MAY METALLIC BIOMATERIALS USED FOR ORTHOPAEDIC IMPLANTS PROMOTE CARCINOGENESIS? PRELIMINARY TRANSCRIPTOMIC RESEARCH ON HUMAN CHONDROCYTES

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Abstract

The aim of this research was to assess the risk of carcinogenesis induced by the metallic materials intended for orthopaedic implants. The report is an analytical summary of changes in the expression of cancer-related genes in human chondrocytes of normal and neoplastic phenotype. Cq values (quantification cycle values) obtained from qRT-PCR reactions (quantitative real-time polymerase chain reactions) were used to count Fc values (fold change values) for each gene. Differences in Fc values obtained for primary and cancer cells grown on the surface of medical steel AISI316L and titanium-aluminum-vanadium alloy Ti6Al4V were then analyzed by t-Student test. The results indicate that for cancer cells grown on the surfaces of both examined materials the fold change greater than 2, usually considered essential, was found for LUM gene involved in sarcoma induction. For FOS gene, also involved in sarcoma induction, the Fc value was also very close to 2 in the primary cells exposed to Ti6Al4V alloy. The remaining observed changes were rather subtle, although they cannot be omitted from further studies because differences in gene expression in primary and tumor cells grown on the same biomaterial were statistically significant in several cases. The compilation of qRT-PCR experiments carried out on primary and cancer cells in parallel allowed to identify possible future contraindications for patients with a genetic predisposition to cancer or with cancer history.

Keywords: *transcriptomics, qRT-PCR, gene expression, orthopaedic implants, cancer, chondrocytes*

[Engineering of Biomaterials 157 (2020) 15-19]

doi:10.34821/eng.biomat.157.2020.15-19

Introduction

Among a wide variety of biomaterials used for implantations, metals and their alloys remain high on the ranking lists of the most commonly used materials. The reason for this lies in their satisfying mechanical properties, second to none in comparison to other classes of biomaterials. In fact, it is only the matter of biocompatibility that may be the critical point for the selection of metals for some clinical applications [1,2]. Still a lot can be done to improve the biofunctionality and biocompatibility of metallic implants, especially through the effective methods of surface modifications. Laser surface modification is one of the most promising approaches to obtain high biocompatibility of metallic implants, which in the case of their orthopaedic applications mainly comes to induction of osteointegration processes [3].

There are numerous reports on the formation of cancer changes adjacent to the implant or in places distant but temporally correlated with the implantation. This phenomenon is strongly marked in dental implantology, where one of the main cancer types located in close proximity of dental implants is squamous cell carcinoma [4]. At the moment there is no indisputable data on the initiating of carcinogenesis by implants used in orthopaedics, although this subject has been often discussed in works in the last three decades. For example, after the total hip arthroplasty, the appearance of malignant neoplasms in the area of endoprostheses, including osteoma, osteosarcoma, lymphoma, or squamous cell carcinoma has been reported [5,6]. However, no mechanism is fully confirmed, and the issue of accelerated tumour induction at the implantation site is still poorly understood and unclear.

A literature review on clinical reports indicates that cancer changes situated in the bone and affecting not typically osteoblasts but chondrocytes and cartilage tissue may be the reason of failure in the orthopaedic implantation process [7]. What is more, some benign tumors like enchodroma, which are very often difficult to diagnose, can transform into chondrosarcoma over the years [8]. Combining all the above disturbing reports became the impetus for selecting cartilage tissue cells (primary chondrocytes and chondrosarcoma cells) to perform this study. The assumption of this work was to verify whether immortalized cell lines with neoplastic phenotype show an altered response to contact with implant material when compared to primary cell lines of the same type.

Transcriptomics techniques seem to be irreplaceable to thoroughly investigate this phenomenon. Methods enabling examining the changes in gene expression have gained popularity over the last decade and become the obvious choice to assess the materials biocompatibility at a molecular level [9,10]. In fact, the possibilities offered by these techniques are huge and can be underestimated. One of them - the qRT-PCR technique - was implemented in this work.

