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INVESTIGATION ON THE DIFFERENCE EXPRESSION OF TYPE IV COLLAGEN α1(IV)- α6(IV) CHAIN MRNA IN NORMAL FIBROBLAST AND IN SKIN CELL MALIGNANT MELANOMA

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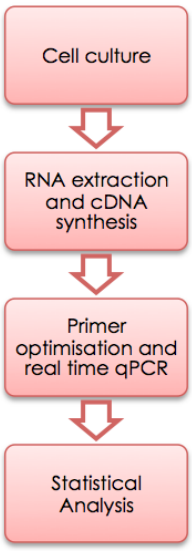
Graphical abstract



Abstract

Collagen IV is the major basement membrane protein that influences adhesion, proliferation, and migration of cells. The collagen composed of a network chains α1 to α6. The characterization of this collagen IV will correlates the relationship of collagen gene expression and cancer. This is important in order to provide more detailed understanding of the expression of collagen in tumor cells. . The aim of this study is to determine the α1 to α6 (IV) mRNA expression in the cell lines obtained from skin and melanoma cell. To investigate the mRNA expression, the RNA was extracted from the fibroblast and melanoma (A375) cell lines. The RNA was subjected to reverse transcription and then synthesized. The mRNA expression levels were measured using real time PCR with related to internal control, *GAPDH*. The study identified that α1, α2, α4, α5 and α6 of the α1-α6 (IV) were expressed in skin fibroblast. This corresponds to the α1α1α2 and α5α5α6 networks. However, in melanoma cell lines the collagen IV α2, α4, α5 and α6 mRNA was observed in low level compared to α1 and this suggested that the tumor has affected the expression of collagen and basement membrane of the cell.

Keywords: Collagen, type IV collagen, melanoma cell lines, qPCR, gene expression

1.0 INTRODUCTION

Basement membranes are universal, thin, sheet-looking structures usually found under epithelial and endothelial cell linings, also found surrounding many cell types including muscle, nerve and fat. They are highly unique structures that act as barriers that separate the epithelium from the underlying extracellular matrix (ECM), besides providing selective filtration barrier for macromolecules in the

kidney, blood-brain barrier and placenta [1]. Basement membranes also store growth factors and cytokines that are utilized upon release by proteases to control many cellular functions [2].

The basement membranes consist of mostly type IV collagen together with laminin, nidogen and heparan sulphate proteoglycans [3, 4]. Type IV collagen exist in three trimeric combinations, σ1σ1σ2, σ3σ4σ5 and σ5σ5σ6 [3], that are critical in embryogenesis, angiogenesis, tumour growth and forming fluid barriers [5, 6]. The σ1σ1σ2 network is most abundant, and is found in vascular membrane throughout the body and in other membranes including in the brain and muscle [3, 7]. The σ5σ5σ6 network is found in the skin, Bowman's capsule, smooth muscle, the distal tubules and testis [5, 8].

Distinct changes in the basement membrane composition accompany maturation of epithelial tissues [9]. The composition of alpha (σ) chain gives rise to different type of collagen and molecular mass been accumulates in the ECM. The defects in basement membrane through modification in its appearance and thickness cause disruption and detachment of the matrix from cell. Any alteration or mutations that disturb the collagen assembly would affect the basement membranes, hence results in disease.

The dissolution of basement membrane is considered a prerequisite for invasive tumor growth [10]. Cancer occurs when the basement membrane that lies under epithelial and endothelial cells gets breached by metastasizing cancer cells at the point of vasculature [11]. Besides that, increase in the density of collagen massively influences the matrix stiffness, stimulate metastatic cell to increase contractile force generation and cell spreading [12].

Change in the collagens has also been identified as an indicator for basement membrane disruption, thus acting as indicator of invasion and metastasis [13]. The lost of their expression upon the invasion of cancer leads to the remodeling of basement membrane assembly. Therefore the over-expression of collagen in different cancers, such as collagen type I in breast cancer and medulloblastoma [14, 15], collagen type IV in pancreatic cancer [16] and collagen type IV and VII in colorectal cancer [17], have made collagen a suitable biomarker for cancer diagnosis, as well as predictor for prognosis [16].

