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Expression of Caveolin-1 in Human Cutaneous and Uveal Melanoma Cells*

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Caveolin-1 can act as a tumour promoter or suppressor depending on the cancer type and stage. In melanoma, information available concerning its expression is ambiguous. In this study, we investigated caveolin-1 mRNA and protein expression levels in human melanoma cell lines of different origin and progression stages. Metastatic cutaneous (WM-266-4, A375), primary cutaneous (WM-115, IGR-39) and primary uveal (mel-202, 92-1) cells were used for quantitative RT-PCR, Western blotting and confocal microscopy. We observed significantly higher expression of caveolin-1 mRNA in cutaneous than in uveal melanoma cells. In accordance, immunostaining of caveolin-1 was stronger in cutaneous cell extracts, while protein bands of uveal origin displayed weak signals. Finally, we detected differences in the caveolin-1 subcellular pattern of distribution between primary and metastatic cells. Overall, this is the first demonstration of caveolin-1 expression in human primary uveal melanoma cell lines and observation that the origin of cells (uveal/cutaneous) has an impact when considering the utility of caveolin-1 as a melanoma cell marker.

Key words: Caveolin-1, cutaneous melanoma, melanoma marker, uveal melanoma.

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Malignant melanoma still remains the most deadly and difficult skin cancer to treat. Great progress in understanding the biochemistry and genetics of human melanoma is concomitant with its increasing incidence. Cutaneous melanoma can be curable if diagnosed before the cancer has spread, however prognosis for patients with distant metastases is very poor (BESARATINIA & TOMMASI 2014). Uveal melanoma constitutes about 5% of all melanoma cases. It often receives similar treatment to its cutaneous equivalent despite the different origin and molecular characteristics of this tumour (CHATTOPADHYAY *et al.* 2016). The awareness of discrepancies between the two cancer types may be important in planning proper clinical interventions.

Caveolin-1 (22 kDa) is a principal component of caveolae, small (50-100 nm) omega-shaped invaginations of the plasma membrane, present in most cell types (CHEN & CHE 2014). It is located predominantly at the plasma membrane, but it has also been found in a number of other cellular compartments (PARTON *et al.* 2006). Its cellular distribution and expression level depends on cell type and physiological condition. The relationship between caveolin-1 expression pattern and function, especially in cancer cells, remains elusive. Caveolin-1 (Cav-1) belongs to a highly conserved protein family consisting of three members: Cav-1, Cav-2 and Cav-3. Caveolin-1 has two isoforms (α and β) which are generated either from different mRNA or by a divergent translation start site. Both isoforms of Cav-1 are assigned to different functions in a cell. Cav-1α forms caveolae more effectively than Cav-1β. Caveolin-1 (α and β) forms a hairpin-like structure which is inserted into the plasma membrane and allows both the N and C terminals of this protein to be oriented towards the cytosol. There are three structural domains and a few functional domains within Cav-1. The most important one is CSD (caveolin scaffolding domain) which is essential for caveolin-1 interaction with signalling molecules. Caveolin-1 is involved in a number of cellular processes such as signal transduction, cholesterol homeostasis, vesicle transport, cell migration, cell cycle, cell death

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and survival, multidrug resistance and angiogenesis. However, Cav-1 has both positive and negative impacts on these processes (GOETZ *et al.* 2008).

Caveolin-1 is a molecule that has two faces and raises many controversies, because it plays an ambiguous role in cancer development (SHATZ & LISCOVITCH 2008). This protein can act as a tumour promoter or suppressor depending on the cancer type, stage and microenvironment (CHEN & CHE 2014). Caveolin-1 expression and function have been investigated in a panel of human cancers. Generally, in mammary, lung, colon and ovarian cancers caveolin-1 displays a role in tumour suppression, while in prostate, pancreatic, renal cancers, it acts as a tumour promoter (QUEST *et al.* 2008). In melanoma, its possible impact on tumourigenicity still remains unclear.

