ADHESION PROTEINS PROFILE AND LOCALIZATION IN OVARIAN CARCINOMA CELL LINES

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Abstract. The three-dimensional structure of tumor tissue and particularly cell-cell and cell-extracellular matrix adhesion is an important factor that can determine the phenotype of tumor cells. In this work, we have investigated the abundance profile of actin-binding adhesion proteins in human ovarian adenocarcinoma cell lines SKOV-3 and SKOV-3.ip. We have investigated levels of total and superficially localized adherens junctions proteins E- and N-cadherin, gap junction protein connexin-43 and cell-extracellular matrix contacting integrin beta-1. Our results indicate a complete absence of epithelial marker E-cadherin, a low level of mesenchymal N-cadherin and high levels of connexin-43 and integrin beta-1. Modest superficial localization of the represented proteins was observed, indicating their mislocalization. SKOV-3 cell line was characterized by higher levels of the total content of studied cell-cell contacts proteins and a lower level of superficially localized integrin beta-1, which is both considered to be associated with lower tumor aggressiveness. The revealed differences in the profile of adhesion proteins are in line with the accepted view on SKOV-3.ip cell line having a more aggressive phenotype than that of SKOV-3. The revealed features of the total abundance of the adhesion proteins and their superficially localized pool made it possible to supplement the information on the nature of phenotypic differences between the studied cell lines.

Keywords: cell–cell contacts, cell-extracellular matrix contacts, cancer cell phenotype, adherens junctions, gap junctions, integrin-based junctions.

List of Abbreviations

SMT – somatic mutation theory
TOFT – tissue organization field theory
ECM – extracellular matrix
RF – relative fluorescence
EMT – epithelial-to-mesenchymal transition
WNT – Wingless-integrated
GJIC – gap junction intercellular communication
EGF – epidermal growth factor
Bax – Bcl2 associated X protein
FAK – focal adhesion kinase

Introduction

Contribution of the three-dimensional structure of the tumor to its development and drug resistance is now a cutting edge in cancer research. Thus the prevailing ‘somatic mutation theory’ (SMT) of carcinogenesis which declares that cancer stems from one compromised cell is now being revised and complemented by elements of ‘tissue organization field theory’ (TOFT) of cancer, which in turn suggests that the development of the tumor process lies in the disorder of the interaction between all of the participants of the tissue microenvironment (Soto, Sonnenschein., 2020; Neophytou *et al*., 2021). Disturbance in cellular communication has been widely described for tumors of various locations and stages of development and is being considered as a potential target for various treatment approaches (Brücher & Jamall., 2014; Song et al., 2019; Dominiak *et al*., 2020).

Communication within the tissue is mediated by cell-cell contacts (adherens, tight, gap junctions and desmosomes) and cell-extracellular matrix (ECM) contacts (mainly integrin-based) comprised by complexes of integral proteins. Adherens junctions are comprised by calcium-dependent proteins cadherins which organize in belt-like adhesion plaques thus providing the organization of the epithelial sheet and participate in maintaining its polarization along with tight junctions and cell-ECM contacts (Harris & Tepass, 2010). Tight junctions are comprised by caludins and occludin

which form the most prohibitive junctions strictly regulating paracellular transport (Tsukita *et al*., 2001). Gap junctions constituted by connexins on contrary act as direct cell connectors as they form hexameric transmembrane channels (Nielsen *et al*., 2012). Desmosomes are formed by desmogleins and desmocollins which are calcium-independent non-classical cadherins (Garrod & Chidgey, 2008). Integrin-based contacts to ECM are dimers of various alpha- and beta-integrin subunits, where the specificity of adhesion is defined by the composition of a dimer (Takada *et al*., 2007). Concerning intracellular connections of integral adhesion proteins, they bind to various components of the cytoskeleton: desmosomes bind to intermediate filaments (Hatzfeld *et al*., 2017), while proteins of adherens and tight junctions bind to the actin cytoskeleton (Campbell *et al.*, 2017; Vicente-Manzanares *et al*., 2009). Emerging data testifies that gap junction proteins connexins are also connected to actin cytoskeleton via adapter proteins which bind to specific sites on their cytoplasmatic C-tail (Herve *et al*., 2007; Ambrosi *et al*., 2016).

