Dipeptidyl Peptidase IV as a Novel Prognostic Marker and Important Therapeutic Target in Melanoma

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Research

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Abstract

Background

There is a lot of evidence which suggests that DPP IV level may correlate with a type of tumor cells, metastatic potential and prognosis for the patient. Bearing in mind that the melanomas are characterized by high heterogeneity and identification of specific phenotypes of cells allows for early and more effective therapy, the aim of our study was to check whether there is a correlation between the DPPIV and the metastatic potential of melanoma cell lines. Additionally, the aim of our research was to evaluate the anti-tumor potential of linagliptin and saxagliptin in melanoma cell lines as well as determining correlation between cytotoxicity of the drugs and DPP IV level.

Methods

The inhibitory effect of tested drugs on the cancer cell growth was assessed using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) while cell cycle analysis and apoptosis were performed using the NucleoCounter® NC-3000™ system (ChemoMetec, Denmark), following the instructions provided by the manufacturer. DPPIV release by cancer cells was measured by DPP4/CD26 ELISA assay kit for biological samples (Cloud-Clone Corp.,Wuhan,China).

Results

Our results showed that DPPIV overexpression promoted cell proliferation of melanoma cells. Our data showed that especially short term treatment with linagliptin is associated with not only decreased expression of DPPIV and inhibition of cell proliferation but also induction of cell cycle disruption and apoptosis in melanoma.

Conclusions

The routine identification of this glycoprotein in melanoma would be fundamental to assessing not only the risk of metastasis/disease progression but also selection of therapy and evaluation of its effectiveness.

1. Background

Cancer is one of the most common causes of mortality in the world [1]. Late-stage presentation of symptoms, untargeted diagnostic procedures leads to late diagnosis and treatment and thus poor prognosis for the patient. The most common types of cancer diagnosed in men are related to prostate, lung, colon and urinary bladder cancer (CRC). While in women, the three most common cancers are breast cancer (accounting for 30% of all cancer diagnoses), lung cancer, and colorectal cancer [2]. Over the past four decades, we observed a dynamic increase in the incidence of liver, kidney and skin cancer [3].

Melanoma is a skin cancer which belongs to the group of most aggressive human cancers having high ability of metastasis to other organs such as lungs, liver, brain, or lymph nodes [4]. It is one of the cancers that is still escalating in incidence [5]. More than more than 50% of cases of this cancer are diagnosed in advanced stages, which in turn leads to poor prognosis and elevated mortality [7]. The hallmarks of these tumors include a high biological heterogeneity of cells, highly infiltrative behavior and resistance to standard treatment [6]. Previous research indicates that melanoma cells present in the primary tumor undergo intense morphologic, genotypic and phenotypic changes which alters their signaling pathways and results in rapid development of cell resistance per applied therapy [9, 10].

The identification of specific cancer cells population by specific proteins or genes may be of high importance in the diagnosis of tumors with high cellular heterogeneity such as melanoma [11–15]. Additionally, the use of selected predictive markers characterized by specific sensitivity or resistance to certain forms of treatment could have practical application in both selection and evaluation of effective therapy. Despite significant progress in research on melanoma heterogeneity, the identification of individual sub-populations of cells is still extremely difficult. These diagnostic limitations are influenced not only by the high genetic diversity of cells, but also acquired heterogeneity, resulting from cell reaction to changes in the environment of growing tumor [16].

Dipeptidyl peptidase IV (DPPIV), also known as CD26 is a multifunctional glycoprotein present on the surface on numerous different cell types such as epithelia and endothelia of the systemic vasculature, lung, pancreas, spleen, kidney, small intestine, heart, hepatocytes and immune system cells [17–19]. Part of the studies shows the participation of DPPIV in the development of neoplastic diseases. Mechanisms that regulate DPPIV gene transcription and enzymatic activity in cancer cells are not fully understood so far. DPPIV expression may be dependent on various factors, including the studied cells type and phenotype, tumor location as well as grade of malignancy also from presence in surrounding of cancer tissues environmental factors [20–25]. DPP-IV can be strongly expressed in some cancer cells, while being absent or present at low levels in others [21, 26–29]. In some forms of cancer, increased cell invasiveness may be correlated with decreased CD26/DPP4 expression [30–35] while in other type of aggressive malignancies, such as ovarian cancer [36], renal cell carcinoma [37], chronic lymphocytic leukemia [38] or thyroid cancer [39] over-expression of DPPIV is observed. The available research shows that DPPIV expression level may play important role in pathogenesis of melanoma [21]. It is speculated that DPPIV expression level significantly affects the regulation of expression of bcl-2 family proteins and hence melanoma cells resistance to apoptotic death [40–43]. As it turns out one of the key factors contributing to changes in DPPIV expression and increased susceptibility of cells to oncogenic transformation is variable level of oxygen [44]. Previous research proved that, in response to hypoxia, HIF-1α is activated which strongly induces DPPIV expression. This process may be correlated with chemoresistance, metastasis and poor prognosis for patients with ovarian cancer [44, 45]. Also, studies on other types of human cells confirmed the significant influence of hypoxia on the level of DPP IV expression [46–48]. Other research also shows that melanocytes are more prone to oncogenic transformation when grown in a low oxygen microenvironment [49]. The available research shows that hypoxia contributes to the metabolic changes inside cancer cells and genomic changes that make it

possible for the cells to adapt to very low oxygen concentrations and limited access to nutrients, thus remaining viable. Also, hypoxia can affect HIF1a or/and transcription factor (MITF) level in melanoma cells which determines differentiated and invasive this cancer cells [50–53]. So, the ratio of specific cell phenotypes and imbalance in both oxygen supply and consumption in a melanoma tumor may have fundamental meaning to DPPIV expression and hence disease progression as well as effectiveness of the therapy.

Given that the DPP-IV may play an important role in the pathogenesis of various human cancers, the aim of this study was to examine the level of DPPIV release by selected cancer cell lines characterized by high invasiveness and resistance to available therapies. Bearing in mind that the melanomas are characterized by high cellular heterogeneity and that early reliable identification of specific phenotypes of cells allows more efficient therapy, three different melanoma cell lines characterized by differential expression of DPPIV: SK-MEL-28, A-375 and G-361 were selected for the next stage of our research. Considering the fact that the tumors consists of cells with different metabolic phenotype and different sensitivity to periodic hypoxic states which may result in developing resistance to chemo- and radio-therapies, the aim of our research was to assess impact of hypoxia on the viability and DPPIV release by melanoma cells. In this study, we tested the hypothesis that linagliptin and saxagliptin, drugs belonging to DPPIV inhibitors by down regulation of DPPIV, may contribute to the growth control in melanoma cells by the inhibition of cell proliferation, cell cycle arrest and induction of apoptosis.