The aim of this study was to analyse caveolin-1 expression in different primary and metastatic human melanoma cell lines of cutaneous and uveal origin. We used Real-time RT-PCR, Western blotting and confocal microscopy techniques to evaluate the expression and localization of this protein in six human melanoma cell lines.

Material and Methods

BSA – bovine serum albumin, Cav-1 – caveolin-1, CSD – caveolin scaffolding domain, ER – endoplasmic reticulum, RPII – RNA polymerase II, SDS-PAGE – polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

Antibodies

The following antibodies were used in the study: sheep polyclonal anti-rabbit IgG conjugated with alkaline phosphatase (Chemicon International), polyclonal rabbit IgG anti-caveolin-1 (catalog no. C4490), monoclonal mouse IgG anti-beta-tubulin (clone AA2), polyclonal goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma-Aldrich), monoclonal mouse IgG anti-caveolin-1 (clone 2297) (BD Transduction Laboratories), donkey anti-rabbit IgG conjugated with Alexa Fluor 488 (Molecular Probes Life Technologies).

Cell culture

IGR-39 (ESTDAB-037), WM-115 (ESTDAB-066), WM-266-4 (ESTDAB-076), 92-1 (ESTDAB-127), mel-202 (ESTDAB-128) cell lines were obtained from ESTDAB Melanoma Cell Bank (Tübingen, Germany). A375 cell line was purchased from ATCC. Human primary cutaneous (WM-115, IGR-39), metastatic cutaneous (WM-266-4, A375), and primary uveal (92-1, mel-202) melanoma cell lines were maintained in RPMI 1640-L-glutamine culture medium with 25 mM HEPES (Gibco Life Technologies) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin; Sigma-Aldrich) supplemented with 10% fetal bovine serum (Gibco Life Technologies) in a CO_2 incubator (37°C, 5% CO_2). Cells were routinely tested for mycoplasma contamination by PCR and DAPI staining.

RT-PCR

RNA isolation and reverse transcription was performed as previously described (BUBKA *et al.* 2014) with the use of High Pure Isolation Kit and Transcriptor High Fidelity cDNA Synthesis Kit with 2.5 μM anchored-oligo$(dT)_{18}$ (Roche Diagnostics) according to the supplier's protocols. Primers were synthesized at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences. *CAV-1* expression was evaluated by Real-time RT-PCR (Applied Biosystems StepOnePlus Real-time PCR system). The following primers were used (300 nM each): CAV-1 forward 5'-TGGTTTTACCGCTTGCTGTCTG-3', CAV-1 reverse 5'-GCAAGTTGATGCGGACATTGCT-3' (DIAZ-VALDIVIA *et al.* 2015). The general conditions of experiment were the same as described by BUBKA *et al.* (2014). *RPII* endogenous control was used for data normalisation according to the equation: $\Delta Ct = Ct_{\text{control gene}} - Ct_{\text{gene of interest}}$.

Cell lysate preparation

Cells were collected as reported (BUBKA *et al.* 2014) and homogenized in 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 150 mM NaCl with protease inhibitors (Sigma-Aldrich), 0.01% protamine sulfate (Sigma-Aldrich) and 1% Triton X-100 (Sigma-Aldrich) with constant shaking (4°C, 1.5 h). Debris was centrifuged (30 min, 4°C, 35000xg), supernatants collected and stored at -70°C. Protein concentration was determined using Total Protein Kit, Micro-Lowry, Peterson's modification (Sigma-Aldrich) according to Peterson's method (PETERSON, 1977).

Western Blotting

SDS-PAGE was performed according to modified Laemmli's method (LAEMMLI, 1970) in the presence of 0.1% SDS (Sigma-Aldrich) in 12% gel (reducing conditions). Western blotting was performed as detailed in BUBKA *et al.* (2014) with modifications. Membranes were probed with rabbit anti-caveolin-1 primary antibody (1:1000) or mouse anti-beta-tubulin antibody (1:1000) for 1 h at RT, washed and incubated with sheep anti-rabbit (1:4000) or goat anti-mouse secondary antibodies conjugated with alkaline phosphatase

(1:4000) for 1 h at RT. Molecular weights were calculated based on Dalton Mark VII-L standards (Sigma-Aldrich). Three experiments were performed with polyclonal anti-caveolin-1 antibody and an additional test with monoclonal anti-caveolin-1 IgG (clone 2297).