Materials and Methods

Cell cultures

Primary chondrocytes line HC-a derived from the human articular cartilage were purchased from ScienCell Research Laboratories (Cat. #4650), together with all the reagents necessary for the culture. The cells were cultured at 37°C and 5% CO_2 in Chondrocyte Medium (CM, Cat. #4651) supplemented with the fetal bovine serum (FBS, Cat. #0025), chondrocyte growth factors (CGS, Cat. #4682) and antibiotics (penicillin-streptomycin solution, Cat. #0503). The medium was changed every three days and the cells were subcultured at 95% confluency.

Secondary chondrocytes line SW 1353 derived from a patient with chondrosarcoma were from ATCC (ATCC-HTB-94). These cells were cultivated at 37°C in Leibovit'z L-15 Medium (ATCC, Cat. #30-2008) supplemented with the fetal bovine serum (Capricorn Scientific, Cat. #FBS-12A) and antibiotics (penicillin-streptomycin solution from ScienCell, Cat. #0503) in free gas exchange with atmospheric air.

Tested materials

The subject of this study were two types of metallic materials shaped as 16-mm diameter discs used for orthopaedic implant production. The metals are medical steel AISI 316L and titanium alloy Ti6Al4V. The materials surface was modified by grinding. Before each experiment, the samples were washed for 15 min in deionized water and for the next 15 min in 70% ethanol in an ultrasonic bath. Then the discs were sterilized with dry hot air.