2. Materials And Methods

2.1. Reagents

Reagents were purchased from the following sources: linagliptin, saxagliptin (Selleckchem, Munich, Germany), thiazolyl blue tetrazolium bromide (MTT; Sigma-Aldrich, Steinheim, Germany), dimethyl sulfoxide (DMSO; POCH S.A. Avantor Performance Materials, Inc., Gliwice, Poland), phosphate-buffered saline (PBS; Mediatech, Inc. Corning Subsidiary, Manassas, VA, USA). The Eagle's Minimum Essential Medium (EMEM, ATCC, Manassas, VA, USA), Dulbecco's Modified Eagle's Medium (DMEM, PAN-Biotech GmbH, Aidenbach, Germany) or RPMI-1640 (PAN-Biotech GmbH, Aidenbach, Germany), foetal bovine serum (FBS) the antibiotics (penicillin, streptomycin, amphotericin B) were purchased from PAN-Biotech GmbH (Aidenbach, Germany). Trypsin solution (0.25% trypsin/2.21 mM EDTA) was obtained from Mediatech, Inc. Corning Subsidiary (Manassas, VA, USA).

2.2. Cell culture

The cancer cell lines: PC-3 (catalog no. CRL-1435™), DU-145 (catalog no. HTB-81™), SK-MEL-28 (catalog no. HTB-72™), A-375 (catalog no. CRL-1619™), G-361 (catalog no. CRL-1424™), Hep G2 (catalog no. HB-8065™) and 769-P (catalog no.CRL-1933™) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in EMEM, DMEM or RPMI-1640 medium with 10% FBS and antibiotics: 100 U/ml penicillin, 100 µg/ml streptomycin and 2.5 µg/ml amphotericin B. The cell lines were routinely

screened for mycoplasma and maintained in a humidified atmosphere with 5% CO_2 at 37°C in a cell incubator. Cells were grown in 75 cm^2 tissue culture flasks (EasYFlasks™ Nunclon™ Δ; Nalge Nunc International, Penfield, NY, USA). Before the experiment, cells were trypsinized (0.25% trypsin/2.21 mM EDTA) and seeded in 96-well or 6-well plates (SPL Life Sciences, Pocheon, Korea) at a density of $1x10^5$ cells/ml. The prepared plates were incubated for 24 h in order to achieve cell adhesion. After this time, drugs were added to the cells in the right concentrations and incubated for 24 hours. Control cells were incubated only with the appropriate medium to the line.

2.3.Identification of DPPIV of cancer cell lines

After treatment under various conditions cancer cells with or without DPPIV inhibitors for 24 h, the culture medium was harvested and cells were washed with phosphate-buffered saline(PBS) and subsequently removed and were lysed by sonication with extract buffer (Lysis Buffer1; Cloud-Clone Corp.,Wuhan,China) and each lysate was then centrifuged at 9000×g for 20 min at 4°C. The cell culture supernatants were collected, and the DPPIV release by cancer cells was measured by DPP4/CD26 ELISA assay kit for biological samples (Cloud-Clone Corp.,Wuhan,China). The assays were performed in duplicates according to the manufacturer's instructions. The measurement was performed spectrophotometrically using a microplate reader at 450nm wavelength. The protein concentrations were determined with the Bradford method using bovine serum albumin as a standard [76]. The DPPIV values were normalized by the amount of protein (Bradford method) and then leveled in proportion to the control.

2.4. Selection of cancer cell lines

To the next stage was selected melanoma cell lines on the basis of the differential expression of DPPIV with emphasis on high level of DPPIV in SK-Mel-28 cell line as compared to other cancer line. The melanoma cancer cell lines selected SK-MEL-28, A-375, G-361 represent cells with high and medium expression of DPPIV and diversified metastatic potential.

2.5. Cell sensitivity of drugs and changing conditions/MTT assay

The MTT assay was performed to investigate the cell proliferation and viability. It was made in accordance with DB-ALM Protocol no. 17 (European Centre for the Validation of Alternative Methods, Database Service on Alternative Methods to Animal Experimentation). The drugs (linagliptin, saxagliptin) were dissolved in DMSO to prepare a primary stock solutions (concentration of 1250 μM) and subsequently diluted to the final concentration with in appropriate medium for the line EMEM or DMEM medium. The solutions were prepared ex tempore. The final concentration of DMSO did not exceed 0.5% v/v and did not affect cell viability. Log phase SK-MEL-28, A-375, G-361 cells were seeded in 96-well plates were treated for 24 h with linagliptin or saxagliptin (concentration range of 20-1250 μM) under normoxia (21% O2 and 5% CO2) or hypoxia conditions. For hypoxia exposure, cells were incubated and treated in an in a sterilized anoxic-hypoxic chamber (Modular Incubator Chamber MIC-101, Billups-Rothenberg). Cells had access to limited oxygen (1% O2, 5% CO2, N2 to 94%) which is referred to as

hypoxia. Subsequently, 10 µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/ml in PBS) were added to the each well of a microplate and incubated for an additional 3 h at 37˚C each of the conditions. In the next step, free serum culture medium containing MTT was discarded from each well, and the formazan crystals were dissolved by addition 100 µl DMSO. The absorbance was measured at 550 nm using a microplate reader ELx808IU (BioTek Instruments Inc., Winooski, VT, USA). The experiment was performed twice in triplicate. In the MTT assay, the extent of formazan crystal formation correlates with the number of viable cells.

2.6. Cell cycle analysis

Two-step cell cycle analysis was performed using the NucleoCounter® NC-3000™ system (Chemometec, Allerød, Denmark), following the instructions provided by the manufacturer. For cell cycle analysis, the SK-MEL-28, A-375 and G-361 cells were seeded at a density of $1x10^5$ cells/ml in 35-mm 6-well plates, treated with IC50 concentrations of linagliptin and saxagliptin indicated above for 24 h. After treatment, cells were washed with PBS and suspended in 250 µl Solution 10 (lysis buffer) supplemented with 10 µl DAPI, and were incubated for 5 min at 37˚C, next was added 100 µl Solution 11 (stabilization buffer). The suspension of cells was loaded into the chambers of the NC-Slide A8™ in the volume of 10 µl, and subjected to cell-cycle analysis. The results in the form of a histograms represents the percentage division of cells in the different cell cycle phases (sub-G1/G1, S and G2/M).

2.7.Cell apoptosis analysis

Cell apoptosis analysis was conducted using the Annexin V assay for the NucleoCounter® NC-3000™ system, according to the manufacturer's instructions. The SK-MEL-28, A-375 and G-361 cells were seeded in 6-well plates and incubated 24 h with IC50 concentrations of linagliptin and saxsagliptin (78 and 468 µM for SK-Mel-28; 157.59 and 262.43 for A-375, 188.67 and 265.07 for G-361 respectively). After incubation, the cells were harvested by centrifugation at 400g for 5 min. Supernatant was carefully removed and the cell pellet was gently re-suspended in 100 µl Annexin V binding buffer, supplemented with 2 µl Annexin V-CF488A and 2 µl Hoechst 33342, and was incubated for 15 min at 37˚C. Subsequently, stained cells were centrifuged at 400g for 5 min and re-suspended in 100 µl Annexin V binding buffer with 10 µl propidium iodide (PI) solution. The suspension of stained cells was loaded into the chambers of the NC-Slide A2™ in the volume of 30 µl, and subjected to cell-apoptosis analysis. The quantification of early apoptotic cells was based on Annexin V binding and PI exclusion. The obtained histograms were used to demarcate the percentages of PI negative cells with low vitality, PI negative cells with high vitality (healthy cells), and PI positive cells (dead cells).