Immunofluorescence

Cells ($4x10^4$) were plated on each sterile glass coverslip in a 4-well plate (Nunc) and incubated until they reached subconfluency. Cells were fixed in 4% paraformaldehyde (Sigma-Aldrich) in PBS (20 min, RT) and washed in PBS (3 times, RT). Nonspecific binding sites were blocked with 10% normal donkey serum (Sigma-Aldrich) in PBS (30 min, RT). After washing (3 times), cells were stained with rabbit anti-caveolin-1 antibody in 2% BSA in PBS (1:100, ON, RT), washed three times and incubated with donkey anti-rabbit secondary antibodies conjugated with Alexa Fluor 488 (1:100, 2 hours, RT). After washing (4 times), coverslips were mounted in Vectashield with DAPI (Vector Laboratories) and analysed using a laser scanning confocal microscope LSM 510 META, Axiovert 200 M, ConfoCor 3 (Carl Zeiss MicroImaging GmbH, Jena, Germany). Two independent experiments were performed.

Statistical analysis

The statistical analysis was performed with the use of the Statistica program (StatSoft Poland). Single factor ANOVA and post-hoc Scheffe's Test was performed. $\alpha = 0.05$, $P<\alpha$ was considered significant.

Results and Discussion

Caveolin-1 expression is maintained or even up-regulated in different human cancers suggesting its role as a marker. Nevertheless, its prognostic significance depends on the type of tumour and its state of development. In the case of melanoma there is ambiguous information concerning caveolin-1 expression. Therefore, the goal of the present study was to compare caveolin-1 expression at the mRNA and protein levels in six melanoma cell lines in different progression stages (primary/metastatic) and of different origins (uveal/cutaneous).

Firstly, using the qRT-PCR technique we analysed *CAV-1* expression in two metastatic (WM-266-4, A375), two primary (WM-115, IGR-39) cutaneous and two primary (mel-202, 92-1) uveal human melanoma cell lines. All chosen melanoma cells expressed readily detectable levels of *CAV-1* mRNA. There were some differences in the levels of *CAV-1* mRNA between cell lines of cutaneous origin, whereas both primary melanoma cell lines of uveal origin displayed significantly lower expression of *CAV-1* mRNA compared with other tested cell lines of cutaneous origin (Fig. 1A).

To gain further insight into caveolin-1 expression in melanoma cells, we then explored the level of caveolin-1 protein in total cellular lysates of all chosen cell lines. We detected protein bands ranging from 21 to 25 kDa equivalent to caveolin-1. We observed marked anti-caveolin-1 antibody staining in extracts of cutaneous melanoma cells. The intensity of this immunoreaction was stronger than that for cell lines of uveal origin, which displayed weak signals suggesting low abundance of caveolin-1 protein (Fig. 1B). We obtained similar results using a monoclonal antibody for Western blotting (data not shown). To our knowledge, this is the first report concerning the presence of caveo-