Actin cytoskeleton is recognized as one of the central components orchestrating cancer progression and metastasis (Galluzzi & Thomas, 2020 a,b), thus profile assessment of actin-connected adhesion proteins may be a relevant tool in cancer research. Integral proteins in general are the first-line contributors to adhesion and communication and in these terms their representation profile serves as a reflection of interactions in tumor microenvironment in a certain time point and becomes a potent characteristic which is appropriate to rely on both in fundamental cancer research and drug efficiency evaluation (Farahani *et al*., 2014; Kutova *et al*., 2020). In this study we focus on representative integral actin-binding proteins of adherens junctions, namely epithelial marker E-cadherin and mesenchymal marker N-cadherin (Loh *et al.*, 2019); gap junctions, namely connexin-43 which is the most widely represented connexin in mammalian tissues (Bonacquisti & Nguyen, 2019); and integrin-based cell-ECM contacts, namely integrin beta-1, which is the most preferential partner in α-β integrin dimerization and thus may be considered as the basic member of the majority of integrin-based contacts (Hynes, 1992). A summary of the studied proteins and their common binding partners among adapter proteins are represented in Fig. 1.

It should be noted that to date a considerable amount of data was obtained indicating mislocalization of adhesion proteins (Wang & Li, 2014; Alaga *et al.*, 2017; Seraya-Bareket *et al.*, 2020) which shifts cancer cells towards aggressiveness. Our work aims to investigate the expression profile and localization of actin binding integral adhesion proteins representing cell-cell and cell-matrix contacts in two lines of human ovarian adenocarcinoma cells with different aggressiveness.

Materials and Methods

Cell lines. Cells of human ovarian adenocarcinoma cell lines SKOV-3 and SKOV-3.ip were used. Cells were cultured in DMEM medium containing 2 mM glutamine (PanEco, Russia), 10% (v/v) fetal bovine serum (HyClone, USA), 50μg/ml of Penicillin and 50 μg/ml of Streptomycin (PanEco, Russia) at 37 °C in 5% CO_2. For passaging, cells were detached with Versene solution (PanEco, Russia).

Cell preparation protocol to assess localization of adhesion proteins. In order to perform flow cytometry analysis, monolayer culture was detached from the substrate by incubation with TrypLE solution (Thermo Fischer Scientific, USA) for 20 min at 37 °C in 5% CO_2. Cells were fixed in 4% formaldehyde (Applichem, Germany) for 20 min at room temperature to exclude a possibility of protein profile changes during the analysis. In order to assess the simultaneous superficial and intracellular abundance of studied proteins fixed cells were permeabilized with 0,02% Triton X-100 (VWR Life Science, USA) for 20 min at room temperature. Cells intended for assessing the abundance of superficially located proteins of interest proceeded to staining directly after fixation. Cells were thoroughly washed with PBS to remove formaldehyde and Triton-X100 before staining.

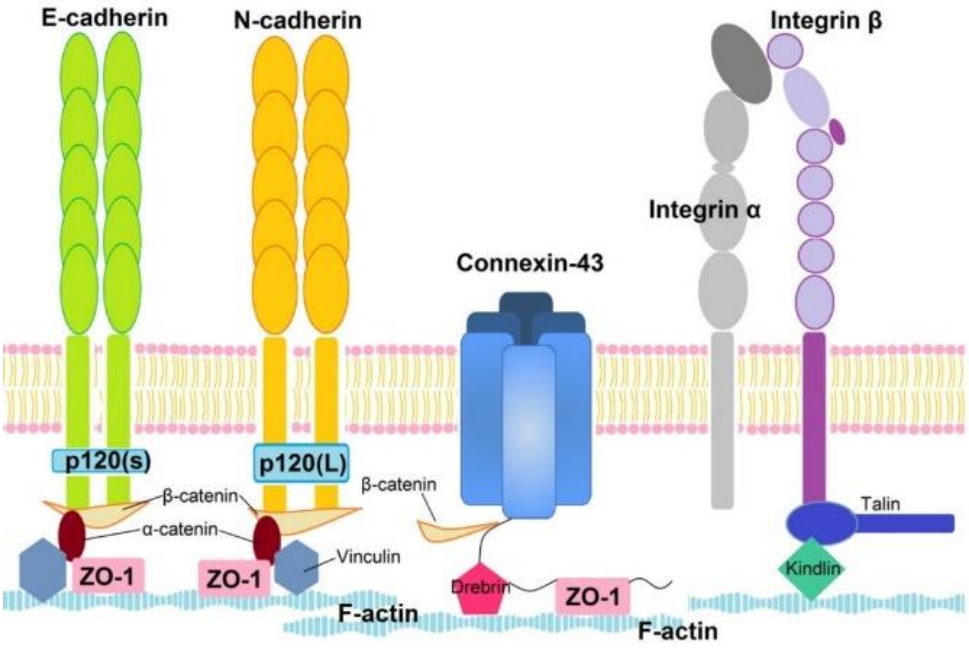

Fig. 1. Summary of studied proteins: E-cadherin, N-cadherin (cell-cell adherens junctions); connexin-43 (cell-cell gap junctions); integrin beta-1 (cell-matrix junctions) with common adapter proteins

