. CAV1 possibly affect the cancer cell invasion.

2. INTRODUCTION

Genetic alterations of chromosome 7 are of great importance in tumor progression and known to be associated with poor prognosis in different cancers, including malignant melanoma (1). FRA7G, a common fragile site at 7q31, contains several genes, including CAV1 and TES that are possible targets of genetic alterations during tumorigenesis similarly to FHIT and WWOX genes (2, 3).

Human CAV1 is the main component of membrane lipid rafts and, consequently, plays an important role in vesicular transport, cholesterol homeostasis, receptor internalization and the regulation of signal transduction via interactions with several proteins, including EGFR, H-RAS and eNOS (4). Overexpression of the CAV1 protein has been shown to inhibit the growth of a laryngeal squamous cell carcinoma cell line by inactivating EGFR/MAPK signaling (5) and, through the inhibition of c-SRC and MET signaling, it reduces the ability of osteosarcomas to form metastases (6, 7). CAV1 has both apoptotic and anti-apoptotic effects during tumor progression, depending on the cancer type and/or stage (4, 8, 9). Nakashima et al. found that CAV1 overexpression

Table 1. Clinical-pathological parameters of primary melanomas

Variables	No. of tumors analyzed using FISH[1]	No. of tumors analyzed using QRT-PCR	No. of tumors analyzed using IHC[2]
All patients	75	33	60
Histological subtype[3]			
SSM	43	21	34
NM	32	12	26
Gender			
Female	34	17	39
Male	41	16	21
Age (years)			
Under 50	22	9	11
Over 50	53	24	49
Breslow thickness (mm)[4]			
Less than 2	21	16	29
2-4	13	5	8
More than 4	41	12	23
Clark's stage			
I-III	24	17	34
IV-V	51	16	26
Localization			
Extremity	32	19	30
Trunk	35	11	22
Head	8	3	8
Metastasis formation[5]			
Absent	20	16	31
Present	48	17	25
Patient's survival[6]			
Alive	28	23	35
Dead	40	10	21
Ulceration			
Absent	30	17	34
Present	45	16	26

[1]Fluorescence *in situ* hybridization; [2]immunohistochemistry; [3]SSM: superficial spreading melanoma, NM: nodular melanoma; [4]thickness categories are based on the current staging system; [5]metastasis formation within 5 years after removal of the primary tumor (only patients with follow-up periods of at least 5 years were included); [6]only patients with follow-up periods of at least 5 years were included

reduced cell growth and motility in the SK-MEL-28 melanoma cell line through the regulation of GD3-mediated signals (10). However, the expression pattern of CAV1 was not investigated in primary melanoma tissues yet.

TES is a putative tumor-suppressor gene positioned close to FRA7G. According to recent studies, TES is an important part of focal adhesions, and therefore has a large impact on cell motility (11-13). Although reduced or absent gene expression has been associated with poor prognoses in hematological and breast cancer cell lines (14), its role in cancer development has not yet been examined.

Because the role of FRA7G-associated genes is not well characterized in melanoma genesis, we aimed to define 7q31 locus copy number alterations in a large set of primary melanoma samples using interphase fluorescence *in situ* hybridization (FISH) and to correlate the results with the clinical-pathological parameters of melanoma patients. We also aimed to determine the mRNA and protein levels of locus-related CAV1 and TES genes and determine their role during melanoma progression.

3. MATERIALS AND METHODS

3.1. Melanoma tissue samples

Melanoma tissues were obtained from the Department of Dermatology, University of Debrecen (Debrecen, Hungary). The patients did not undergo therapy before surgical removal of the primary lesions. The study was approved by the Regional and Institutional Ethics Committee of the University of Debrecen and performed according to the relevant regulations. A written informed consent was obtained from the patients. Lesions were diagnosed on the basis of formalin-fixed paraffin-embedded (FFPE) tissue sections stained with haematoxylin-eosin. The average age of patients with primary melanoma was 61.9 +/- 13.3 years (range: 33-87 years), and the survival time after surgery for the primary tumor until the end of the study was 4.1 +/- 3.0 years (range: 0.04-14.5 years). A total of 75 tumors were examined using fluorescence *in situ* hybridization (FISH), 33 tumors were analyzed using real-time QRT-PCR (28 overlapped with FISH analysis) and 60 lesions were analyzed using immunohistochemistry. Ten tumors were analyzed using all three methods. The clinical-pathological parameters of the tumors are summarized in Table 1.

3.2. Cell lines

Human melanoma cell lines [WM35 (15), WM983A, WM983B (16, 17), A2058, HT168, HT168M1 (18) and HT199 (19)] were maintained in RPMI medium supplemented with 10% foetal bovine serum, 300 mg/l L-glutamine and antibiotics. All chemicals were obtained from Sigma-Aldrich (Sigma-Aldrich Inc., St. Louis, MO, USA).