2.8. Statistical Analysis

The results are expressed as mean ± SD. The data represents the mean of 2 independent experiments, each consisting of 3 replicates. Data was analysed using the Student's t test or one-way analysis of variance (ANOVA) wherever required using the program Graph Pad Prism software, version 8.0. IC_{50}

values for the tested drugs were derived from the concentration-response curves. A *p*-value of < 0.05 was considered to indicate a statistically significant result.

3. Results

3.1. Identification of DPPIV in cancer cell lines

DPPIV was identified in seven cancer cell lines: PC-3, DU-145, SK-Mel-28, A-375, G-361, 769-P, HepG2, representing four distinct tumor types (Fig. 1). The selected cancer cell lines represent different cells characterized by high, medium and low level of DPPIV. The results of the analysis showed that the DPPIV level was the highest in SK-MEL-28 cells and the lowest in human prostate carcinoma cell lines (PC-3 and DU-145). To the next stage of research, on the basis of the differential expression of DPPIV, the melanoma cell lines were selected with emphasis on high level of DPPIV in SK-Mel-28 as compared to other cancer lines. Selected melanoma cell lines (SK-Mel-28, A-375, G-361) represent cells with high and medium expression of DPPIV.

3.2. Effect of hypoxia on viability of melanoma cells and level of DPPIV release

As Fig. 2A shows, tested melanoma cell lines were sensitive to hypoxia. The G-361 line turned out to be most sensitive to hypoxia in the contrary to the least sensitive SK-Mel-28 cells. After 24 hours the cell viability was decreased by 60% ($p < 0.001$; $t = 14.171$; $F = 1.351$) and 15% ($p > 0.05$) respectively when compared to viability of normoxic cells. Given the high viability of SK-Mel-28 cells oxygen deprived, it can be concluded that these cells may be characterized by the glycolytic phenotype. There was not significant statistically difference in the viability of SK-Mel-28 cells under normoxic and hypoxic conditions ($p > 0.05$; $t = 2.24$; $F = 3.08$).

Among the other examined melanoma lines, A-375 line was slightly less sensitive to the hypoxia than G-361 line. The viability of this cell line was decreased by 32% under hypoxic conditions compared to cells grown under normoxic conditions ($p < 0.01$; $t = 6.640$; $F = 1,678$).

In Fig. 2B, it can be seen that DPPIV level depends on not only culture conditions but also type of cell line. Under hypoxia conditions DPPIV level is down-regulated especially in SK-Mel-28 melanoma cell line. The hypoxia leads to significant decrease in DPPIV release by cancer cells (almost half; $p < 0.001$; $t = 39.202$; $F = 8.733$) compared to the level of this parameter under normoxic conditions. In the case of A-375 cells and G-361 line, the significant differences in DPPIV levels released by these cell lines under hypoxic and normoxic conditions weren't observed ($p > 0.05$, the results aren't statistically significant).

Melanoma cells (SK-Mel-28, A-375 and G-361 lines) were cultured under normoxic or hypoxic (1% O2 was referred as hypoxia) conditions for 24 hrs and then measured: (A) The viability of cells by the MTT assay. Cell viability in hypoxia was calculated vs. the same line of cells in normoxia which was set to 100% viability; (B) DPPIV level was measured in the cell lysates by DPP4/CD26 ELISA assay kit. DPPIV level in

hypoxia was compared with level of this parameter in the same line of cells in normoxia. The data represent the mean of 2 independent experiments, each consisting of 3 replicates. The bars represent the means ± SD. (*$p < 0.05$, **$p < 0.01$, *** $p < 0.001$ compared to normoxic cells).

3.3. Evaluate of sensitivity of melanoma cell lines to linagliptin and saxagliptin under hypoxia and normoxia

In general, linagliptin was significantly more active in evaluated melanoma cell lines in hypoxic environment than saxagliptin (Fig. 3A-F). It was most noticeable for SK-Mel-28 line. Linagliptin use in concentrations above 100 μM led to a very dynamic decrease in the viability of this cancer cells line under hypoxic conditions compared to the viability of normoxic cells (Fig. 3A). Linagliptin at a concentrations ≥ 250 μM showed 95% cytotoxicity against the SK-Mel-28 line ($p < 0.001$; t = 9,313; F = 25.584). Saxagliptin showed significantly the lowest efficiency in relation to this cancer line under hypoxia compared to normoxia (Fig. 3D). It only in a concentration above 750 μM significantly decreased the viability of SK-Mel-28 cells compared to the viability of normoxic cells. Furthermore hypoxia significantly increased resistance of SK-Mel-28 line to low concentrations of both linagliptin and saxagliptin (Fig. 3A, 3D).

Exposure of A-375 melanoma line to drugs in hypoxic conditions effectively reduced their viability (Fig. 3B, 3E). Linagliptin and saxagliptin significantly inhibited A-375 cell survival in a wide concentration range (20-1250 μM, $p < 0.01$, $p < 0.001$) under hypoxic conditions compared to cells treated under normoxic conditions. Nearly identical results were observed for G-361 line. The aforementioned cells were sensitive to the cytotoxic effect of drugs already at the lowest concentrations compared to the viability of normoxic cells (Fig. 3C and 3F).

All the melanoma cells tested - SK-Mel-28, A-375, G-361, were incubated with different concentrations (20-1250 μM) of linagliptin (A, B and C) or saxagliptin (D, E and F) for 24 hours under hypoxic (red lines) or normoxic conditions (blue lines). Sensitivity of melanoma cell lines were evaluated by measuring cell viability by the MTT assay (n = 3 replicate experiments for each cell line with each drug). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to normoxic cells.

3.4. Effect of linagliptin and saxagliptin on DPPIV release by melanoma cell lines

24 hrs after administration of test drugs at concentrations corresponding to their IC_{50} values estimated in appropriate lines, DPPIV level released by the melanoma cells was determined. SK-Mel-28 cells treated with linagliptin in a concentration of 78.26 μM or saxagliptin in a concentration of 468.72 μM remained sensitive to both drugs and showed the decrease of DPPIV levels (Fig. 4A, $p < 0.001$, t = 5.993, F = 9.175; $p < 0.01$, t = 4.586, F = 13.735, respectively). Linagliptin and saxagliptin reduced DPPIV level by 43% and 36% in the SK-Mel-28 cell line, respectively. Also, incubation of A-375 and G-361 cell lines with linagliptin (in concentration 157.59 μM or 188.67 μM respectively) or saxagliptin (262.43 or 265.07 μM, respectively) led to a significant decrease of DPPIV level released by cells compared to DPPIV levels in untreated cells (Fig. 4B $p < 0.01$, t = 3.658, F = 3.268; Fig. 4C $p < 0.05$, t = 2.694, F = 1.657). In all the tested melanoma

lines, it was observed that DPPIV level release in linagliptin-exposed cells was lower than in saxagliptin-treated cells (by 10–15%).