Cell staining and flow cytometry analysis. In order to minimize non-specific binding, cells were blocked in a solution of 3% milk (Applichem, Germany) in PBS for 1 h at room temperature and incubated with antibodies specific to the proteins of interest and antibodies of corresponding isotypic control according to manufacturer's instructions. The following specific antibodies were used: E-cadherin Monoclonal Antibody (67A4), FITC (Thermo Fischer Scientific, USA, Cat#A15757), N-cadherin Monoclonal Antibody (8C11), PE (Thermo Fischer Scientific, USA, Cat#12-3259-42), connexin-43 Monoclonal Antibody (CX-1B1), Alexa Fluor 488 (Thermo Fischer Scientific, USA, Cat#138388), integrin beta-1 Monoclonal Antibody (TS2/16), PE (Thermo Fischer Scientific, USA, Cat#12-0299-42). Excess antibodies were washed out from the specimens with 1% BSA (Sigma-Aldrich, USA) solution in PBS and analyzed by flow cytometry using a CytoFlex S (Beckman Coulter, USA). Fluorescence of FITC, AlexaFuor 488 and PE was excited with a 488-nm laser and the signal was detected with 525/40 (FITC, AlexaFluor488) and 585/42 (PE) band pass filters.

Results

Two ovarian carcinoma cell lines SKOV-3 and SKOV-3.ip were used in this study. SKOV-3 cell line was obtained from the malignant ascites of a 64-year-old Caucasian female (Hung *et al.*, 1992) and SKOV-3.ip cell line was obtained from the malignant ascites of tumor bearing *nu/nu* mice which were intraperitoneally inoculated with SKOV-3 cells. SKOV-3.ip cells were indicated to possess higher DNA synthesis rates, accelerated proliferation, increased colony-formation in soft agar, formation of larger subcutaneous tumors and reduced survival of *nu/nu* mice bearing intraperitoneally inoculated tumors (Yu et al., 1993; Dar *et al.*, 2017).

To evaluate the localization of adhesion proteins in monolayer cultures in ovarian carcinoma cell lines we have conducted flow cytometry analysis of adhesion proteins participating in formation of cell-cell contacts, namely E-cadherin and N-cadherin (adherens junctions), connexin-43 (gap junctions) and cell-ECM contacts namely integrin beta-1. The abundance level of each protein of interest is represented by relative fluorescence (RF) value calculated

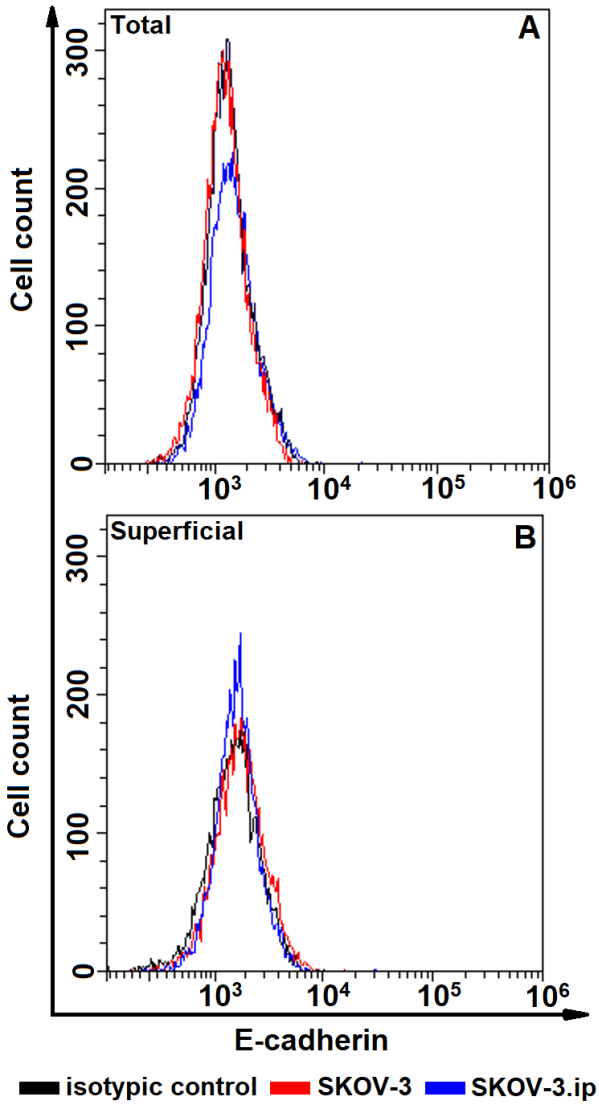

Fig. 2. The distributions of SKOV-3 and SKOV-3.ip cells according to presence of total (A, upper plot) and superficially located (B, lower plot) E-cadherin. Cells were stained with E-cadherin-specific antibodies and analyzed by flow cytometry