3.3. Fluorescence *in situ* hybridization and scoring of the signals

DNA probes specific to the centromere of chromosome 7 (cep7, labeled with SpectrumGreen) and the

7q31 locus (SpectrumOrange) were obtained from Vysis, Inc. (Downers Grove, IL, USA). FISH experiments were performed on imprint preparations made from fresh-frozen tumors using slides treated with 3-aminopropyltrimethoxy-silane (Sigma-Aldrich Inc., St. Louis, MO, USA) according to the manufacturer's protocol, with slight modifications as previously described (20). The probe mixture and target cells were denatured simultaneously as reported previously (21). Nuclei were labeled with 200 ng/ml diaminophenylindole (DAPI) in antifade solution (Vector Laboratories LTD, Peterborough, UK). Fluorescence signals were scored in approximately 100-300 cells per specimen using a fluorescence microscope (ZEISS Axioplan, Carl Zeiss, Jena, Germany). Three-color images were captured using a digital imaging analysis system (ISIS Metasystem GmbH, Altlussheim, Germany). Copy number index (CNI) and categories of the 7q31 copy number alterations were defined as previously described (21).

3.4. RNA extraction and QRT-PCR

The RNeasy Mini Kit (Qiagen GmbH, Hilden, Germany) was used to isolate total RNA from 33 fresh melanoma tissues. The clinical-pathological parameters of the tumors are summarized in Table 1. The quantity of RNA was determined using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). TaqMan one-step RT-PCR was used to determine the mRNA levels of the CAV1 and TES genes. Each reaction contained 150 ng of total sample RNA and was run in triplicate on an ABI-PRISM 7000 instrument (Life Technologies Corporation, Carlsbad, CA, USA). Reverse transcriptase, PCR master mix and assays (Hs00184697_m1 CAV1 targeting the exon 1-2 boundary, Hs00210319_m1 TES targeting the exon 6-7 boundary) were obtained from Life Technologies. PCR data were analyzed using the Livak method (2^{-ddCt}) with glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Hs99999905_m1) as the reference (endogenous control) gene and naevus naevocellularis dermalis from three different individuals and melanocyte as calibrator samples.

3.5. Tissue microarrays and immunostaining

A total of 60 FFPE melanomas were included in the tissue microarray (22) analysis. The clinical-pathological parameters of the tumors are summarized in Table 1. Tissue sections (4 micrometers) of the original blocks were first stained with haematoxylin-eosin and then reviewed by pathologists (MG and BA) to select the area to be punched automatically using a TMA Master (Carl Zeiss, Jena, Germany). Most cases were represented using three 1-mm tumor cores taken from the original blocks. Serial sections of 4 micrometers were excised from the TMA blocks and used for immunohistochemical investigations following haematoxylin-eosin validation of the sample spots. After deparaffinization and blocking of the endogenous peroxidases (1% H_2O_2 for 10 min at RT) and non-specific binding sites we retrieved antigens in 1 mM boiling citrate buffer (pH 6, 3 min). We incubated sections in primary antibody solutions as follows: 1:200 HMB45 (melanoma marker) mouse monoclonal IgG, 1:200 CAV1 (clone N-20) rabbit polyclonal IgG, and 1:50 TES (clone AA-7) mouse monoclonal IgG (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). After incubation with the primary antibodies (1 h at RT), the samples were stained using the Envision/HRP detection system (DAKO Inc., Carpinteria, CA, USA) and the VIP peroxidase substrate kit (Vector Laboratories LTD, Peterborough, UK). Nuclei were counterstained with methyl-green (Vector Laboratories LTD, Peterborough, UK). Primary antibodies were omitted in negative controls. Positive controls were as follows: the A541 human lung cancer cell line and endothelial cells as controls for CAV1 and Jurkat cells for TES. The slides were digitized using a MiraxScan slide scanning device (Carl Zeiss, Jena, Germany) as described previously (23). Digital images were displayed using MiraxViewer software (Carl Zeiss, Jena, Germany) and evaluated by two dermatopathologists (BA and EG) who were blind for the sample data. Reaction positivity was scored on a four-grade scale: 0, no staining; 1+, definite but weak staining; 2+, moderate staining and 3+, strong staining (Figure 1). Scores for the triplicate cores were consolidated to a single value per case.

3.6. Statistical analysis

SPSS 13.0 (SPSS Inc., Chicago, IL, USA) was used for statistical analyses. The Shapiro-Wilk test was used to control the normality of data. Because of the abnormal distribution of data, non-parametric tests were applied for the statistical analysis. Fisher's exact; Kruskal-Wallis and Mann-Whitney-Wilcoxon tests were used to compare the 7q31 alterations to the cep7 copy numbers and the clinical-pathological data of the examined tumors. To analyze the relationship between the QRT-PCR data and the clinical-pathological parameters of melanoma patients, we used Kruskal-Wallis and Mann-Whitney-Wilcoxon tests. Fisher's exact was performed for the statistical analysis of protein expression data. Spearman correlation was used to examine the association between locus genetic alterations and gene expression levels. A p value less than 0.05 was considered statistically significant. In agreement with literature norms, changes in the gene expression level of more than two fold were considered under- or over-expressed.