DPP IV release by cancer cells was measured by DPP4/CD26 ELISA assay kit after treatment; SK-Mel-28 cells (A), A-375 cells (B) and G-361 cells (C) were incubated for 24 hours with linagliptin or saxagliptin. Linagliptin at a concentration of 78.26, 157.59 or 188.67 μM was selected for study as these values represented the IC_{50} for the Sk-Mel-28, A-375 and G-361 melanoma cell lines, respectively. We were guided by the same rationale in selecting saxagliptin concentrations (468.72, 262.43 or 265.07 respectively). Data represents the mean of two independent experiments, each consisting of three replicates. $^{**}p < 0.01$, $^{***}p < 0.001$ compared to untreated melanoma cells.

3.5. Effect of linagliptin or saxagliptin on the cell cycle of melanoma cells

In SK-Mel-28 cells, both linagliptin and saxagliptin slightly increased the percentage of these cells in the G1 phase. The number of cells in this stage of the cell cycle increased by 9–17%, vs. the untreated cells (Fig. 5A). Linagliptin at a concentration of 157.59 μM or 188.67 μM, respectively, resulted in sub-G1 cell cycle arrest in A-375 and G-361 cells (Fig. 5B-C). However, the changes were the most visible in case of G-361 cells where the increase by 38% was observed in above-mentioned cell cycle phase. In turn, saxagliptin used at concentration corresponding to its IC_{50} (i.e. 262.43 μM or 265.07 μM) moderately affect the cell cycle of melanoma A-375 or G-361 cell lines (Fig. 5B-C). In addition, it was observed a decrease by 2–5 fold of cells in the G2 phase in all melanoma lines after the application of appropriate IC_{50} concentrations of linagliptin or saxagliptin (Fig. 5A-C).

3.6. Effect of linagliptin and saxagliptin on the cell apoptosis of melanoma cells

In melanoma cells stained with Annexin V-CF488A and propidium iodide (PI), there was observed an apoptogenic potential of linagliptin and saxagliptin (Fig. 6A-C). Both linagliptin (78.26 μM) and saxagliptin (468.72 μM) induced early and late apoptotic mechanisms in SK-Mel-28 cells (Fig. 6A). As shown in Fig. 6B, in A-375 cell line, a treatment with saxagliptin in a concentration of 262.43 μM led to a 3-fold increase of cell number in the early phase of apoptosis, while after an incubation with linagliptin in a concentration of 157.59 μM almost 2-fold increase was noted when compared to untreated cells. In addition, the population of late apoptotic cells was enhanced by 29% or 25% after treatment with linagliptin or saxagliptin, respectively. In turn, the number of necrotic cells in SK-Mel-28 and A-375 lines was slightly increased after 24-h incubation with drugs.

In the case of G-361 cell line, there was only a significant increase in the number of early apoptotic cells from 7–20% after application of saxagliptin in a concentration of 265.07 μM. In turn, linagliptin in a concentration of 188.67 μM led to a significant increase in the percentage of late apoptotic cells (by 64%) (Fig. 6C) while remaining without any significant effect on the early apoptotic cells' population. Neither

linagliptin nor saxagliptin, significantly enhanced the number of necrotic cells in G-361 line. The number of these cells ranged from 3 to 5%.

The melanoma cells were stained with Annexin V (Annexin V-CF488A) and propidium iodide (PI). Scatter plots demonstrate percentage of viable, apoptotic and necrotic SK-Mel-28 (A), A-375 (B), G-361(C) cells after a 24-h treatment with linagliptin or saxagliptin. The lower left square presents viable cells; right lower square shows early apoptotic cells; right upper square shows late apoptotic cells, and left upper square presents necrotic cells.

4. Discussion

Melanoma is one of the most complex and heterogeneous cancers [9, 10, 54]. Despite significant progress in research on melanoma heterogeneity, the identification of individual sub-populations of cells is still extremely difficult. These diagnostic limitations are influenced not only by the high genetic diversity of cells (in melanoma is the highest frequency of mutation from all cancers), but also acquired heterogeneity, resulting from cell reaction to changes in the environment of growing tumor. The literature data indicate that about 55% of solid tumors (including melanomas) are characterized by areas of hypoxia or anoxia in where the oxygen concentration doesn't excel 1.5% [51, 55, 56].

The available research shows that DPPIV glycoprotein can play an important role in development and progression of neoplastic diseases probably through the enzymatic and non-enzymatic mechanisms. An analysis of the available literature indicates differential CD26/DPP4 expression in different cancer types [26–38]. Our research confirmed differential expression of DPPIV in well-known cancer cell lines PC-3, DU-145, SK-Mel-28, A-375, G-361, 769-P, HepG2 representing 4 distinct tumor types. Our research showed that the greatest changes in the released glycoprotein level were observed between three different melanoma cell lines SK-MEL-28, A-375 and G-361. The human melanoma cell lines used in our studies have been previously described as lines of high and low metastatic potential, respectively [60]. It was already determined that the sensitivity of tumor cells to changes in microenvironmental conditions like oxygen or nutrient deficiency can be diverse (from adaptation up to cells death) [57–60].

Research shows melanoma cells are capable of adapting quickly to changing microenvironmental conditions by leading to cellular metabolism changes (from oxidative phosphorylation to aerobic glycolysis for sufficient ATP production) [51, 56, 58, 59]. Our research indicates diverse sensitivity of melanoma cells to oxygen deficiency both in terms of cell viability and DPPIV glycoprotein levels. The SK-Mel-28 line known for high metastatic potential, was distinguished from other melanoma lines by a high viability but also level of DPPIV under hypoxia conditions. It should be noted that there was no research prior to ours which would evaluate the level of DPPIV in this melanoma cell line under conditions of hypoxic growth that mimics the tumor microenvironment.

Based on the analysis of the available literature, it can be concluded that hypoxia changes the pH in the intracellular and extracellular space. This could trigger an adaptive response among melanoma cells and activate glycolysis which stimulates intense growth of cells despite the seemingly unfavorable

environmental conditions [61, 62]. The glycolytic phenotype of melanoma cells appears to be closely associated with higher metastatic potential and resistance to anti-cancer therapy.

Widmer *et al.* [51] showed that under hypoxic conditions the invasiveness of proliferative melanoma cell may be increased from 2- to 4-fold. Feige *et al.* [52] reached similar conclusions that hypoxia, through HIF1a, alters the gene-expression in proliferative melanoma cells, making them more invasive in *in vitro* assays. Cheli *et al.*[53] showed that hypoxic conditions lead to a decrease in microphthalmia-associated transcription factor (MITF) expression which leads to increase in metastatic potential of melanoma cells *in vivo*. On the other hand, significantly lower cell viability of the A-375 and G-361, known for their low metastatic potential [60, 67], may suggest that hypoxia is not a factor which can trigger these cells adaptive switch to highly proliferative phenotype.