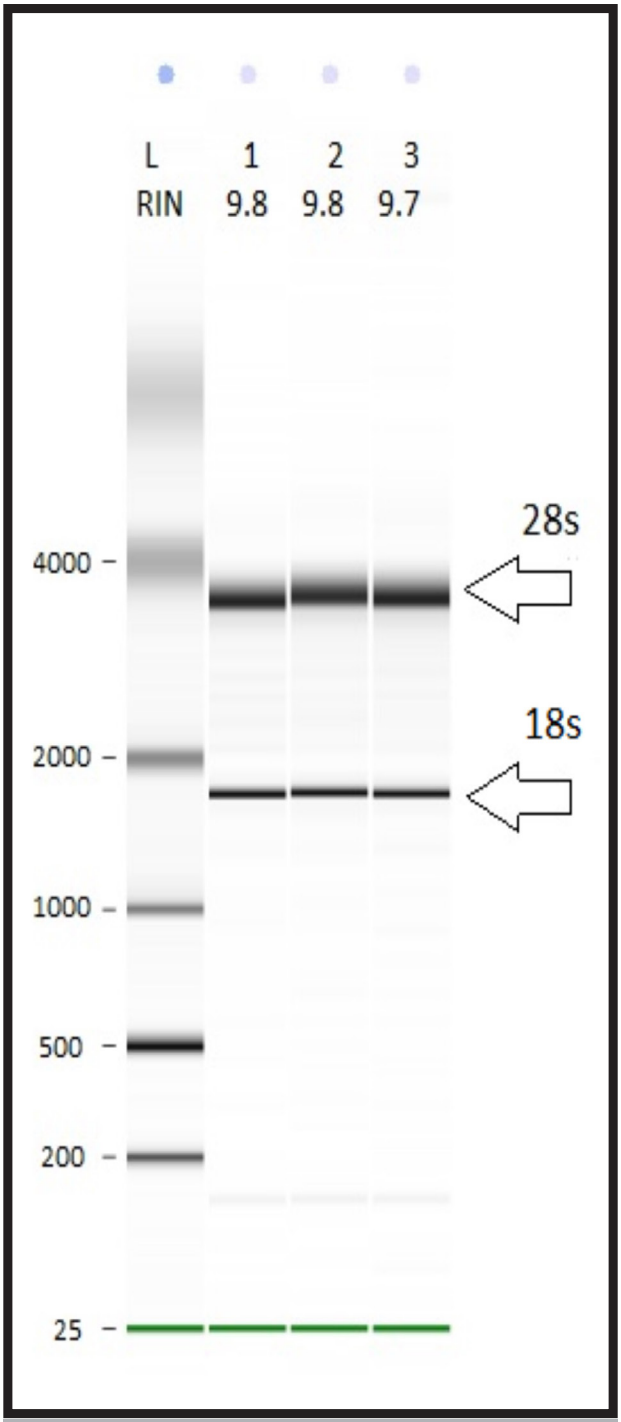

FIG. 1. Capillary electrophoresis carried out by Agilent's 2100 bioanalyzer for HC-a cells (L - ladder, 1 - control, 2 - AISI316L, 3 - Ti6Al4V). Two main ribosomal fractions (subunits 28s and 18s) are clearly marked. The RIN values (RNA integrity number) for all samples (1-3) close to 10 indicate perfect integrity of isolated material suitable for qRT-PCR.

RNA isolation and purification

For the RNA isolation experiment, the HC-a and SW 1353 cells were seeded on the surface of sterile samples placed in 12-well plates with the density of 100 000 cells/ml and incubated for 48 h in 37°C and 5% CO_2 atmosphere (HC-a) or without the additional CO_2 supply (SW 1353). The control was the cells grown on the surface of the standard well of a culture plate.

After the 48 h incubation the cells were harvested by trypsinization with 0.25% Trypsin-EDTA solution (ScienCell, Cat. #0103) and their quantity and viability were measured with trypan blue (ScienCell, Cat. #0203) in an automatic cell counter. Then the cells were transferred onto the system of columns from GeneMATRIX Universal RNA Purification Kit (EURx Ltd, Cat. #E3598) and the isolation and purification of total RNA was proceeded according to the procedure described in the manufacturer's protocol. In the next step, using Agilent's 2100 bioanalyzer, the quality and purity of isolated RNA were assessed by the means of capillary electrophoresis. An example image of the capillary electrophoresis performed for HC-a cells is presented in FIG. 1.

qRT-PCR reaction

Finally, the reverse transcription was performed with the use of the iScript cDNA Synthesis Kit (BIO-RAD, Cat. #1708891). The newly synthesized cDNA was used to carry out qRT-PCR reaction using the CFX96 Touch thermal cycler (BIO-RAD) and 2xSsoAdvanced Universal SYBR Green Supermix reagent (BIO-RAD, Cat. #1725274) on 96-well custom plates. The custom plates contained primers of 19 genes associated with the development of tumorigenic processes chosen basing on the literature review. The selected genes are listed in TABLE 1. GAPDH and AKTB were set as the reference genes.

The above experiment, from the seeding of cells, through the RNA isolation and purification, ending with the qRT-PCR reaction, was performed in seven independent repetitions for each cell line. The scheme of the standard gRT-PCR experiment is shown in FIG. 2.

Results and Discussions

A key parameter to analyze the gene expression changes via the qRT-PCR technique is the Cq value (quantification cycle value). Cq can be defined as a number of cycles after which the signal exceeds the detection threshold and, in fact, it is a measure of the gene expression. The Cq values obtained for every single gene from each repetition of the qRT-PCR reaction were analyzed using the comparative method, based on the Livak mathematical model. This method allows to calculate the relative difference of the expression level of a given gene between the test samples (RNA from the cells cultured on the biomaterials' surface) and control samples (RNA from the cells not stimulated by the presence of materials) [12].

GAPDH and AKTB were set as the reference genes. They were in constant expression in cells, yet at fluctuating levels. The difference between the Cq values of the qRT-PCR reaction running on the template of the test gene and the reference gene (ΔCq) was calculated for individual samples (test and control). Then, ΔΔCq (the difference between the ΔCq of the test and the ΔCq of the control sample) was indicated. Finally, the Fc value (fold change value) was calculated - the normalized value of the expression level of a given gene in the test sample in relation to the expression level of the same gene in the control sample ($Fc = 2^{-\Delta\Delta Cq}$). For the purposes of the analysis, it was assumed that $Fc \geq 2$ ($\log_2 Fc \geq 1$) means gene overexpression, while $Fc \leq 0.5$ ($\log_2 Fc \leq -1$) may be interpreted as gene suppression [12,13].