4. RESULTS

4.1. Chromosome 7 and 7q31 copy number alterations in primary melanomas

Seventy-five primary melanoma samples were analyzed by FISH (Table 1). Centromere 7 and 7q31 signals were counted simultaneously in all samples. Representative images of FISH results are shown in Figure 2A-D. The copy number of cep7 and 7q31 varied from 1 to more than 10 copies/cell. Cep7 aneusomy was observed in 79% of primary melanomas, whereas 95% showed 7q31 copy number alterations. The copy number index (CNI) was between 1.8 - 13.0 for cep7 and between 1.2 - 13.3 for 7q31. Amplification of 7q31 was associated with chromosome 7 polisomy (p-value less than 0.001), whereas deletion was accompanied by chromosome 7 di- or monosomy (Figure 2E). Extra copies of 7q31 relative to cep7 were observed in 71% (53/75) of primary tumors, whereas only 19% (14/75) exhibited deletion in this region. High-level amplification of 7q31 was observed in nine

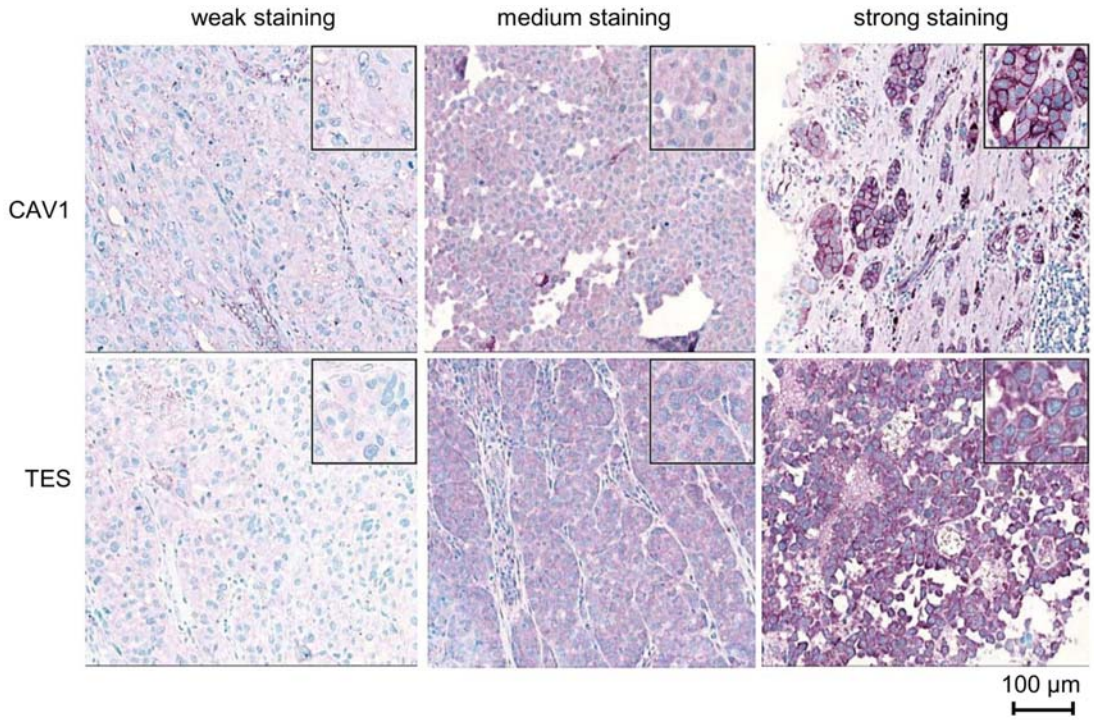


Figure 1. Scoring system for immunostaining intensity of primary melanoma tissues. The staining intensity of CAV1 (A) and TES (B) proteins in melanoma samples with weak (1+), medium (2+) and high (3+) level protein expression.

cases (12%). Except for one case, these tumors were in a more advanced stage with an aggressive phenotype, and eight out of nine melanomas metastasized mainly to distant locations (brain and lung, Table 2).

4.2. Correlation of 7q31 alterations with clinical-pathological parameters of patients

Examining the correlation of the average locus copy number with the different clinical-pathological parameters, we found a significant association with metastasis formation, tumor surface ulceration, patients' survival and age. The calculated 7q31 locus copy number index was significantly higher in primary tumors with metastatic property ($p=0.012$; average CNI=4.6 +/- 2.6 vs. 3.1 +/- 1.5), ulcerated surface ($p=0.002$; average CNI=4.7 +/- 2.6 vs. 3.0 +/- 1.3), and causing the patient's death ($p=0.048$; average CNI=4.5 +/- 2.7 vs. 3.5 +/- 1.7). Patients older than 50 years were characterized as having a slightly higher number of 7q31 copies compared to patients in the younger age group ($p=0.038$; average CNI=4.4 +/- 2.5 vs. 3.1 +/- 1.6).

4.3. Alterations of 7q31 in primary melanomas and their corresponding metastasis pairs

We examined the 7q31 locus genetic alterations in 7 primary melanomas and their corresponding lymph node or cutan metastasis (Figure 3). No significant differences in the 7q31 copy number were noticed between the primary tumor and its corresponding metastasis, except for three tumor pairs: numbered as 1, 5 and 7. In case of pairs No.1 and 5 the 7q31/7c ratio was significantly increased in the metastatic tumor, whereas in pair No.7 both the primary tumor and its metastatic lesion showed a high ratio. Metastases of tissue No. 5 and 7 exhibited a higher 7q31 copy number, whereas in case of tumor No. 1 the decrease of chromosome 7 copy number resulted in an increased ratio in the metastasis, but similar pattern of 7q31 was found in primary tumor and its metastasis.