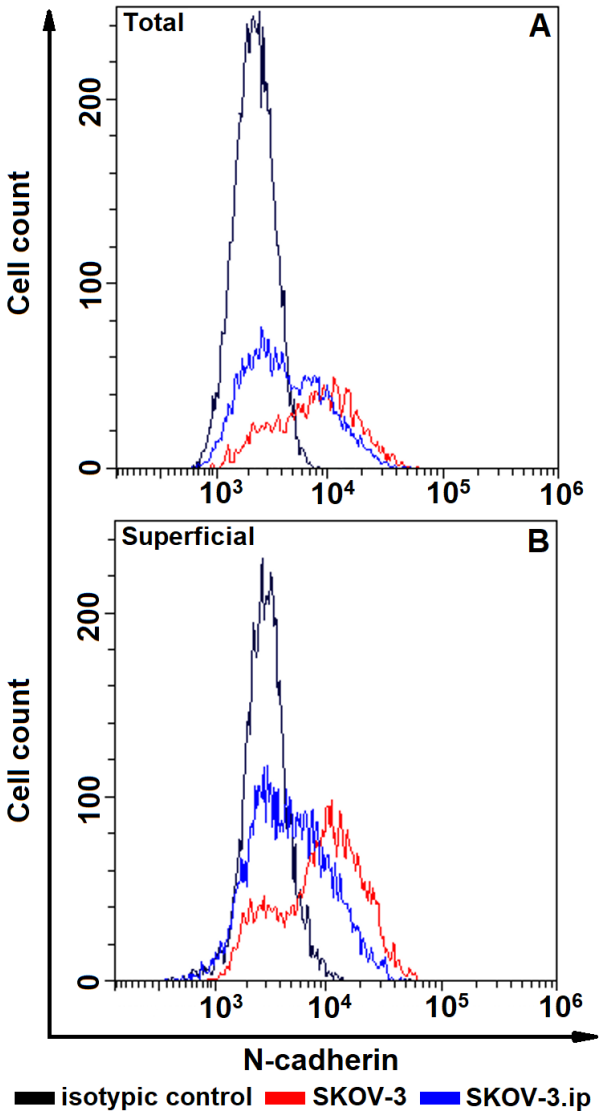

Fig. 3. The distributions of SKOV-3 and SKOV-3.ip cells according to presence of total (A, upper plot) and superficially located (B, lower plot) N-cadherin. Cells were stained with N-cadherin-specific antibodies and analyzed by flow cytometry

as a ratio of mean fluorescence intensity of cells stained with antibodies specific to proteins of interest to mean fluorescence intensity of cells stained with antibodies of corresponding isotypic control. RF value equal to 1 indicates that no analyzed protein is present in the cells.

E cadherin. The obtained data indicate that the studied cells do not possess E-cadherin at all; relative fluorescence values were equal or close to 1 (Figs 2, 6, 7).

N-cadherin was represented at very low level in both cell lines. Along with that, population heterogeneity was detected: a considerable part of the cells demonstrated the complete absence of N-cadherin (Fig. 3). Only a modest part of total N-cadherin was superficially located (Fig. 6). Of note, the total abundance of N-cadherin in N-cadherin-positive cells was significantly higher in SKOV-3 cells in comparison to SKOV-3.ip cells (Fig. 7,B).