TABLE 1. 19 genes promoting cancer formation selected for the experiment to design a custom PCR plate [11].

Gene symbol		Name of encoded protein	Which process the gene regulates?
1.	BCL2	apoptosis regulator Bcl-2	negative apoptosis regulation
2.	CASP3	caspase-3	
3.	ABL1	tyrosine-protein kinase ABL1	
4.	BAK	Bcl-2 homologous antagonist/killer	positive apoptosis regulation
5.	TNF	tumor necrosis factor	
6.	RB1	retinoblastoma-associated protein	cell cycle
7.	CHEK2	checkpoint kinase 2	
8.	VEGFa	vascular endothelial growth factor A	angiogenesis
9.	ABCB1	multidrug resistance protein 1	drug resistance
10.	ATM	serine-protein kinase ATM	
11.	CDKN1A	cyclin-dependent kinase inhibitor 1	
12.	NFKB1	DNA-binding factor KBF1	transcription
13.	JUN	transcription factor AP-1	
14.	TRF1	telomeric repeat-binding factor 1	cell aging
15.	PINX1	PIN2/TERF1-interacting telomerase inhibitor 1	
16.	MMP1	matrix metalloproteinase-1	proteolysis
17.	LUM	lumican - keratan sulfate proteoglycan lumican	sarcoma induction
18.	FOS	proto-oncogene c-Fos	
19.	NOS-2	nitric oxide synthase	hypoxia