4.4. CAV1 and TES mRNA and protein expression in primary melanomas

Thirty-three primary melanomas were available for gene expression analysis of these genes. Table 3 summarizes the results of the gene expression analysis. CAV1 mRNA level was significantly lower in thick tumors (more than 4.0 mm, $p=0.002$). TES mRNA levels were different in the primary tumors depending on the location of the corresponding metastasis ($p=0.011$). TES overexpression was found only in samples with cutan or lymph node metastasis (6/17). Tumors that formed cutan and/or lymph node and distant organ metastasis (6/17) were characterized by TES mRNA downregulation. Lesions with only lung, brain, bone and liver metastasis (5/17) had similar TES mRNA level as naevus samples.

We could examine the protein expression levels of CAV1 and TES genes in 60 primary melanomas using tissue microarray. Thick (more than 4.0 mm) and advanced-stage (Clark IV-V) melanoma samples (n=6) showed high intratumor staining heterogeneity regarding the morphologically different cell areas (i.e., foam cells or spindle cells). The other lesions were homogenous both histologically and with respect to protein staining. The examined naevus samples did not show CAV1 and TES positivity.

Table 2. Clinical-pathological parameters of primary melanomas with high-level 7q31 locus amplification (N=9)

Sample no.	7q31/7c ratio	TNM stage[1]	Survival[2]	Metastasis	Ulceration
1	1.02	T4bN3M1c	5.9	brain, lung, ly[3]	yes
2	1.25	T4bN0M0	2.4	lung	yes
3	1.71	T1aN0M0	alive[4]	no	no
4	1.82	T4bN0M0	0.7	ly	yes
5	2.35	T4bN0M0	5.9	brain	yes
6	2.38	T3bN0M1c	2.4	brain, lung	yes
7	2.39	T4bN2bM0	alive	lung, ly	yes
8	2.48	T4bN0M0	4.9	brain	yes
9	2.62	T3bN0M0	1.4	lung	yes

[1]TNM stage at the time of surgery of primary tumor; [2]survival in years after surgery; [3]lymph node; [4]more than 5 years

Table 3. Correlation between the relative CAV1 and TES mRNA levels and the clinical-pathological parameters of primary melanomas

Variables		No. of tumors analyzed	CAV1	p[1]	TES	p
Subtype[2]	SSM	20	4.706 +/- 2.895	0.207	1.640 +/- 0.413	0.870
	NM	13	2.947 +/- 1.687		1.494 +/- 0.486	
Gender	Female	17	3.025 +/- 1.182	0.292	1.545 +/- 0.413	0.986
	Male	16	5.062 +/- 3.674		1.622 +/- 0.480	
Age	Under 50 years	9	1.259 +/- 0.562	0.414	1.792 +/- 0.624	0.677
	Over 50 years	24	5.046 +/- 2.531		1.504 +/- 0.364	
Thickness	Less than 4.0 mm	21	5.651 +/- 2.844	**0.002**	1.966 +/- 0.429	0.063
	More than 4.0 mm	12	1.147 +/- 0.882		0.911 +/- 0.348	
Clark's stage	I-III	17	5.478 +/- 3.390	0.068	1.797 +/- 0.468	0.444
	IV-V	16	2.499 +/- 1.378		1.354 +/- 0.411	
Metastasis[3]	Absent	16	2.619 +/- 0.697	0.067	2.092 +/- 0.514	0.191
	Present	17	5.416 +/- 3.602		1.135 +/- 0.344	
Survival[3]	alive	23	3.010 +/- 0.961	0.125	1.956 +/- 0.418	0.057
	dead	10	6.475 +/- 5.925		0.778 +/- 0.236	
Location of metastasis	lymph node/cutan	6	3.960 +/- 3.384	0.702	1.887 +/- 0.901	**0.011**
	distant organ	5	12.13 +/- 11.91		1.098 +/- 0.207	
	complex	6	1.274 +/- 0.561		0.415 +/- 0.116	

[1]Mann-Whitney-Wilcoxon or Kruskal-Wallis test; [2]SSM: superficial spreading melanoma, NM: nodular melanoma; [3]within a 5-year follow up period; significant p-values are indicated in bold

Melanoma tissues in general showed strong membrane and weak, diffuse cytoplasmic CAV1 immunostaining. The TES protein often formed plaques in the membrane, however, weak plasma positivity was also observed. CAV1 protein expression was detected in 83% (41/51) of primary lesions. The remaining 10 samples did not show any CAV1 immunostaining. Breslow thickness was the only parameter showing different CAV1 protein levels between the two groups (p=0.038; Table 4). Thicker melanomas have a reduced CAV1 expression, however, it is important to note that a subpopulation of the thinner lesions exhibited no protein staining. TES expression was found in 76% (42/55) of primary lesions. However, we did not find any significant associations between the TES protein expression and the clinical-pathological parameters of the primary melanomas.

