Analysis Of Candidate Genes Expected To Be Essential For Melanoma Surviving

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Abstract

Background

New approach for treating cancer is to influence on vital genes for tumors. A number of attempts have led to discovery candidate genes that are mean to be essential for surviving of tumor cells of different types. In this work, we tried to analyze genes that were previously found to be essential for melanoma surviving. We performed transient siRNA-mediated knockdown of UNC45A, STK11IP, RHPN2 and ZNFX1 in melanoma cell line A375. After that, we checked cell viability, proliferation and migrating rate in vitro.

Methods

For knockdown of the genes we used siRNA mediated interference. To deliver siRNA in cells we used transfection by Metafectene PRO. Cell viability and proliferation assessed by MTT assay. Cell migration was assessed by wound healing assay. Knockdown of genes was evaluated via RT-qPCR analysis.

Results

Our results indicate that knockdown of the genes leads to no statistically significant changes in cell survival according to viability assay. However, mobility assay shows that knockdown of each of the target genes accelerates the speed of cells migrating.

Conclusion

Our results do not confirm the hypothesis. Possible explaining of such controversial results could be in insufficient bioinformatical analysis or in need of multiplex knockdown of the genes.

Background

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body [1]. In USA, cancer is the second leading cause of death in both men and women of all ages on average [2]. Many efforts are directed against cancer: there are chemotherapy, immunotherapy, radiation, surgery, stem cell or bone marrow transplantation, hormone therapy and palliative care and their combinations [3] fighting different types of cancer. However, most of these approaches have severe side effects and/or are expensive. Since cancer is a result of spontaneous mutations and a pattern of such mutations is unique in each case, a common care for all cancers is must be limited by approaches that affect healthy cells too [4, 5]. One of the most promising approaches in treating cancer is to cure each patient individually, or at least to treat each type of cancer in a special way. Approaches in medicine that are tailored to the individual patient now are called the personalized medicine [6]. Nowadays, US sales of chemotherapies and especially targeted cancer therapies are expanding, and the large number of anticancer drug candidates currently in clinical trials suggests that the market is likely to be increasingly crowded in the future [7]. A strategy when a common cytotoxic therapy is combined with target molecular therapy is highly promising too [8].
One of the important parts of the personalized medicine is genetics. With regard to oncology it means genetics of cancer cells which define their resistance to drugs, aggressiveness and ability to metastases. In particular, it is critical to know the difference between cancer and normal cells in respect of genome to successfully distinguish them and to selectively destroy only tumor cells.

Nowadays most researches investigate cancer development via search for genes that mutated [9] or differently expressed [10] in cancer cells compared to adjacent normal tissues. Thus they find oncogenes, known as genes that play a role in cancer emergence and development. An extended statistical study reported of detection more than 200 potential oncogenes across 21 cancer types [11]. That approach can discover genes that drive cancerogenesis, but not the genes essential for cancer surviving. In evolutionary process that takes place in tumor progression those cells which have mutations in the genes will soon perish due to cell circle arrest or immune response of the organism. On the contrary, cells with intact “essential” genes will survive, divide and may reach DNA or RNA sequencing in a lab. Therefore, it is important to recognize those genes to target them during cancer therapy. For that purpose earlier our colleagues have used an Abraham Wald's aircraft approach and identified genes and corresponding proteins that are essential for surviving of cancer cells [12]. Their approach was more applicable to tumors triggered by point mutations [13], such as lung tumors or melanomas. Cutaneous malignant melanoma is one of the most aggressive and deadly skin cancers [14–16] and the rate of melanoma incidence is continuing to rise [17]. In addition, this cancer provides much more statistics on protein sequences to make the prediction robust [12]. Furthermore, melanoma is characterized by the highest somatic mutation frequency compared to other cancers [18]. Based on groundbreaking approach which aggregates evolutional dN/dS parameter as a measure of negative selection, gene expression and functional impact of amino acid changes, our colleagues have predicted 91 protein-coding genes to be essential in melanoma cancer cells.

In our work, we analyzed the importance of distinct genes on survivability of melanoma cell line. For this we performed siRNA-mediated knockdown of the most candidate genes to investigate influence on the cell viability, proliferation and migrating rate in vitro. We hypothesized that melanoma cells lacking transcripts of essential genes would show decrease in proliferation and viability.