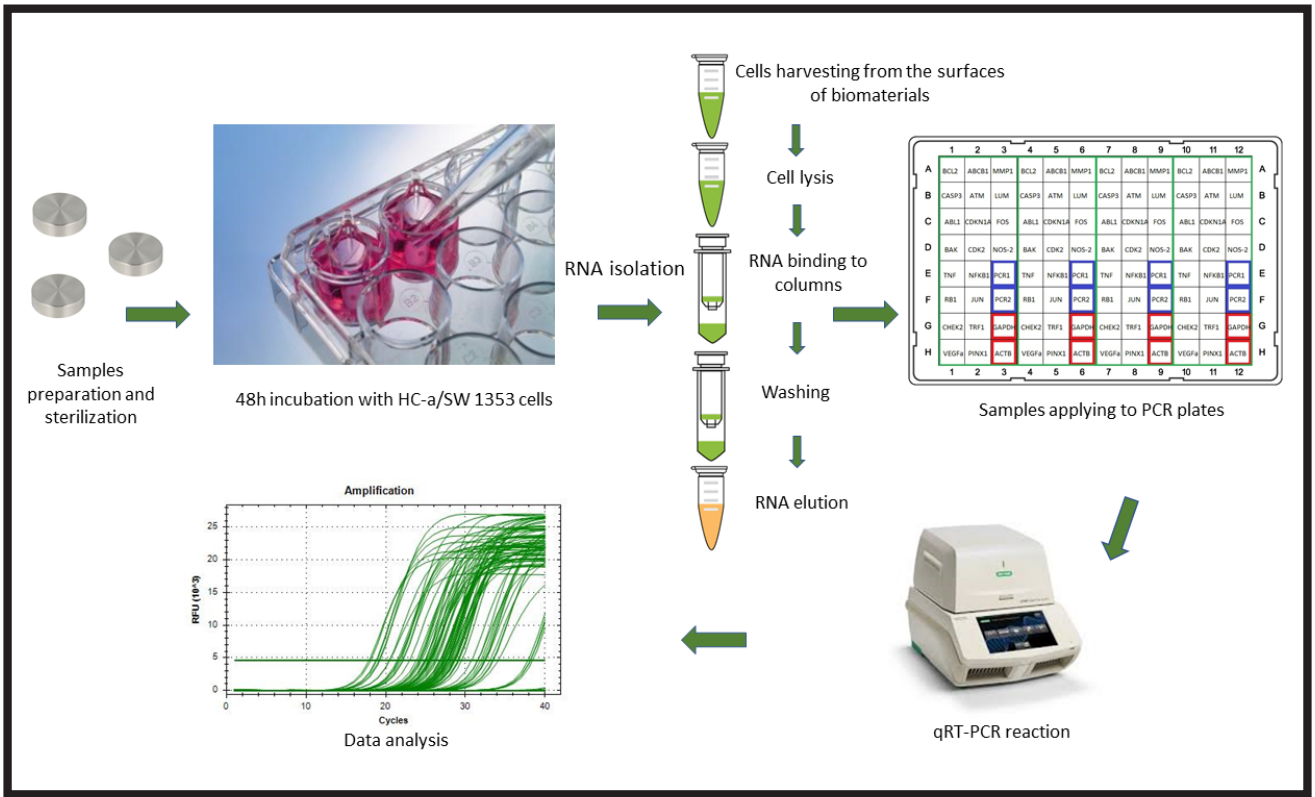

FIG. 2. The scheme of the standard qRT-PCR experiment: starting with sample preparation, through RNA isolation and purification, ending with qRT-PCR reaction.

Below, the graphs present mean values of log_2Fc obtained from seven independently performed qRT-PCR reactions for each examined gene and for both tested materials. The mean values are given with standard deviations (FIGs 3 and 4).

The t-Student test was used for the statistical analysis of Fc values of each gene in the primary and neoplastic chondrocytes grown on the examined surfaces (AISI 316L and Ti6Al4V). The results of statistical analysis are marked in FIGs 3 and 4 (VS - very significant, S - significant, MS - marginally significant).