In our studies we noticed that, DPPIV level may depend not only on type of melanoma cell line but also on culture conditions. The change in the microenvironmental conditions for SK-Mel-28 cells determined the level of glycoprotein released by them. We didn't observe such dependencies for other tested melanoma lines. Regardless of the aforementioned observations, we were surprised with high level of glycoprotein released by examined melanoma cells. The analysis of limited number of available previous studies pointed to decrease or loss of CD26/DPPIV expression in the course of neoplastic transformation of melanocytes [21–23, 43]. In turn, the noticeable differences in the level of DPPIV released by SK-Mel-28 in the environmental conditions tested, may be due to activation of glycolysis by hypoxia which leads to extracellular acidosis of melanoma. This process could eventually result in lower DPPIV level compared to the level of this parameter under normoxic conditions (the optimal pH for DPPIV is 7.8) [61, 62].

Our studies found that DPPIV inhibitors, especially linagliptin, can inhibit melanoma cell proliferation in hypoxia conditions. Already the lowest concentrations of drugs dynamically reduced viability of the most sensitive to hypoxia cell lines: A-375 and G-361. Linagliptin at a concentration of 157.59 μM or 188.67 μM, respectively, significantly inhibited recruitment of the above-mentioned melanoma cells from sub-G1 phase to further stages of the cell cycle. Interestingly, the most hypoxic-resistant SK-Mel-28 line has undergone cytotoxic effects of linagliptin (IC_{50} 78.26 μM) considered to be the most potent and selective dipeptidylpeptidase IV (DPPIV) inhibitors in this class of antidiabetic agents [71]. Linagliptin triggered early and late apoptosis of the above-mentioned melanoma cells. A 2- fold increase of early apoptotic cells and over a 10-fold enhancement of late apoptotic cells were noticed after treatment with this gliptin. Also, Li *et al.* [72] revealed, the cytotoxic action of linagliptin in colorectal cancer cell line. The cytotoxic effect of linagliptin was dependent on the dose and the time of exposure of cancer cells. Linagliptin significantly inhibited HCT116 cell proliferation by cell cycle arrest at G2/M and S phase and the induction of cell apoptosis. In our study, we observed the increased population of SK-Mel-28 cells in G1 phase after treatment with aforementioned gliptin. Yang *et al.*[73] confirmed the anti-cancer activity of sitagliptin in their research. Sitagliptin limited cell proliferation and invasiveness of endometrial cancer through regulation of HIF-1α and VEGFA signaling dependent on DPPIV expression.

Our results showed that lowering the level of DPPIV in all melanoma cell lines correlated with apoptogenic potential of linagliptin and saxagliptin. Despite extensive research into the new molecules which can initiate and regulate apoptosis in melanomas, still there is no enough information on how to effectively limit their chemoresistance. The gliptins we tested, especially linagliptin, turned out to be strong inducers of apoptosis in melanoma cells which are known not only from apoptotic pathway evasion strategy, but also from the unchecked proliferation. Linagliptin and saxagliptin in melanoma cells, induced both early and late apoptotic mechanisms [43, 75]. Moreover, the activation of apoptosis by DPPIV inhibitors led to a significant decrease in the number of viable melanoma cells. This may suggest that DPPIV is a promising therapeutic target for melanomas treatment.

In the future study, we want to provide information on the role of DPPIV inhibitors in modulation of melanoma cells resistance in available anti-cancer therapies. We want to check whether modulation of DPPIV expression translates to delay of melanoma cells resistance of anti-cancer therapy.

5. Conclusions

In our humble opinion, DPPIV should strongly be considered as a prognostic marker in screening tests of melanoma. The presence of high DPPIV level in SK-Mel-28 cell line under normoxia and hypoxia makes it possible to conclude that thanks to this glycoprotein it is possible to identify this particular cell line in a heterogeneous sample tumor, regardless of oxygen availability for this cells. Finally, routine identification of this glycoprotein in melanoma cells would be fundamental to assess not only the risk of metastasis/disease progression but also selection of therapy as well as evaluation of its effectiveness.

Our results also show that DPP IV may represent a potential novel therapeutic target for melanoma. Perhaps, in the future gliptins turn out to be important components of anti-cancer therapies to enhance their cytotoxic effect or they will constitute an alternative form of therapy when conventional treatment does not provide satisfactory results.

Declarations

Ethics Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

Not applicable.

Competing interests

The authors declare no conflict of interest.

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Authors' Contributions

Conceptualization, IPC; Methodology, IPC, MGG; Formal Analysis, DNC and MH; Data Curation, MH, MI; Investigation, IPC, MI and DNC; Visualization, DNC, MI; Supervision, MH and AKP; Writing – Original Draft Preparation, IPC; Writing – Review & Editing, MGG, AKP and JD. All authors read and approved the final version of manuscript.