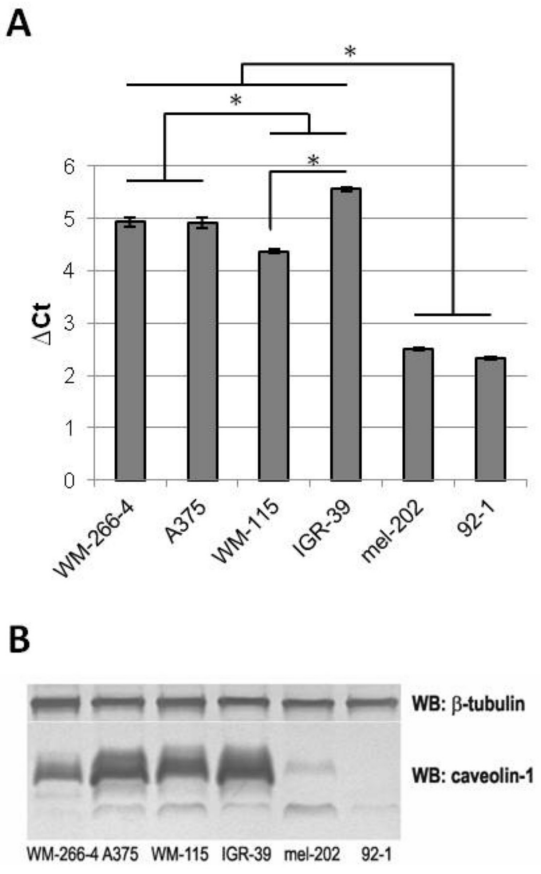

Fig. 1. Expression of caveolin-1 in human melanoma cell lines. Two metastatic cutaneous (WM-266-4, A375), two primary cutaneous (WM-115, IGR-39) and two primary uveal (mel-202, 92-1) melanoma cell lines were used for the experiments. Real-time RT-PCR analysis of *CAV-1* mRNA expression was performed using *RPII* as a reference control. Means of three measurements +/- SEM; *, $\alpha = 0.05$, $P = 0.00$, $P<\alpha$, single factor ANOVA, Post-hoc Scheffe's Test (A). Total protein extracts were separated by SDS-PAGE (10 μg of protein per lane) and subjected to Western blotting analysis using anti-Cav-1 polyclonal antibody. β-tubulin was a loading control (B).

lin-1 in human primary melanoma cell lines of uveal origin (mel-202, 92-1). Caveolin-1 expression has been studied in rodent retinas (SCHERER *et al.* 1997). BERTA *et al.* (2007) used immunocytochemical staining to demonstrate caveolin-1 distribution within different layers of human retina affected by melanoma malignum but without identification of specific cell types. STENZEL *et al.* (2015) have shown that caveolin-1 was expressed in all studied paraffin sections of human primary uveal melanoma. The authors suggested correlation of caveolin-1 expression with tumour size and stage as well as a predicted association with the occurrence of metastatic disease during patient follow-ups. Interestingly, in our study immunoblot analysis revealed that caveolin-1 expression was lower in mel-202 and 92-1 cell lines as compared to cutaneous cell lines. These results were inconsistent with the theory that caveolin-1 expression is elevated in primary melanoma cell lines (FELICETTI *et al.* 2009; LOBOS-GONZÁLEZ *et al.* 2011), however, this relates to cells of cutaneous origin. On the other hand, LOBOS-GONZÁLEZ *et al.* (2014) have shown that caveolin-1 expression is lowest in melanocytes and increases with progression of human cutaneous melanoma.

Data concerning caveolin-1 expression and function in tumour progression is in conflict. The abundance of evidence summarized previously indicates tumour suppressor ability of caveolin-1 hinted by its expression down-regulation that has favoured lung (HO *et al.* 2002) and mammary hyperplasia and tumour formation (WIECHEN *et al.* 2001a; WIECHEN *et al.* 2001b). There is also evidence of an opposite role for caveolin-1 in prostate cancer, where caveolin-1 is known to promote tumour formation and its expression is correlated with poor prognosis and survival ability (BARTZ *et al.* 2008; KARAM *et al.* 2007). In cutaneous melanoma caveolin-1 plays a dual role depending on the presence of its protein partners (LOBOS-GONZÁLEZ *et al.* 2013). According to a phenotype-switching model (HOEK & GODING 2010), it acts as a tumour suppressor during the early stages of the disease while promoting metastasis later on (LOBOS-GONZÁLEZ *et al.* 2014).

Here we reported the presence of caveolin-1 in human cutaneous melanoma cell lines. Our observations were in accordance with the results obtained by FELICETTI *et al.* (2009) who demonstrated caveolin-1 expression in a panel of melanoma cell lines including A375 cells.