4.5. Copy number alterations, mRNA and protein expression levels of the CAV1 and TES genes in primary melanomas and melanoma cell lines

In case of 14 primary melanomas we had the possibility to analyze the 7q31 copy number alterations and the expression levels for both CAV1 and TES genes in the same tumor samples (Table 5). The results clearly shows that amplification of the locus is not or weakly correlates with mRNA and protein expressions of CAV1 and TES genes. The alterations of CAV1 and TES mRNA expressions had an impact on protein levels in some cases. However, we did not find any correlation between the genetic alterations, gene- and protein expression levels. In order to get clearer view of this phenomenon we analyzed 7 melanoma cell lines (WM35, WM983A, WM983B, HT199, A2058, HT168 and HT168M1) with different biological behavior for 7q31 locus copy number aberrations, mRNA and protein (CAV1 and TES) expressions (all of the cell line data are summarized on Figure 4).

All cell lines exhibited heterogeneous copy number distribution for chromosome 7 centromere and 7q31 copy numbers. Low-level amplifications and deletions were present in all cell lines with different frequencies. Locus deletion was observed with high frequency in three (WM983B, HT199 and WM35) and low level amplification was present in two (WM983A and HT168), whereas high level amplification was detected in two cell lines (A2058 parental cell line and HT168M1 cell line selected in vivo from the A2058 with a high liver metastatic capacity).

Table 4. CAV1 (N=51) and TES (N=55) protein staining distributions of primary melanomas regarding clinical-pathological parameters

Variables		No. of samples (%)	Staining category				
			0	1+	2+	3+	p[1]
Ulceration							
CAV1	no	27 (52.9)	6 (22.2)	0 (0.0)	12 (44.5)	9 (33.3)	0.089
	yes	24 (47.1)	4 (16.7)	5 (20.8)	7 (29.2)	8 (33.3)	
TES	no	29 (52.7)	8 (27.6)	6 (20.7)	12 (41.4)	3 (10.3)	0.206
	yes	26 (47.3)	5 (19.2)	12 (46.2)	6 (23.1)	3 (11.5)	
Metastasis[2]							
CAV1	no	24 (51.1)	7 (29.2)	0 (0.0)	9 (37.5)	8 (33.3)	0.053
	yes	23 (48.9)	2 (8.7)	5 (21.7)	9 (39.1)	7 (30.5)	
TES	no	27 (51.9)	9 (33.4)	5 (18.5)	10 (37.0)	3 (11.1)	0.128
	yes	25 (48.1)	4 (16.0)	12 (48.0)	6 (24.0)	3 (12.0)	
Exitus[2]							
CAV1	no	27 (57.4)	6 (22.2)	1 (3.8)	10 (37.0)	10 (37.0)	0.327
	yes	20 (42.6)	3 (15.0)	4 (20.0)	8 (40.0)	5 (25.0)	
TES	no	31 (59.6)	8 (25.8)	8 (25.8)	12 (38.7)	3 (9.7)	0.409
	yes	21 (40.4)	5 (23.8)	9 (42.9)	4 (19.0)	3 (14.3)	
Breslow thickness							
CAV1	Less than 4.0 mm	29 (56.9)	7 (24.1)	0 (0.0)	13 (44.8)	9 (31.0)	**0.038**
	More than 4.0 mm	22 (43.1)	3 (13.6)	5 (22.7)	6 (27.3)	8 (36.4)	
TES	Less than 4.0 mm	32 (58.2)	9 (28.1)	9 (28.1)	11 (34.4)	3 (9.4)	0.482
	More than 4.0 mm	23 (41.8)	4 (17.4)	9 (39.1)	7 (30.4)	3 (13.1)	
Clark stage							
CAV1	I-III	26 (51.0)	6 (23.1)	0 (0.0)	11 (42.3)	9 (34.6)	0.119
	IV-V	25 (49.0)	4 (16.0)	5 (20.0)	8 (32.0)	8 (32.0)	
TES	I-III	29 (52.7)	9 (31.0)	7 (24.2)	11 (37.9)	2 (6.9)	0.263
	IV-V	26 (47.3)	4 (15.4)	11 (42.3)	7 (26.9)	4 (15.4)	
Histological subtype							
CAV1	SSM	26 (51.0)	5 (19.2)	0 (0.0)	12 (46.2)	9 (34.6)	0.099
	NM	25 (49.0)	5 (20.0)	5 (20.0)	7 (28.0)	8 (32.0)	
TES	SSM	29 (52.7)	10 (34.5)	7 (24.2)	9 (31.0)	3 (10.3)	0.196
	NM	26 (47.3)	3 (11.5)	11 (42.3)	9 (34.6)	3 (11.5)	