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Figures

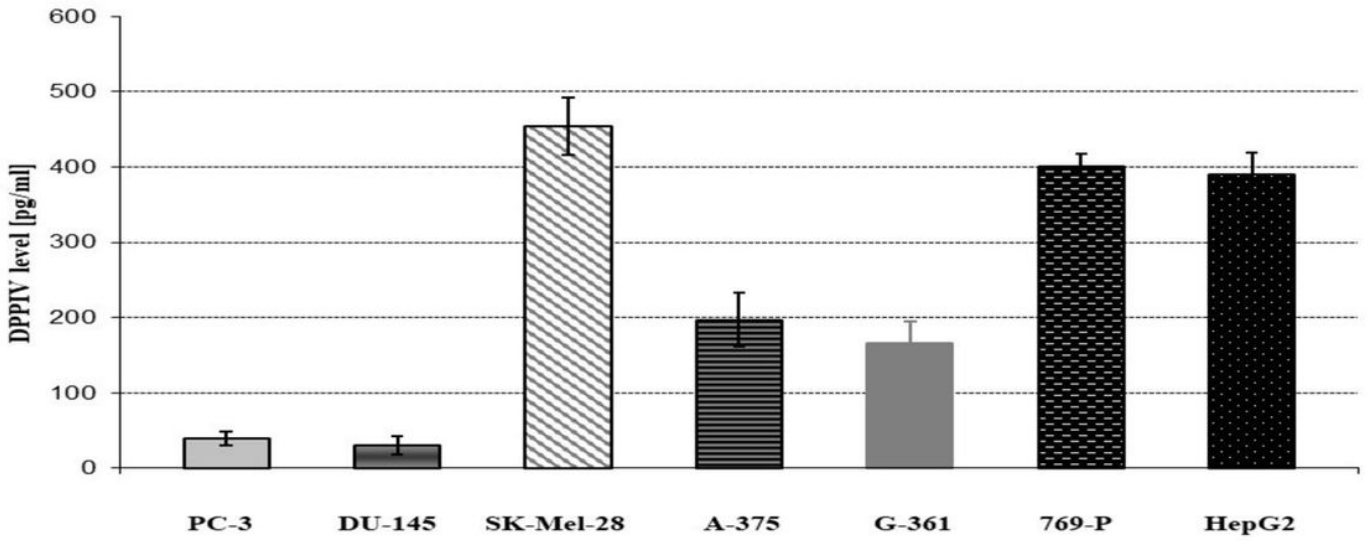
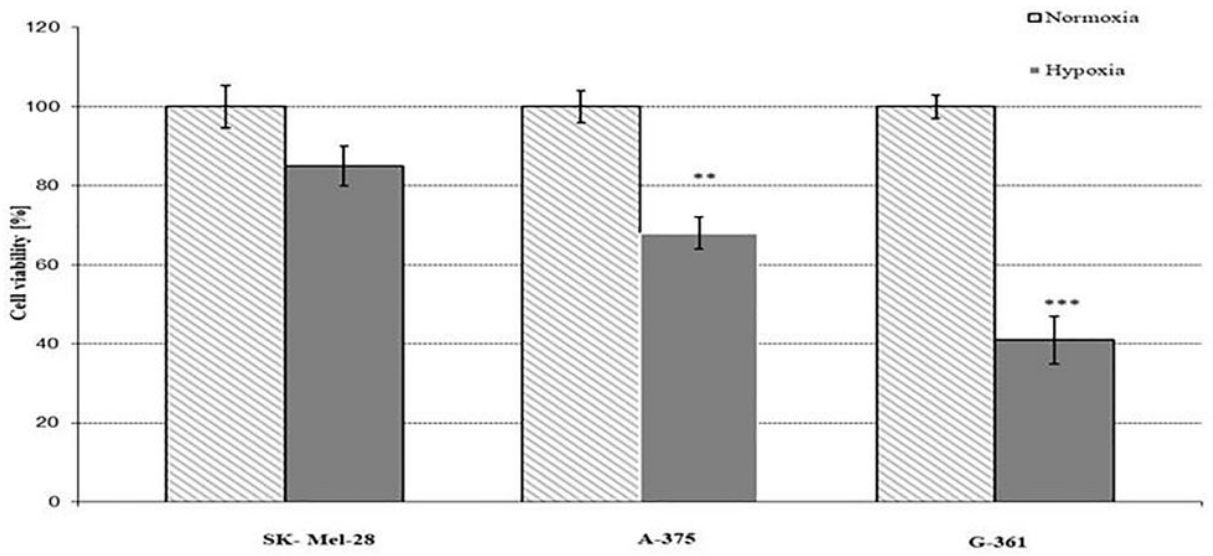


Figure 1

Level of DPPIV in selected human cancer cell lines. Level of DPPIV was measured in cancer cell lysates by DPP4/CD26 ELISA assay kit. The data represent the mean of 2 independent experiments, each consisting of 3 replicates. The bars represent the means ± SD.

2A



2B

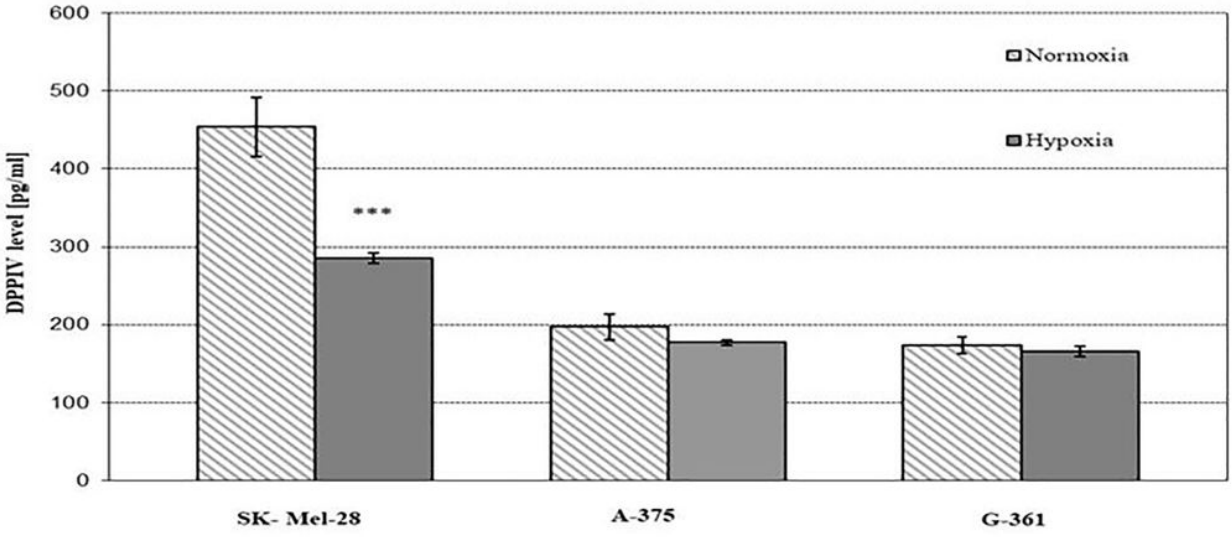


Figure 2

Effect of hypoxic conditions on viability of human melanoma cells and level of DPPIV release.

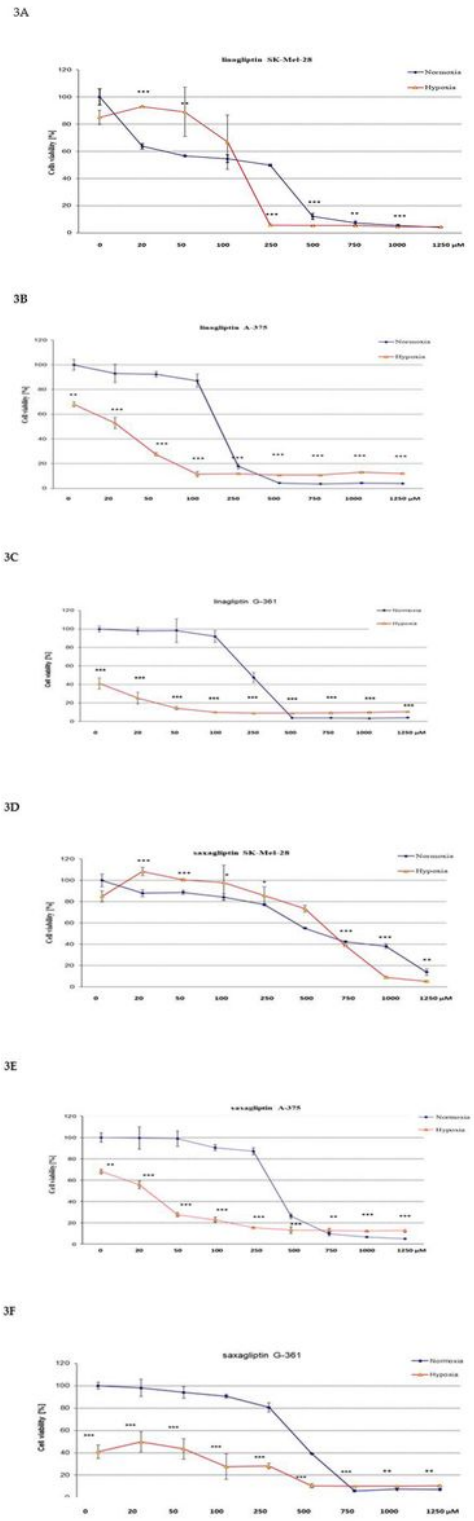
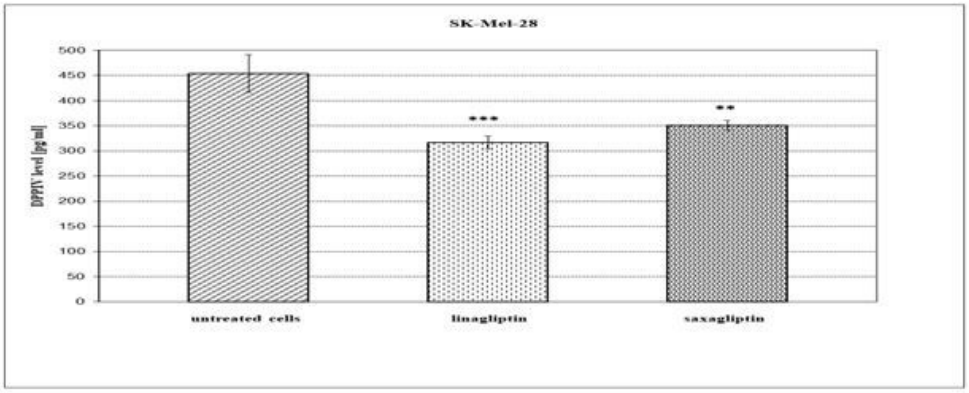