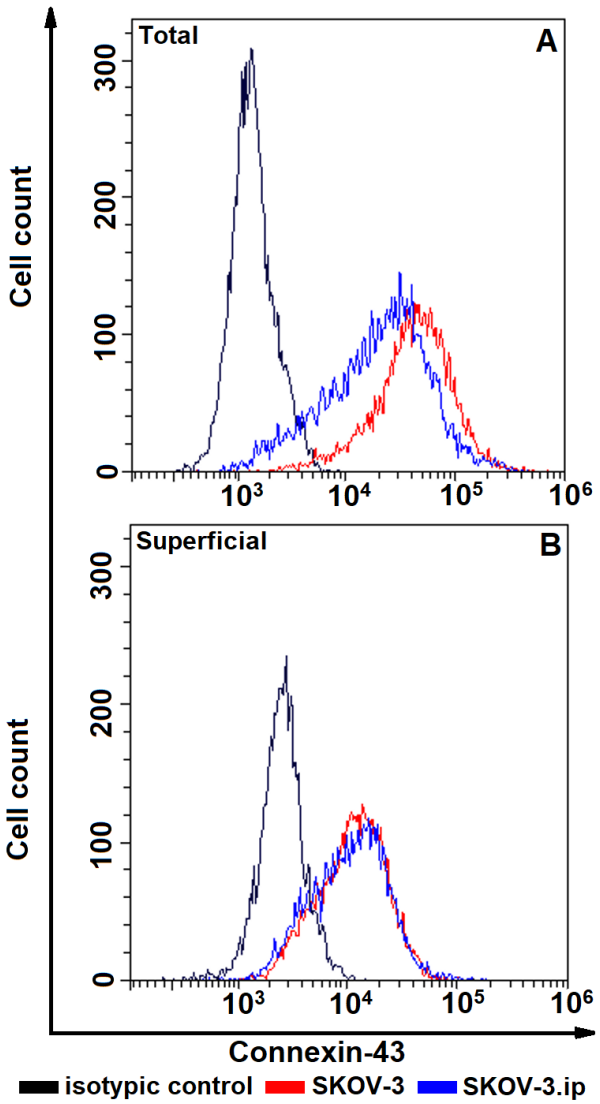

Fig. 4. The distributions of SKOV-3 and SKOV-3.ip cells according to presence of total (A, upper plot) and superficially located (B, lower plot) connexin-43. Cells were stained with connexin-43 -specific antibodies and analyzed by flow cytometry

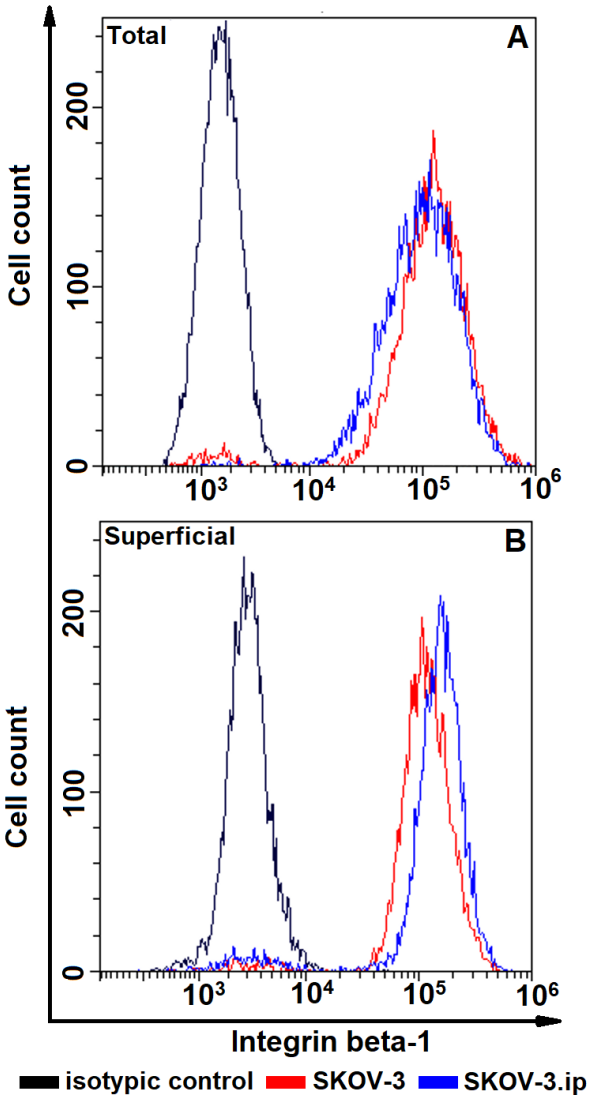

Fig. 5. The distributions of SKOV-3 and SKOV-3.ip cells according to presence of total (A, upper plot) and superficially located (B, lower plot) integrin beta-1. Cells were stained with integrin beta-1-specific antibodies and analyzed by flow cytometry

Connexin-43 total representation was at a relatively high level both in SKOV-3 and SKOV-3.ip cell lines, at the same time similarly to N-cadherin, only a modest level of connexin-43 was represented at the cell surface (not more than 20% of total amount) (Figs 4, 6) and also similarly to N-cadherin, connexin-43 was significantly more abundant in SKOV-3 cell line compared to SKOV-3.ip (Fig. 7, B).