[1]two-sided Fisher's exact test; [2]within 5-year follow up period

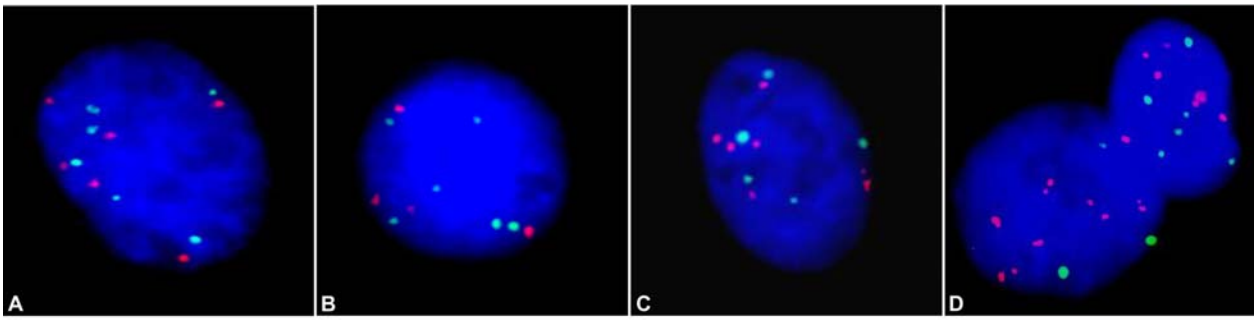
The relative mRNA expression of the CAV1 gene was similar in the WM35, WM983A, WM983B, HT199 and HT168 melanoma cell lines (Figure 4). A significant association between high level amplification of the locus and the relative mRNA level was observed for two aggressive cell lines (A2058 and HT168M1). All cell lines showed CAV1 protein expression, but the level was different. High protein expression was found in the invasive but not metastatic WM35 and the metastatic WM983B, HT199, A2058, HT168 cell lines and low expression in the WM983A cell line (Figure 4). No CAV1 protein expression was observed in the HT168M1 cell line.

The mRNA level of TES was higher in the cell lines compared to the mRNA expression in melanocytes and was associated with strong protein expression as detected by immunohistochemistry (Figure 4).

5. DISCUSSION

In this study our aim was to define 7q31 locus copy number aberrations in a relatively large number of primary melanoma samples using FISH and correlate the genetic results with the clinical-pathological parameters of patients. The strength of this study is that beside the copy number changes at site of the large fragile site, FRA7G, we also determined the mRNA and protein expression levels of the important locus-related genes (CAV1 and TES) surrounded the fragile site and defined their role during melanoma progression. Using FISH, we found deletions and different levels of amplification of the 7q31 locus. Deletion was associated with a favorable prognosis in contrast to the amplification that resulted in a poor clinical outcome and made primary tumors liable to form metastases. Increased CAV1 protein level statistically showed a high association with tumor thickness in several cases. The influence of TES expression during melanoma progression requires more detailed investigation.

As previously found, CAV1, a multifunctional scaffolding protein has a controversial role in the development of several human tumors (24). The upregulation of CAV1 gene expression was associated with an elevated level of CAV1 protein, and had an influence mainly on tumor thickness in our melanoma samples. On the other hand, downregulation of CAV1 was also observed in a subpopulation of thinner samples and melanoma cell lines with poor clinical outcome suggesting that CAV1 may



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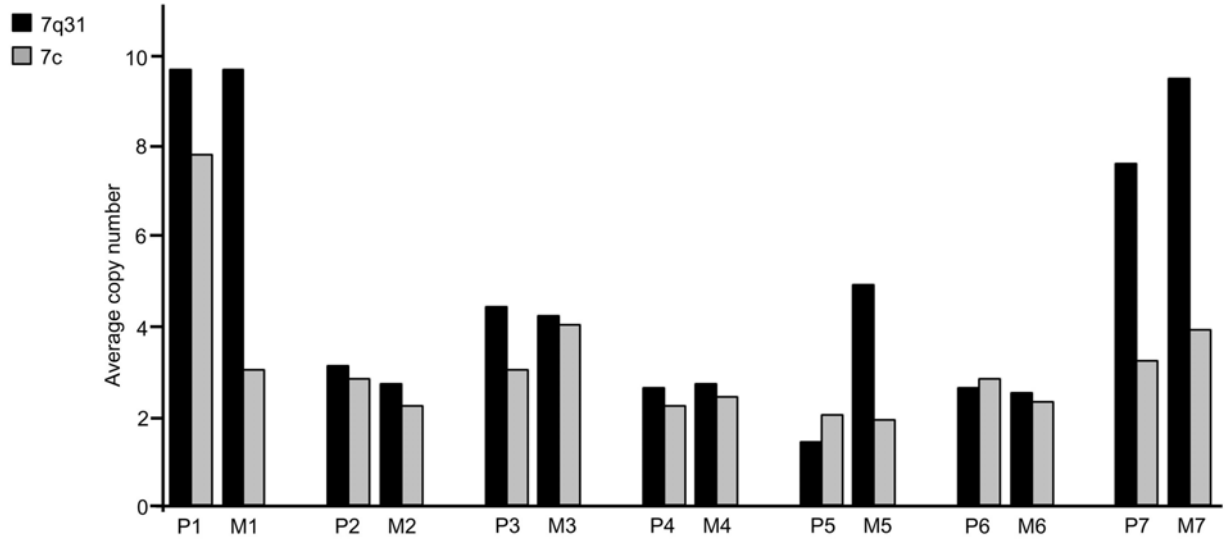
Chromosome 7 ploidy	No. of samples (%[1])	7q31 locus copy number category				
				Amplification status		
		Deletion N (%)	Normal N (%)	Apparent N (%)	Low level N (%)	High level N (%)
Monosomy	7 (9.3)	6 (85.7)	0 (0.0)	0 (0.0)	1 (14.3)	0 (0.0)
Disomy	17 (22.7)	3 (17.7)	4 (23.5)	0 (0.0)	9 (52.9)	1 (5.9)
Polisomy	51 (68.0)	5 (9.8)	0 (0.0)	4 (7.8)	34 (66.7)	8 (15.7)

[1]Frequency is given within a copy number category of chromosome 7.