Figure 3

Effect of linagliptin or saxagliptin on viability of melanoma cell line in normoxia and hypoxia conditions. All the melanoma cells tested - SK-Mel-28, A-375, G-361, were incubated with different concentrations (20-1250 µM) of linagliptin (A, B and C) or saxagliptin (D, E and F) for 24 hours under hypoxic (red lines) or normoxic conditions (blue lines). Sensitivity of melanoma cell lines were evaluated by measuring cell

viability by the MTT assay (n = 3 replicate experiments for each cell line with each drug). *p<0.05, **p<0.01, ***p<0.001 compared to normoxic cells.

4A



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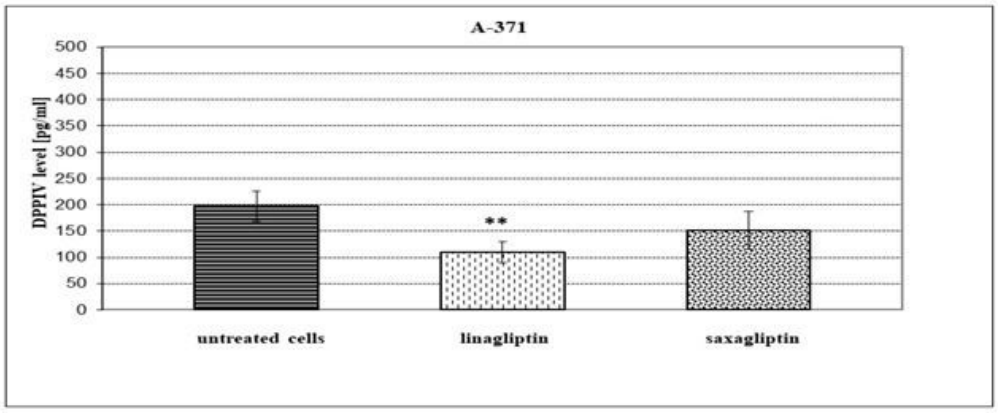

4C

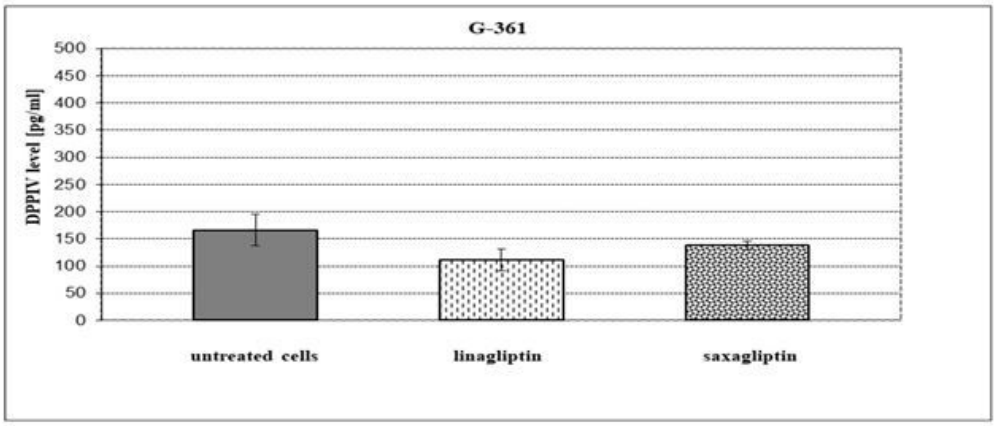

Figure 4

Effect of linagliptin and saxagliptin on DPP IV release by melanoma cells.