Integrin beta-1 was the most abundant of the studied proteins both in SKOV-3 and SKOV-3.ip cell lines and again only a part of the total integrin beta-1 content was superficially localized. Also the studied cell lines showed heterogeneity as a minor part of the population represented loss of integrin beta-1 (Figs 5, 6). Statistically significant differences between the studied cell lines were detected, moreover, the peculiar phenomenon was revealed for integrin beta-1: SKOV-3 cell line possessed higher total integrin beta-1 level (Fig. 2,A) while superficially located integrin beta-1 prevailed in SKOV-3.ip cell line (Fig. 2,B).

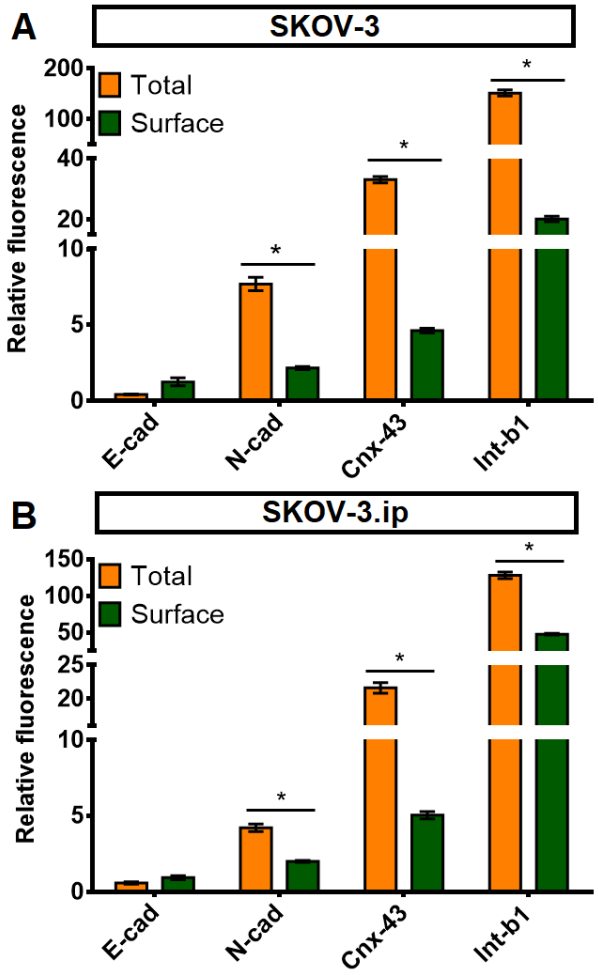

Fig. 6. The abundance of total and superficially located adhesion proteins in SKOV-3 (**A**) and SKOV-3.ip (**B**) cell lines. Expression levels of E-cadherin, N-cadherin, connexin-43 and integrin beta-1 denoted as relative fluorescence values calculated as a ratio of mean fluorescence intensity of cells stained with specific antibodies to mean fluorescence intensity of cells stained with antibodies of corresponding isotypic control. Data are presented as mean ± SD (n = 3). "*" indicates significant difference in RF between total and superficially located protein (Unpaired t-test with Welch correction, $p < 0.05$)