We observed a stronger immunoreaction of caveolin-1 protein in the IGR-39 cell line derived from primary tumour of cutaneous origin, consistent with many previous reports indicating that the caveolin-1 level is elevated at the beginning of the disease in comparison to melanocytes and metastatic cells (FELICETTI *et al.* 2009; LOBOS-GONZÁLEZ *et al.* 2011). Two of the analysed cutaneous cell lines, namely WM-115 and WM-266-4, were derived from primary and metastatic sites, respectively, and originated from the same patient. The comparison of results obtained for these cell lines showed lower caveolin-1 immunostaining in WM-266-4 metastatic melanoma cells - supporting the observation of TRIMMER *et al.* (2010), who analysed the expression as well as function of caveolin-1 in melanoma tumour growth and metastasis. By immunoblot analysis, they found higher caveolin-1 expression in primary human melanoma cell lines (WM35, WM-115) compared with reduced expression in metastatic melanoma cell lines including WM-266-4 and A375. Also SENETTA *et al.* (2013) compared the presence of caveolin-1 between A375 and WM-266-4 cell lines, but in this study A375 was described as a non-metastatic cell line. In contrast to our results they detected stronger immunoreaction of caveolin-1 in WM-266-4 in comparison to A375.

Moreover, our results showed different caveolin-1 mRNA/protein expression patterns in WM-115 and WM-266-4 cells. Similarly, the results obtained by Western blot for WM-266-4 and A375 cells did not directly reflect the results achieved by Real-time RT-PCR. This may suggest the occurrence of translational control of caveolin-1 but further research is needed to address this issue. Additionally, the discrepancies between mRNA and protein level of caveolin-1 may be partly explained by the fact that Cav-1 can be regulated by ubiquitination and trafficking to endolysosomes (HAYER *et al.* 2010) or secreted from cells in exosomes (LOGOZZI *et al.* 2009).

Finally, we analysed the cellular distribution of caveolin-1 in the cells under investigation by fluorescence confocal imaging. We observed a different pattern of caveolin-1 subcellular distribution between primary and metastatic cells. All metastatic melanoma cells had rather diffuse cytoplasmic staining of caveolin-1 (Fig. 2A,B). In contrast, primary cells, regardless of their origin (uveal/cutaneous) showed fluorescence in the form of micropatches inside the cells (Fig. 2C-F). It was surprising that only a small part of caveolin-1 was present in the cell membrane. Caveolin-1 is the integral membrane protein found to be associated with endoplasmic reticulum (ER), Golgi and plasma membrane that reflects the transit of caveolin-1 from ER to the plasma membrane via Golgi during synthesis (QUEST *et al.* 2008). Several studies indicate that caveolin-1 is not only an integral membrane protein but, in addition, is also found within the cell associated with mitochondria (LI *et al.* 2001), lumen of ER, in secretory vesicles, as well as in the cytoplasm as a soluble protein, and even in the nucleus (LIU *et al.* 1999; PELKMANS