**Methods**

**Cell lines and cell culture conditions**

Cell line A375 (human malignant melanoma) was cultured under standard conditions. In brief, Dulbecco’s Modified Eagle’s Medium (DMEM) + 10% fetal bovine serum (FBS) and l-glutamine in humidified atmosphere with 5% CO2 at 37 °C. Prior transfection cells were trypsinised, resuspended, diluted to 1 ml, and counted by Partec Flow Cytometer to obtain indicated number of cells per well.

**Knocking down the expression of the genes in cell lines**
Prior transfection, siRNAs were designed according to recommendations [19] with the use of in-home software. Efficiency of each of siRNAs were checked by qPCR in HEK cells. The sequences of siRNAs are listed in Table 1. The knockdown was performed as described in [20]. In brief, cells were grown up to 80% confluency, trypsinised, resuspended in antibiotics-free medium, and transfected with Metafectene (Biontex) according to manufacturer’s instructions: a 15 min incubated mixture of 0,4 mkl of Metafectene and 15 ng of siRNA in 60 mkl of PBS were added to cells in 96-well plates. Transfection efficiency was monitored by flow cytometry of cells transfected with FAM-tagged siRNA (siFlu) and was > 70% in all experiments.
Table 1
Sequences of primers and siRNAs used in the work.

| Primers of target genes |  |
|------------------------|--|
| RHPN2 (forward)        | GGGCTGAACATCTCGGTGG |
| RHPN2 (reverse)        | CCGGCTAGGCGTCCGACA |
| UNC45A (forward)       | CTCCACTCTCAAACCTGGCTAA |
| UNC45A (reverse)       | GTCGGCATCAAAGGTCAGGT |
| ZNFx1 (forward)        | TTGGAATTCTGCCAGCGAAC |
| ZNFx1 (reverse)        | CCTGCGGAGAGATTTGCTCA |

| HK genes primers and probes |  |
|-----------------------------|--|
| B2M (forward)               | GAGTATGCCTGCCGTGTG |
| B2M (reverse)               | AATCCAAATGCGGCATCT |
| B2M (probe)                 | FAM-CCTCCATGATGCTGCTTACATGTCTC-BHQ1 |
| TFRC (forward)              | GCCAACTGCTTTCATTTGTG |
| TFRC (reverse)              | ACTCAGGCCATTTTCTTTA |
| TFRC (probe)                | ROX-AGGGATCTGAACATACAGAGCAGACA-BHQ1 |
| HPRT (forward)              | TCAGGCAGTATAATCCAAAGATGGT |
| HPRT (reverse)              | AGTCTGGCTTATATCCAACACTTCG |
| HPRT (probe)                | TAMRA-CAAGCTTGTCTGGTAGAAACGGACCCC-BHQ1 |
| TBP (forward)               | CACGAACCACGGCACTGATT |
| TBP (reverse)               | TTTTTTCTGTGCCAGTCTGGAC |
| TBP (probe)                 | VIC-TGTGCACAGGAGCCAGAGTGAAGA-BHQ1 |

| siRNAs                     |  |
|-----------------------------|--|
| siZNFx1#1                   | 5'- GAUGGAGAGUUACAC AC dTdT - 3 |
|                             | 3'- dTdTTCUAC UCUC UGGUGGUU - 5 |
| siZNFx1#2                   | 5'- GG AC GGAGC AC CAGU AC dTdT - 3 |
|                             | 3'- dTdTUCUUC UCGUUGUCACUUU - 5 |
| siZNFx1 #3                  | 5'-GAGCAAAAGUUACACAAAUCdTdT3' |
|                             | 3'-dTdTUCGUUUCAAUUGUUAAGA-5' |
RT and qPCR analysis

Total RNA was extracted from cells using phenol-chloroform method according to Chomczynski and Sacchi [21]. RNA was treated with DNaseI (Thermo Fisher Scientific, USA) and reverse transcribed using ImProm-II™ Reverse Transcription System (Promega, USA). qPCR experiments were performed using EvaGreen® Dye (Biotium) and SmarTaq DNA Polymerase (Dialat) in StepOne instrument (Applied Biosystems). All PCR amplification reactions were run in triplicates for each cDNA sample. For normalization, we used expression of four reference genes (B2M, HPRT, TFRC, TBP). Primer sequences for reference genes and genes of interest are listed in Table 1.