In fact, few studies have identified the relationship of collagen gene expression and cancer. A study by Mario *et al*. (2012) [18] reported an increased amount of interstitial fibrillar collagens type I and type III were found in pancreatic cancer. In addition, it was shown that over-expression of type I collagen is able to cause pancreatic ductal adenocarcima cells to override gemcitabine-induced checkpoint arrest, leading to over-proliferation of cells and thus, cancer [19].

However, little is known about the expression of type IV collagen in skin malignant melanoma. Therefore, we used real time qPCR to identify the expression of type IV collagens mRNA in melanoma cell lines. For comparison, we also studied the distribution of these collagens in normal fibroblast cells.

Table 1 Intra- and inter-assay coefficients of variation (CV) for mRNA quantitation of COL4A1-COL4A6 (IV) in normal fibroblast cell lines and skin melanoma cell lines. All the values obtained were less than 10%, which show that the quantitation of the mRNA was highly reliable and reproducible

Collagen type IV	Fibroblast cell lines		Skin cell melanoma lines	
	CV of intra-assay (%)	CV of inter-assay (%)	CV of intra-assay (%)	CV of inter-assay (%)
COL4A1	2.28	2.28	0.62	0.35
COL4A2	2.04	2.60	1.05	0.85
COL4A3	4.79	5.94	2.77	1.20
COL4A4	1.46	1.25	0.69	0.40
COL4A5	1.23	1.43	1.17	1.10
COL4A6	2.27	2.89	2.62	3.31
GAPDH	2.21	1.76	0.62	0.35

2.0 EXPERIMENTAL

2.1 Samples and Cell Cultures

The malignant melanoma cell line, (A375) and the normal fibroblast cell line (3T3-L1), was obtained from iMolec, ICRACU laboratory, IIUM Kuantan and the Research Laboratory of Kulliyah of Pharmacy, IIUM Kuantan respectively. The cell lines were cultured and maintained in DMEM supplemented media and was harvested upon confluence for extraction of RNA.

2.2 RNA Extraction and cDNA Synthesis

Total RNA was extracted from cell lines using an RNeasy® Mini Kit (Qiagen, Germany) and its concentrations identified spectrophotometrically using Nanodrop Technologies. The RNA was

subjected to cDNa using SensiFAST™ cDNA Synthesis Kit (Bioline).

2.2 Real time qPCR

Samples were then assayed for expressions, using the fluorescent intercalating agent SensiFAST™ SYBR® & Fluorescein Kit (Bioline) and CFX96 Real-Time System (BioRad). Individual reactions comprised 5 µl of 2x SensiFAST™ SYBR® & Fluorescein Kit (Bioline), 0.7 µl of each 20 ng/µl sense and antisense primer and 2 µl of 100 ng/µl cDNA template, in a total volume of 10 µl. The Cq value was calculated at the end of each run using *GAPDH* as the internal control, and software provided by the manufacturer (Biorad). Primer pairs for the genes whose mRNA expression was determined were purchased from Integrated DNA Technologies and been optimized before used in the experiments. Each sample was examined in triplicate and the assays performed in duplicate.

2.3 Statistical Analysis

The data were collected and coefficient variation (CV) of the experiments was determined. The results were compared statically using standard t test.

3.0 RESULTS AND DISCUSSION

The results on expression of α1-α6 (IV) mRNA chains expression in fibroblast and melanoma cell lines are summarized in Figure 1 and Figure 2 respectively while the Intra- and inter-assay correlation coefficients of variation (%CV) for mRNA quantitation on both assay were included in Table 1.

Both intra- and inter-assay %CV values were assessed to analyze the reproducibility and repeatability of the qPCR assays. The values obtained were less than 10%, which show that the quantitation of the mRNA was highly reliable and reproducible [20].