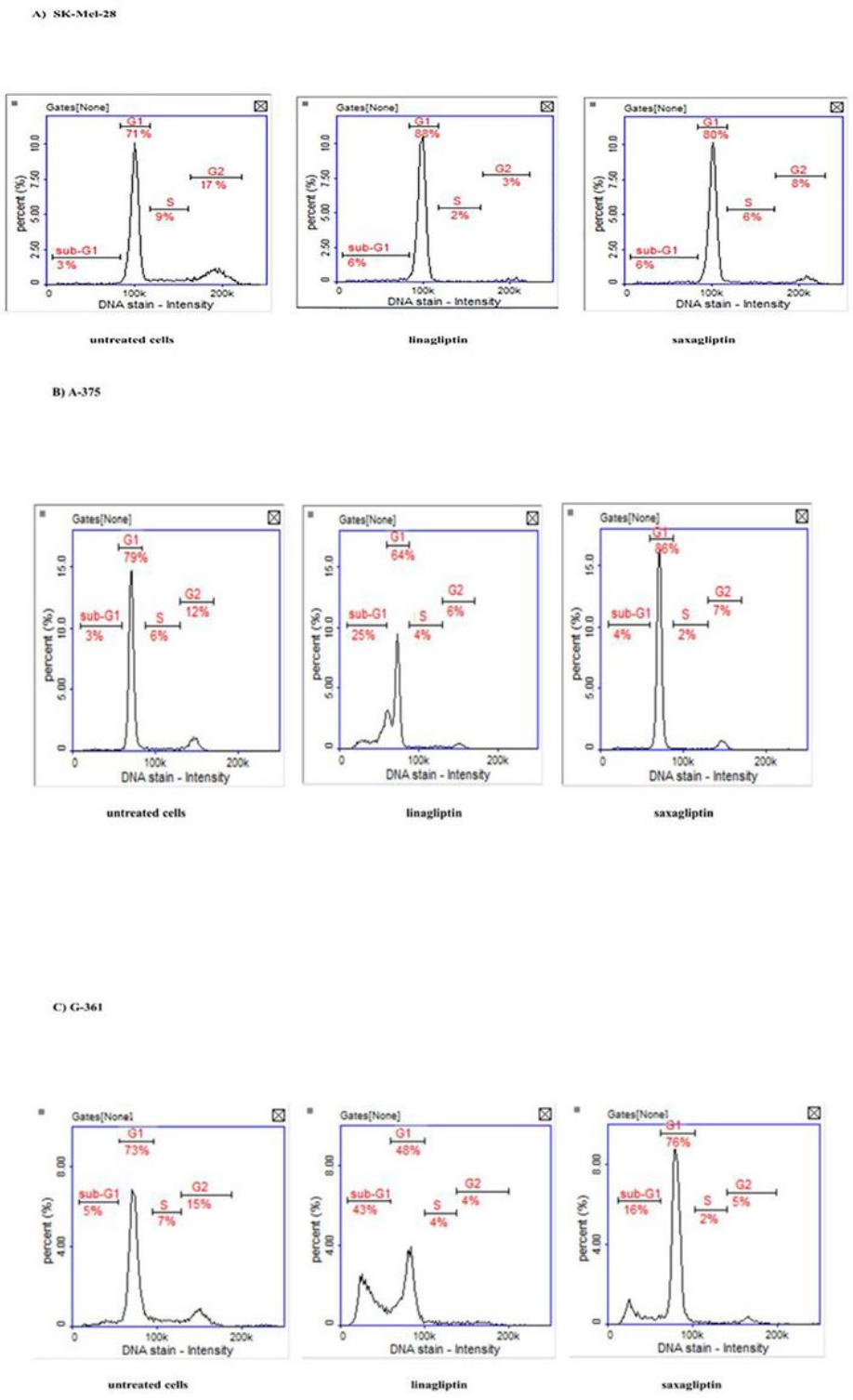

Figure 5

Cell cycle analysis of melanoma cell line SK-Mel-28 (A), A-375 cells (B) and G-361 cells (C) following a 24-h incubation with linagliptin or saxagliptin. The determination of the percentage division of SK-Mel-28 cells (A), A-375 cells (B) and G-361 cells (C) in cell cycle phases was investigated by an image analysis using the automated NC-3000™ system based on DAPI (4',6-diamidino-2-phenylindole). The values indicate the percentage of cells in the indicated phases of the cell cycle (sub-G1/G1, S and G2/M).

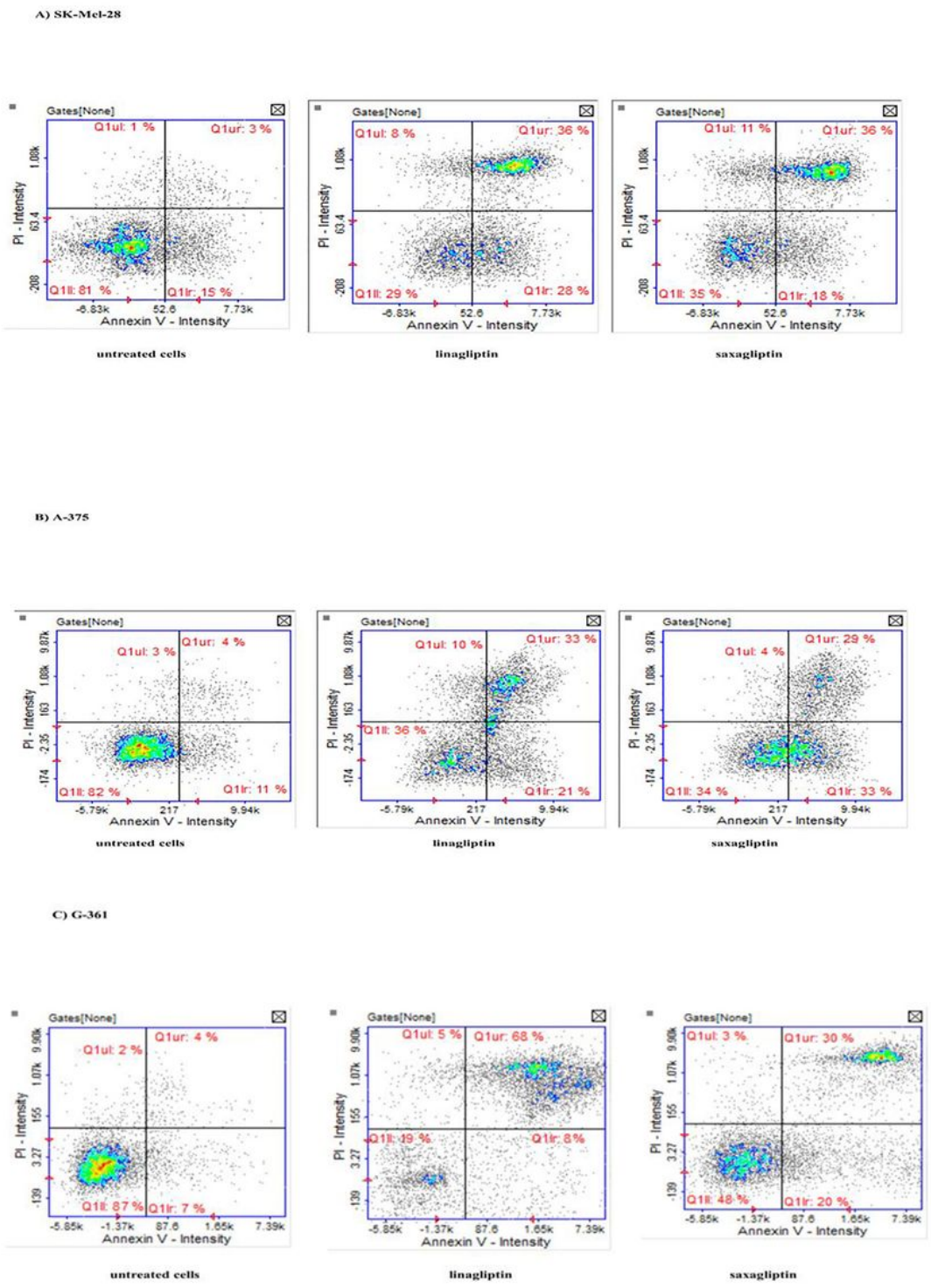

Figure 6

Effect of linagliptin or saxagliptin on cell apoptosis of melanoma cells The melanoma cells were stained with Annexin V (Annexin V-CF488A) and propidium iodide (PI) . Scatter plots demonstrate percentage of viable, apoptotic and necrotic SK-Mel-28 (A), A-375 (B), G-361(C) cells after a 24-h treatment with linagliptin or saxagliptin. The lower left square presents viable cells; right lower square shows early

apoptotic cells; right upper square shows late apoptotic cells, and left upper square presents necrotic cells.