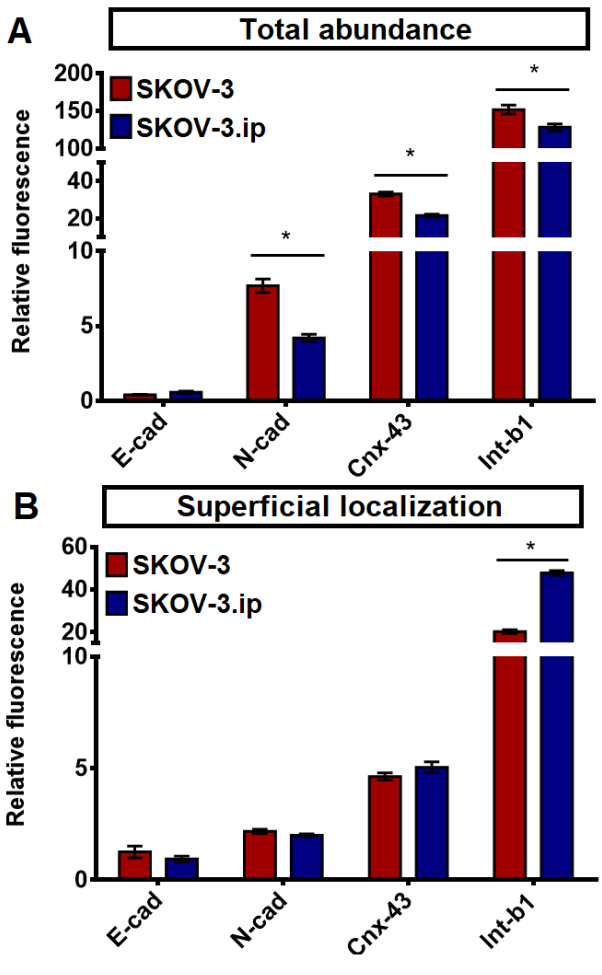

Fig. 7. The abundance of adhesion proteins in ovarian adenocarcinoma cell lines totally represented (**A**) and located superficially (**B**). Expression levels of E-cadherin, N-cadherin, connexin-43 and integrin beta-1 denoted as relative fluorescence values calculated as a ratio of mean fluorescence intensity of cells stained with specific antibodies to mean fluorescence intensity of cells stained with antibodies of corresponding isotypic control. Data are presented as mean ± SD (n = 3). "*" indicates significant difference in RF between protein abundance in studied cell lines (Unpaired t-test with Welch correction, $p < 0.05$)

Discussion

Intercellular contact proteins are usually considered to be tumor suppressors, but there is more and more conflicting evidence of their involvement in the formation of an aggressive phenotype and triggering tumor progression and invasion. An intriguing potential reason of such controversy is the context in which these proteins function, e.g. a protein which is considered to be proliferation suppressor might drive cell invasiveness or motility (Moh & Shen, 2009; Tang et al., 2018; Mulkearns-Hubert et al., 2020).

In present work we have analyzed the total abundance and the abundance of superficially localized representative proteins of actin-connected cell-cell and cell-ECM contacts in two ovarian carcinoma cell lines with different aggressiveness. In case of adherens junctions we have showed that E-cadherin was absent in

both studied cell lines. Concerning N-cadherin the studied cell cultures showed heterogeneity as a part of the cells did not express N-cadherin and the rest of the population expressed N-cadherin at a relatively low level. Loss of E-cadherin, which is widely recognized as an epithelial marker, triggers the Epithelial-to-Mesenchymal Transition (EMT) of cells and contributes to the development of an aggressive phenotype. During EMT, there is a switch from the synthesis of E-cadherin to the synthesis of N-cadherin, which is a mesenchymal marker and is involved in intracellular signaling promoting invasion and metastasis (Gravdal *et al.*, 2007; Loh *et al.*, 2019). It is worth noting that the cells of the studied lines are characterized by the formation of loose three-dimensional aggregates in vitro; which is in line with an assumption of their mesenchymal state (Winner *et al.*, 2016; Sokolova *et al.*, 2019; Kutova *et al.*, 2020).

Currently, more and more evidence is accumulating that EMT is not a binary process, but a continual one, implying a huge number of variants of the intermediate states of the cell, when it can express epithelial and mesenchymal markers simultaneously, which contributes to the development of cancer plasticity (Sha *et al.*, 2019). It has been hypothesized and supported by mathematical modeling that such intermediate states can accelerate EMT and exacerbate its consequences (Goetz *et al.*, 2020). The data on the intermediate position occupied by the SKOV-3 and SKOV-3.ip lines vary, because despite the fact that these lines are called intermediate mesenchymal, this can mean both the simultaneous presence of E-cadherin and N-cadherin (Rosso *et al.*, 2017; Teng *et al.*, 2015) or the absence of E-cadherin with presence of N-cadherin (Klymenko *et al.*, 2017), which was observed in our work. Such inconsistency of the data might be due to the high plasticity of tumor cells, which results in subtle differences in the phenotype evoked by different cultivation conditions. It should also be noted that the representation of N-cadherin in our experiments was rather low. Simultaneous loss of adherens junctions' proteins has been described for very poorly differentiated hepatocellular carcinoma, which was accompanied by extremely low patient survival (Liu *et al.*, 2015) and in exceptional cases of invasive lobular breast cancer characterized by tubular elements formation (Christgen *et al.*, 2020). In addition, the observed difference in the amount of total N-cadherin and superficially localized can be explained by the transition of N-cadherin into a soluble form, which has pro-angiogenic properties and is at high level detected in biological fluids of cancer patients (Derycke *et al.*, 2006 a, b).