Figure 2. Copy number distributions of the 7q31 region in interphase melanoma cells. A chromosome 7 centromere-specific probe was labeled with SpectrumGreen (green signals), and a 7q31 locus-specific probe was labeled with SpectrumOrange (red signals). Nuclei were stained with DAPI (blue fluorescence). 7q31 apparent amplification (A), 7q31 deletion (B), low-level 7q31 amplification (C) and high-level 7q31 amplification (D). Significant association (p-value less than 0.001; two-sided Fisher's exact test) between alterations of the 7q31 locus copy number and chromosome 7 ploidy (E).



Average 7q31/7c ratio	No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	No. 7
Primary	1.25	1.10	1.47	1.18	0.69	0.94	2.39
Metastasis	3.28	1.26	1.05	1.11	2.64	1.10	2.47

Figure 3. Average copy number of the 7q31 region and chromosome 7 in primary cutaneous melanomas and their corresponding lymph node metastasis. P – primary melanoma, M – metastasis. Numbers above the columns represent the 7q31/7c ratio.

Table 5. Comparison of 7q31 locus copy number status and the expression level of CAV1 and TES genes in cutaneous melanoma

Tumor number	Copy number index of 7q31	Main 7q31 locus copy number category	Percentage of cells with 7q31 alteration	Relative mRNA level		Immunohistochemical score	
				CAV1	TES	CAV1	TES
10	2.1	Deletion	97.2	0.153	0.092	2+	1+
11	2.6	Low level ampl.	39.5	2.528	0.484	2+	1+
12	3.5	Low level ampl.	50.0	0.634	2.174	1+	1+
13	3.6	Low level ampl.	69.3	0.076	0.137	1+	1+
14	3.6	Low level ampl.	90.3	0.526	0.096	2+	3+
15	4.5	Low level ampl.	98.5	0.607	1.663	2+	3+
16	4.9	Low level ampl.	91.4	59.760	0.607	2+	0
17	5.0	Low level ampl.	99.1	0.139	0.107	3+	2+
8	6.4	High level ampl.	70.7	0.212	0.762	2+	2+
1	13.3	High level ampl.	100.0	0.223	0.319	1+	1+
18	1.7	Deletion	37.1	nd	nd	1+	1+
19	2.6	Deletion	68.0	nd	nd	2+	1+
20	3.3	Apparent ampl.	84.0	nd	nd	0	1+
21	3.4	Low level ampl.	74.4	nd	nd	nd	2+
22	nd	nd	nd	0.076	1.503	3+	2+
23	nd	nd	nd	1.418	0.128	2+	2+
24	nd	nd	nd	3.693	0.690	3+	nd

