Keratin 8 and 18 knockdown increases cisplatin-induced apoptosis and invasive potential through claudin-1/Pi3K/NFkB up-regulation in epithelial carcinoma cells

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Keratins are epithelial-specific intermediate filament (IF) proteins, which are expressed in a tissue- and differentiation state-specific manner. As part of the cytoskeleton, keratins are important for the mechanical stability and integrity of epithelial cells and tissues. Moreover, a number of keratins are involved in intracellular signalling pathways which regulate many diverse functions, including motility, tissue homeostasis, and protection against injury and non-mechanical stresses [1, 2]. Cell growth [3-5], cell death [6-9] and cancer progression [10-12]. Keratins 8 and 18 (KB18) are typically co-expressed as the primary keratin pair in simple epithelial cells and their expression are maintained during malignant transformation, hence their use as diagnostic marker in tumor pathology. However, in recent years different studies have shown that IF should not be considered only as markers proteins but also as regulators of cancer cell signaling and that they might play an active role in malignant transformation. In the present study, we addressed the question as to whether KB18 expression affects tumor initiation and progression.

Our results showed that KB18 stable knockdown using shRNA increases cisplatin sensitivity in different epithelial cancer cell lines. Indeed, western blot analysis of caspases activation and flow cytometry analysis of Annexin V/PI staining show that KB18 knockdown sensitizes cells to cisplatin-induced apoptosis. Increased Fast, expression and FasR membrane targeting suggest that apoptosis is enhanced via the death receptor pathway.

Moreover, in vitro wound healing and transwell invasion assays, we observed that KB18-deficient cancer cells display an increased motility and invasion. Interestingly, we observed that these cells present higher PIP3 levels in the plasma membrane as determined by flow cytometry analysis. Consequently, the KB18-shRNA clones show P38kA and NFkβ pathways hyperactivation and increased MMP-9 expression. Furthermore, these processes are shown to be partially regulated by the tight junction’s protein Claudin-1, which is highly increased in KB18-shRNA clones.

To our knowledge, these results represent the first indication that KB18 can influence the phenotype of epithelial cancer cells. Knockdown of KB18 increases cisplatin sensitivity and invasive potential of epithelial cancer cell lines through the regulation of different cell signaling pathways, involving claudin-1-dependent PI3k activation and NFkβ transcription activity. Several studies demonstrate that over-expression of claudin-1 protein is associated with increased invasiveness and metastatic behavior [13-15], partly through the up-regulation of MMPs [16-18]. Moreover, NFkβ is shown to regulate MMPs expression in some epidermal studies [19-22]. Our results demonstrate that KB18 constitute a signaling platform capable of modulating cell invasion/survival-dependent signal transduction in tumor cells. KB18 knockdown induces hyperactivation of the NFkβ and several downstream effectors (NFkβ) that seems to be key steps in invasion. Moreover, our results suggest that KB18 could also play a key role in the location of cell death receptors to the plasma membrane so that the cisplatin-induced apoptosis is improved. These results support the hypothesis that keratins 8 and 18 play an active role in cancer progression.

Model cell lines—We used two epithelial cancer cell lines: endometrial carcinoma KLE cells (expressing constitutively activated Akt isoforms) and hepatocellular carcinoma HepG2 cells.

Transfections with shRNAs—Stable transfection of the cells was carried out with keratin 8, keratin 18 or scrambled negative control (NC) shRNAs (SA Biosciences) using FuGene 6 reagent. G418 was applied to isolating resistant clones.

Targeting of Claudin1 by siRNA—Cells were transfected for 24h with 100nM claudin1 or scrambled negative control (NC) shRNA (Ambion) using TransIT-TKO reagent (Mirus).

qRT-PCR—Quantitative real-time PCR was performed with Mx3000P (Stratagene) in duplicates from at least three independent experiments. RNA 18s and β-actin was used as reference genes.

Invasion and wound-healing assay—Invasive properties were measured using 2mg/mL of Matrigel-coated Transwell inserts. Invasive cells that had adhered to the porous insert were fixed in methanol and nuclear staining was performed with Hoechst dye. Epithelial cell motility, cells were grown to near confluence and a wound was created with the blunt end of a yellow tip. Each experiment was performed in duplicates and repeated three times.

Cell fractionation—Separation and preparation of cytoplasmic, membrane and nuclear extracts from cells were done with the Subcellular Protein Fractionation Kit (Thermo Scientific).

Apoptosis analysis by flow cytometry—Cells were dual stained with propidium iodide and Alexa Fluor 488-annexin V using Dead Cell apoptosis kit (Life Technologies) according to the manufacturer’s protocol. Stained cells were analyzed by FC 500 MCL system (Becton Coulter).

PPI3 levels analysis by flow cytometry—Cells were fixed in 2% paraformaldehyde, blocked with 10% normal goat serum in PBS, and incubated at 4°C with mouse anti-Pi3K monoclonal antibody (Echelon). Primary antibody was detected with Alexa488-conjugated donkey anti-mouse antibody (Molecular Probes). Samples were acquired on a FC 500 MCL system (Becton Coulter).

Luciferase Reporter Assay—Cells were transfected with NFκB-Luc reporter plasmid and TK-H1Luc, followed by dual luciferase assay (Promega). Each experiment repeated three times.

References