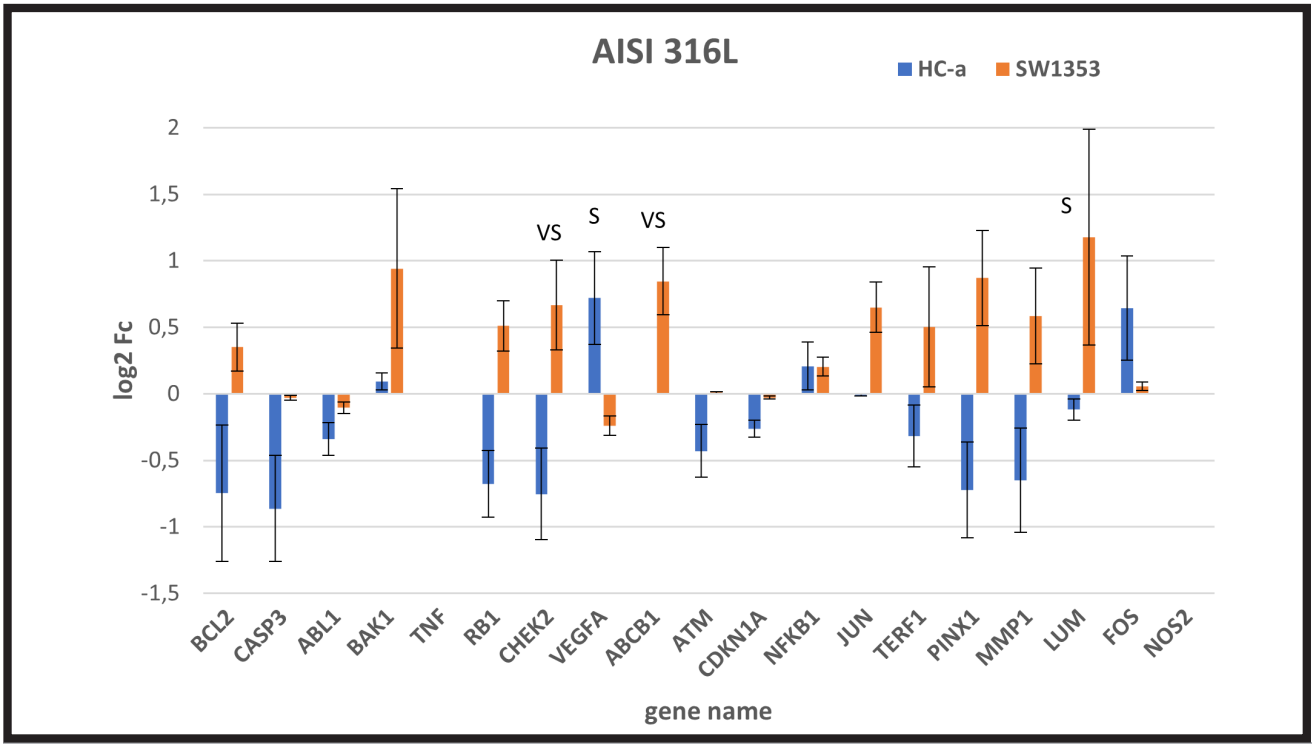

FIG. 3. The mean values of expression changes of individual genes expressed as log_2Fc and standard deviations obtained from 7 qRT-PCR reactions for HC-a and SW1353 chondrocytes grown on the surface of medical steel AISI 316L with statistical significance (MS - marginally significant for $p < 0.07$, S - significant for $p < 0.05$, VS - very significant for $p < 0.005$).