nd: no data available

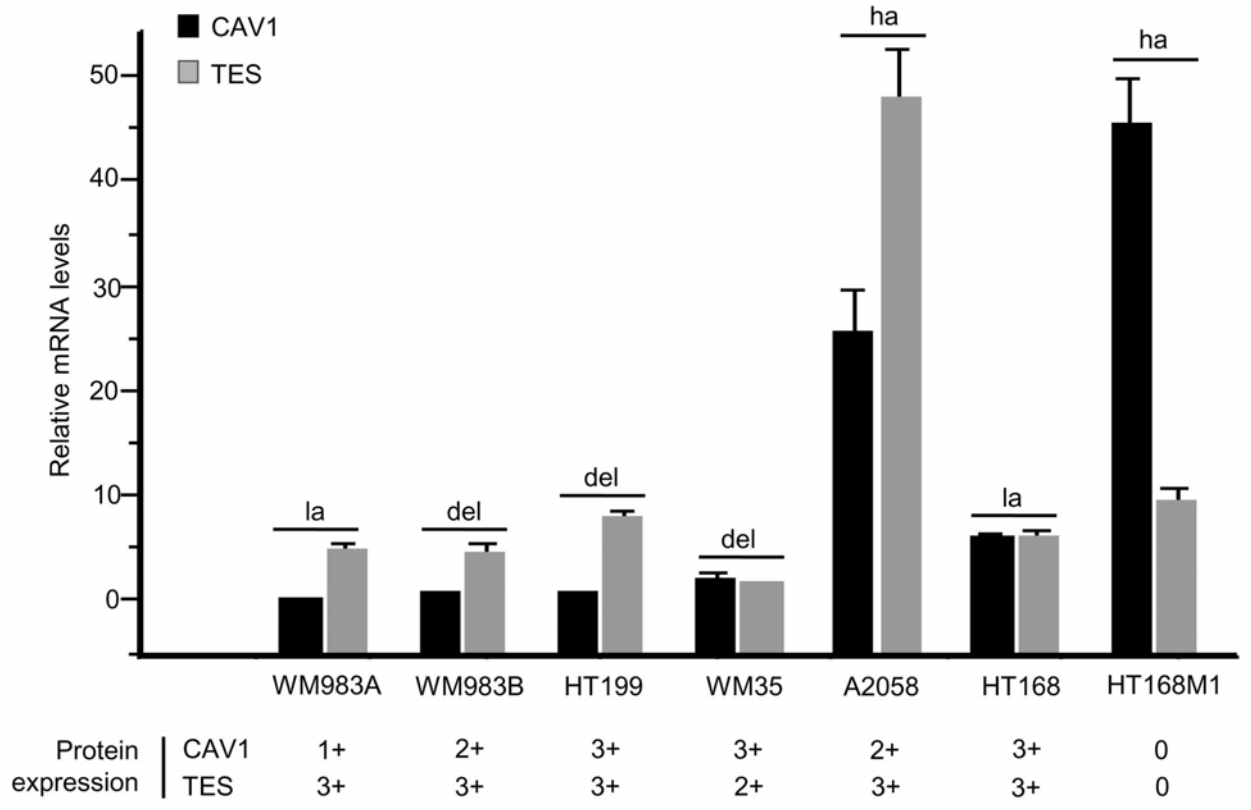


Figure 4. Relative mRNA and protein expression in human melanoma cell lines. The main 7q31 copy number categories are labeled on the top of the columns: la = low level amplification, del = deletion, ha = high level amplification. Error bars represent +/- SD. The lower part of the figure summarizes the staining intensities of the CAV1 and TES proteins: no staining (0), weak (1+), medium (2+) and strong (3+).

play a dual role in melanoma progression. Furthermore, the lack of CAV1 protein in the HT168M1 cell line, despite the high CNI and mRNA level, raises an issue that it would be worthwhile to extensively examine the epigenetic processes affected this molecule. These are important observations, because it has recently been tested that CAV1 protein is a potential biomarker for melanoma prognosis, since it contributes to tissue invasion and metastasis formation (25, 26). The reduced expression of CAV1 may be associated with an increased proliferative ability of tumor cells or the loss of tumor cell apoptosis through caspase-3 consequently leading to the development of metastatic

disease (27). Furthermore, a recent melanoma study provided evidence for the possible anti-metastatic function of the CAV1 protein (28). Total loss of the protein may result in decreased tumor neovascularisation, tumor suppression through the inhibition of cytokine receptor signaling or diminished tumor cell motility through the regulation/stabilization of focal adhesion domain organization (29, 30). It is also possible that CAV1 protein has a dose-dependent effect on cancer cells. Another reason might be that CAV1 has multiple interacting partners; therefore, the expression levels of these interacting molecules may have a significant impact on CAV1 function during cancer progression (24).

Downregulation of TES, which has previously been observed in head and neck squamous cell carcinomas (31) and gastric cancers (32) at both mRNA and protein levels, was frequently found in our primary melanoma samples. Although TES did not exhibit a significant association with metastasis formation, the mRNA level was significantly altered between lesions with different preference of site to form metastasis, and might have a predictive value in the future. These observations support the idea that TES may play an important role in primary melanomas. However, further studies, including metastatic melanomas, are required to clarify the exact role of TES in cancer progression.

The deletion of 7q31 locus was followed by the downregulation of the CAV1 and TES genes in some cases, but we did not find significant associations. One reason could be the relatively small available set of paired samples to perform the correlation analysis. Another reason can be that we observed only heterozygous 7q31 deletion; therefore, the remaining copy may make uninterrupted mRNA synthesis possible. However, amplification might not affect the entire locus, and the size of the altered region is difficult to determine. It is also difficult to define whether the alterations are included in a definite gene (7). Furthermore, an error in the epigenetic regulation of genes could also affect the mRNA and protein levels (14, 33),

In conclusion, 7q31 amplification is associated with unfavorable prognosis and reduced CAV1 protein level could have an impact on melanoma cell invasion. Whereas TES may play a part in the anchoraging of circulating tumor cells, defining the location of metastasis. This study shows that in the future it is worthwhile to examine deeper the role of these genes in melanoma genesis. Because cancer development is a complex process that alters a large number of genes and molecular pathways, it is also important to consider these alterations on the basis of their own complexity.

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Abbreviations: FRA7G: fragile site 7G; CAV1: caveolin 1; TES: testin; FISH: fluorescence *in situ* hybridization; FFPE: formalin-fixed paraffin-embedded; H&E: haematoxylin-eosin; QRT-PCR: quantitative real time polimerase chain reaction; DAPI:diaminophenylindol; CNI: copy number index; EGFR: endothelial growth factor receptor; H-RAS: Harvey rat sarcoma viral oncogene; eNOS: endothelial nitric oxide synthase; MAPK: mitogen-activated protein kinase; c-SRC: v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog

Key Words: Malignant melanoma, 7q31, Caveolin 1, Testin, Fluorescence *In Situ* hybridization, Tissue microarray

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