3.1 Collagen α1-α6(IV) mRNA Expression in Fibroblast Cell Lines

The study identified that α1, α2, α4, α5 and α6 IV) mRNA were expressed in skin fibroblast cell lines (Figure 1). This corresponds to the α1α1α2 and α5α5α6 networks. In the endoplasmic reticulum (ER) of normal cell, the three polypeptide chains of collagen type IV will interact to form triple helical promoters of the networks [21]. Thus, the level of α1(IV) would be the highest with the presence of α1α1α2 network [22, 23] and that the selective increase in α1(IV) mRNA is convenience for the accumulation of basement membrane components and reduplication of basal lamina in the skin [24]. Previous study has also suggested that α1(IV) and α2(IV) are highly conserved across species and present in all basement membranes [25].

3.2 Collagen α1-α6 (IV) mRNA Expression in Skin Cell Malignant Melanoma Lines

In the study, α1(IV) and a low level of α2(IV) mRNA were observed whereas α4, α5, and α6 (IV) mRNA were detected in a very low expression compared to α1(IV) ($p < 0.01$) (Figure 2).

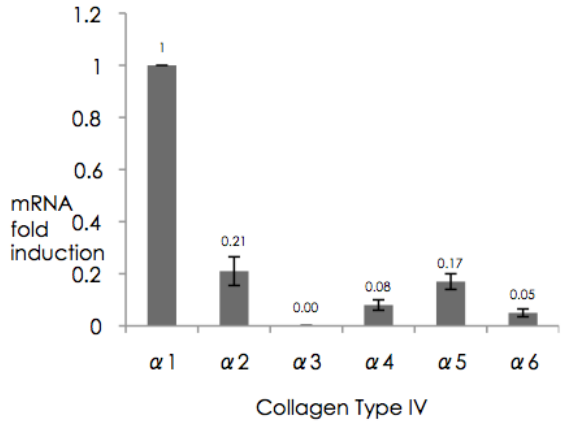


Figure 1 Levels of collagen α1-α6(IV) mRNA expression in skin fibroblast cell lines

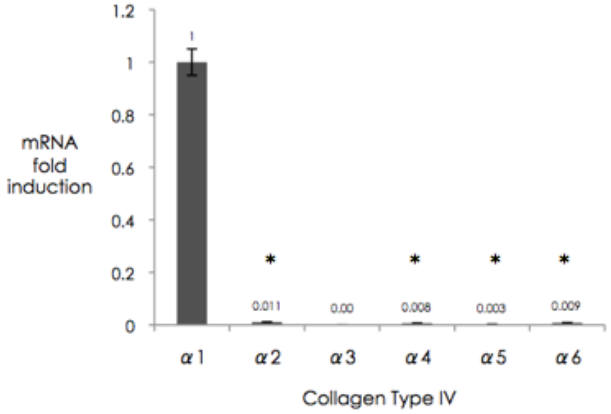


Figure 2 Levels of collagen α1-α6 (IV) mRNA expression in skin cell melanoma lines. (*$p < 0.01$)

Overall, the level of collagen type IV α2, α4, α5, α6 mRNA appeared to be low in melanoma cell lines compared to α1. The very low amount of the collagen type IV mRNA in melanoma cell lines suggested that the tumor had their effect through the ECM.

The results indicated that the presence of cancer in the skin has affected the level of collagen type IV mRNA and possibly is in its primary stage of melanoma. Previous study has found that the expression of collagen type IV was higher in metastatic melanoma compared to primary

melanoma due to the rising of its aggressive behavior [26]. No definitive reason for this but unlimited exposure of radiation from sun could cause mutation in the gene that code for these collagen [27]. The abnormal collagen formed in the skin would damage the normal function of the epidemic cell and result in the disruption of the cell cycle that lead to abnormal growth of cell which become cancer in the late stage [28].

4.0 CONCLUSION

This study has characterized the expression of collagen type IV α1-α6 mRNA expression in fibroblast cells and in skin cell melanoma lines using real-time PCR.

The results of the study demonstrate that the collagen expression was low in the cancer cell lines because of the low level of type IV α2, α4, α5, α6 chain mRNA been expressed in the melanoma cell lines. This study also reveals possible association of the collagen and cancer in cell lines as well as the effects of cancer on collagen type IV mRNA expression.

A correlation of this study with collagen type IV protein chains analysis would be useful for future reference and could contributes to a more detailed understanding of the effect of tumour on collagen expression.

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