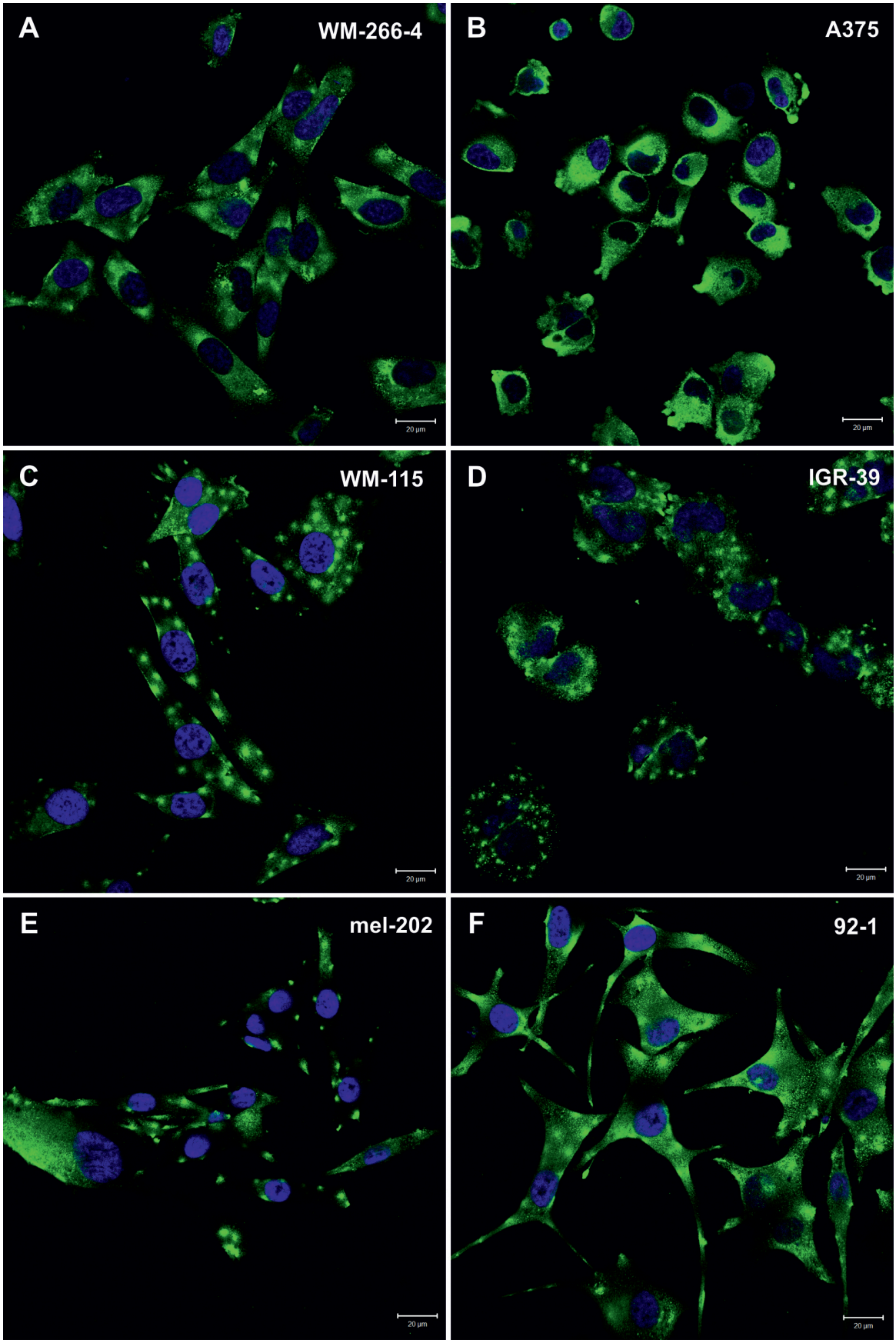

Fig. 2. Immunofluorescence staining of human melanoma cell lines.
Two metastatic cell lines: WM-266-4 (A), A375 (B) and four primary cell lines: WM-115 (C), IGR-39 (D), mel-202 (E), 92-1 (F) were analysed using anti-Cav-1 antibody (green). Nuclei were stained with DAPI (blue). Bars = 20 µm.

et al. 2001; SANNA *et al.* 2007). What determines the distribution of caveolin-1 remains unclear, but it is very important to understand how it affects cell function and contributes to human disease states including cancer (QUEST *et al.* 2008).

In summary, our studies on human melanoma cell lines have shown that caveolin-1 expression was altered in melanoma cell lines of cutaneous origin in relation to the corresponding cells of uveal origin. Importantly, we observed that the level of caveolin-1 in primary melanoma cells of uveal origin was substantially reduced compared to primary cutaneous melanoma. We believe that these findings can give new insight on caveolin-1 expression profiles in melanomas of distinct origin showing that these two melanoma types may differ in many aspects.

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