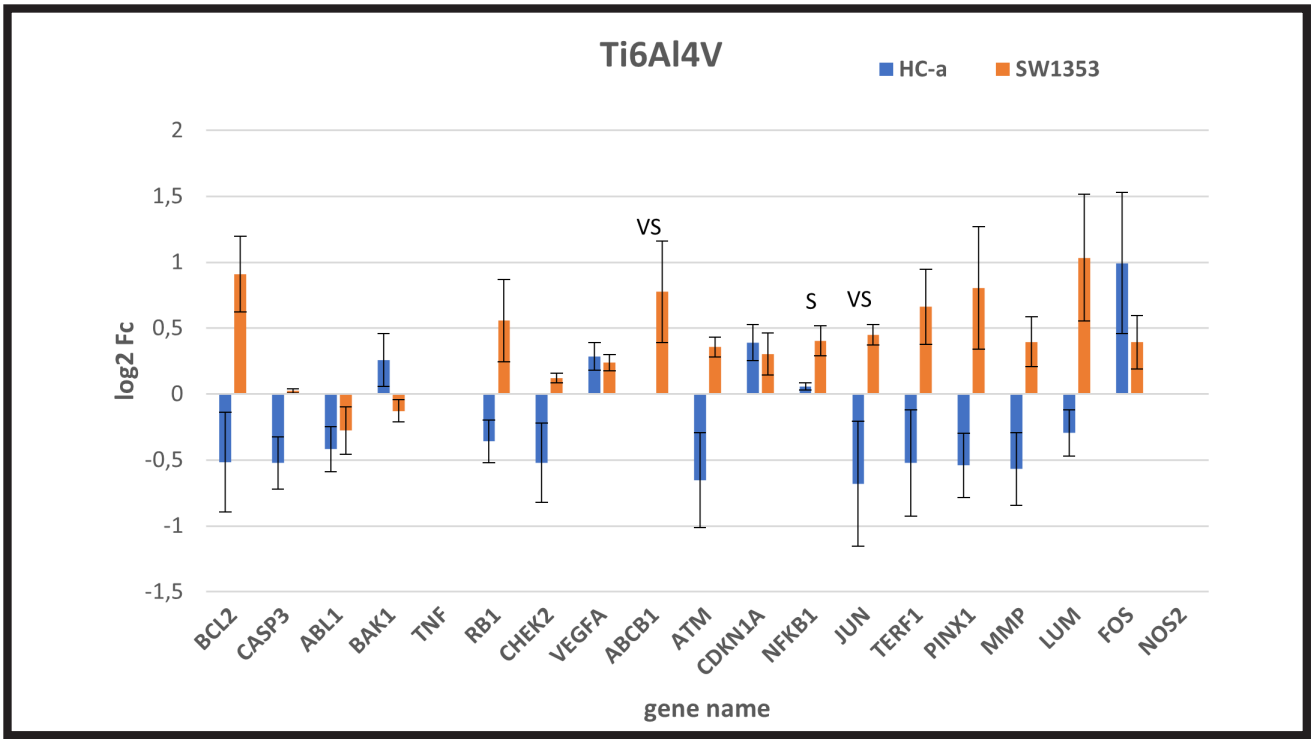

FIG. 4. The mean values of expression changes of individual genes expressed as log_2Fc and standard deviations obtained from 7 qRT-PCR reactions for HC-a and SW1353 chondrocytes grown on the surface of Ti6Al4V alloy with statistical significance (S - significant for $p < 0.05$, VS - very significant for $p < 0.005$).

Out of the 19 examined genes, the changes in gene expression were observed for 16 and 17 of them, for the HC-a and SW1353 cells, respectively. In both cell lines two genes – TNF and NOS-2 – were not expressed at all for any of the tested materials. TNF gene is responsible for positive apoptosis regulation, whereas NOS-2 – for the process of hypoxia. Another gene, ABCB1 gene, coding the multi-drug resistance protein that participates in failures in many different therapies, was active only in chondrosarcoma cells, not in primary ones. This confirms the well-known phenomenon of the tumor cell resistance to pharmacological treatment [14].

The predominant direction of changes for HC-a cells was the gene suppression (negative values of $\log_2$Fc), whereas for SW1353 tumor chondrocytes the direction of changes was exactly opposite, and in most cases, the gene overexpression could be observed (positive values of $\log_2$Fc). However, out of all examined genes for both cell lines and both types of the tested materials only one gene – LUM – was properly overexpressed according to the assumptions of this analysis, i.e. only this gene reached the value of $\log_2$Fc greater than 1. This situation was observed only in two cases – for the SW1353 cells after contact with AISI316L and Ti6Al4V. The LUM gene codes the proteins related with degradation of extracellular matrix and keratin metabolism. In this way, they may limit the tumor progression by preventing extracellular matrix collagen proteolysis. In cancer cells, these proteins are usually down-regulated. The LUM gene overexpression in cancer chondrocyte cells was statistically significant in comparison to the primary chondrocytes (very significant difference for AISI316L and Ti6Al4V). The results of the experiments may suggest that these two materials tend to limit tumor growth [15]. It is also worth paying attention to the FOS gene, for which the Fc value is very close to 2 in HC-a primary cells grown on the surface of the Ti6Al4V alloy. This gene encodes the c-Fos protein which is the human homologue of retroviral oncogene v-Fos and has an oncogenic activity [16].

The remaining observed differences in the expression of individual genes in primary and neoplastic cells are not so spectacular, but even these subtle differences should be taken into account in further research. Certainly, the number of genes examined in this study is not enough to draw far-reaching conclusions, it was only a starting point to find some possible tendencies. Gaining more knowledge in this area requires further intensive research, the results of which may be of paramount importance in choosing a safe biomaterial, especially for patients with a cancer history.

Conclusions

The analysis of changes in the expression of cancer-related genes is the most accurate approach to assess the risk of implants inducing or intensifying carcinogenesis. The compilation of qRT-PCR experiments carried out on primary and cancer cells in parallel allowed to identify possible future contraindications for patients with a genetic predisposition to cancer or with cancer history. What is more, this approach may be a crucial step to select the right biomaterial for a specific patient, which is the goal of personalized medicine.

Acknowledgements

This work has been supported by the National Centre for Research and Development, project number POIR.04.01.04-00-0058/17-00 "Projektowanie i wytwarzanie spersonalizowanych implantów medycznych" Custom-ITAP.

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