According to the obtained data gap junction protein connexin-43 was represented at relatively high level in both studied cell lines yet significantly prevailed in SKOV-3 cell line. Functional connexin-based gap junctions being properly organized provide Gap Junction Intercellular Communication (GJIC), which in turn maintains coordinated work of the cells within the tissue. Thus, connexins are considered to hinder tumor progression via GJIC (Krutovskikh *et al.*, 2002, Zefferino *et al.*, 2019). Another plausible mechanism of connexin-mediated tumor suppression is the participation of connexins in intracellular signaling interfering with proliferative and invasive signals (Aasen *et al.*, 2019; Mulkearns-Hubert *et al.*, 2020). Concerning tumor suppressing potencies of connexin-43 it is reported that it can inhibit proliferation by affecting the Wingless-Integrated (Wnt)/β-catenin pathway (Shima *et al.*, 2006), decreasing the activity of proto-oncogene tyrosine-protein kinase Src (acronym of 'sarcoma') or epidermal growth factor (EGF) (Herrero-Gonzalez *et al.*, 2010; Qui *et al.*, 2016) or by triggering apoptosis via binding to pro-apoptotic Bcl2-associated X protein (Bax) (Sun *et al.*, 2012). Of note, our data indicate that the level of superficially localized connexin-43 did not exceed 20% of total abundance. It is possibly due to the disrupted connexin-43 trafficking to plasma membrane. Translocation to the cytoplasm was reported for connexin-43 and may be mediated by Wnt signaling pathway (Hou *et al.*, 2019); it was also shown for other connexins (Krutovskikh *et al.*, 1994; Thiagarajan *et al.*, 2018). Aberrant localization of connexins which was observed in our study is reported to be associated with the triggering of EMT (Crespin *et al.*, 2016; Kotini *et al.*, 2018).

The most abundant of all observed proteins was integrin beta-1. The majority of research testifies that high levels of integrin beta-1 are represented in tumor cells compared to normal epithelium (Min *et al.*, 2020). Up-regulated integrin beta-1 is associated with increased cancer cell survival, proliferation, migration and colony formation in vitro (Pardo *et al.*, 2002; Chang *et al.*, 2019) and with advanced stage of the tumor and lower life expectancy in patients (Lin *et al.*, 2014; Lawson *et al.*, 2010). Our data indicate that superficial localization of integrin beta-1 is low compared to total protein. It is interesting to note that higher total abundance of integrin beta-1 was detected in SKOV-3 cell line, yet superficially localized integrin beta-1 was more represented in SKOV-3.ip cell line. Superficially located integrins being activated are shown to promote tumors towards increased malignancy mainly via focal adhesion kinase (FAK) signaling axis (Yang *et al.*, 2014; Zhang & Zou, 2015; Xu *et al.*, 2017).

The profile of adhesion proteins expression appears to be more adequate means of tumor phenotype identification compared to assessment of individual proteins due to their complex crosstalk and reciprocal regulation. For example, it was shown that diapedesis-mediated tumor invasiveness was registered in tumors with simultaneous loss of E-cadherin and high expression of connexin-43 (Mol *et al.*, 2007) or integrin beta-1 (Shu *et al.*, 2013, Symowicz *et al.*, 2007). It should be noted that protein expression and their representation on the cell surface is a dynamic process which is highly dependent on the interplay of microenvironmental context and ongoing physiological processes. For example, along with high expression of connexin-43, E-cadherin abundance can continuously change over time during different phases of an invasion process (Mol *et al.*, 2007; Xu *et al.*, 2008).

Conclusions

In this work we have analyzed total content and superficially localized pool of adhesion proteins in two ovarian carcinoma cell lines. The absolute loss of epithelial marker E-cadherin and overall mislocalization of the rest analyzed proteins and hence their impaired adhesive function, allow us to speculate about mesenchymal characteristics of the studied cell lines. At the same time, we have detected heterogeneity of the studied cell cultures in terms of N-cadherin and integrin beta-1 abundance. This is in line with the inconsistency of published data on SKOV-3 and SKOV-3.ip phenotype and thus may be a strong evidence of cancer cells plasticity even in controllable conditions of laboratory maintenance. According to the obtained data SKOV-3.ip cell line possessed lower overall adhesion proteins abundance which is in line with a mainstream notion that loss of cellular adhesion underlies tumor aggressiveness. At the same time higher representation of superficially localized integrin beta-1 might be an additional sign of SKOV-3.ip superlative aggressiveness. Our data may be useful to expand an understanding of the nature of the exceeding aggressiveness of SKOV-3.ip cell line in comparison to SKOV-3.

Acknowledgments

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