Cell growth assay

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) was used to determine cell viability. In brief, cells were transfected at 10^3 cells/well in 96-well plates (five replicates). After 24 h cells were trypsinised, counted and seeded at 2*10^2cells/well. Every 24 h a 5 wells with cells were treated with MTT solution and incubated 3 h at 37 °C. After remove of media, in each well formazan pellets were dissolved in 200 µl of DMSO. Absorbance of formazan solution was analyzed at 570 nm in Multimode Plate Reader (PerkinElmer).

Cell migration assay

Cell migration was assessed by wound-healing assay as described in [22]. In brief, cells were transfected in five replicates at 12*10^3 cells/well in 96-well plates. 24 h post transfection cell mat was wounded by 200 µl pipette tip and photographed every 2–5 h for up to 24 h in total. Photos were processed with software ImageJ according to [23]. The slope of linear fit curve was used to compare the speed of cell migration.
Statistical analysis

All statistical analysis was performed using STATISTICA 8.0 software. Measurement data were obtained at least in triplicates. Results are reported as the mean ± standard deviation. The Mann–Whitney U and the Kolmogorov–Smirnov tests were used to differentiate groups of samples. p < 0.05 was considered as statistically significant.

Results

Analysis of essential genes for melanoma surviving

We performed analysis of the genes which were found previously to be essential in skin melanoma [12]. From 91 gene according to Fantom5 data only 44 were expressed in melanoma cell lines. If a gene is essential for cell surviving, it would rather be generally intact in human genome. We examine the list of genes according to the deCODE data of association between diseases and genes. Among 44 genes only 18 were not causing any known disease of Icelanders according to deCODE genetics research. Next, we analyzed information about function of the 18 selected genes from articles and GeneOntology. We evaluated literature data referred to functions of the genes and to existing knockdown experiments in any cell lines. Among the genes there were significant part described as plasma membrane-associated ones. Since this kind of genes may be involved in immune response \textit{in vivo} [23] and therefore may have no effect on surviving of melanoma cells \textit{in vitro}, we discarded such genes from our analysis. As a result, we have 10 genes that can potentially be essential for melanoma progression and survival. 7 genes out of the 10 were the most interesting and were not still verified to confirm our theory and had no knockdown data in melanoma cells.

After that we performed qPCR analysis for the 7 genes on RNA isolated from melanoma cell lines: A375, G361, Sk-mel-1 and Sk-mel-5. Only the genes that were expressed at detectable level at least in one cell line were chosen.

Among these genes there were ones that were demonstrated to play a role in other cancers (UNC45A, STK11IP), as well as uncharacterized in such perspective (RHPN2 and ZNFX1). These genes were selected for further analysis and validation.

Knockdown experiments

To performed knockdown experiments we designed 1–3 siRNA for each of the genes ZNFx1, RHPN2, STK11IP and UNC45A, and tested them on melanoma cell line A375 (Fig. 1). For designing we used our own script which parses every 20 nt of the mRNA sequence of the gene by tiling and analyze it by a number of rules listed in [19]. mRNA level of the genes was confirmed using RT-qPCR as compared with cells transfected with non-specific siRNA. The most effective siRNA for each of the genes was used to check the effect of knockdown of the genes in melanoma cell line A375.
Next, we assessed viability and proliferation of the cells. For viability assay we transfected cells in low number (1K cells per well in 96-well plate) and cultivated them in standard conditions for 6 days. Every day we assessed the viability by an MTT test. Proliferation assay was performed every 12 h during 45 or 60 h range by flow cytometry. Cells were transfected the same way as for MTT assay. Prior cytometry cells were tripsinised and resuspended in PBS. Proliferation assay showed no significant differences in cell counts between knockdowned and control cells (data not shown). Viability assay also revealed no statistically significant changes in cell survival (Fig. 2).

Then, we analyzed the cell migrating rate in cells transfected with target and control siRNAs by using a wound-healing assay. Our results show that knockdown of all of the target genes, ZNFx1, RHPN2, STK11IP and UNC45A, accelerates the speed of cells migrating (p < 0.05) (Fig. 3).

**Discussion**

Recent advantages in DNA sequencing rises new approaches of analyzing cancer genomes. Instead of looking at a cancer as a result of a series of point mutations in oncogenes or tumor suppressor genes researches assume tumors as completely different type of cells [24]. Cancer cells pass through lots of cycles of natural selection accompanied by genome reorganization and DNA repair system modulation [25, 26]. Most of mutations in cancer genome are not driver ones but passenger. They do not improve tumor surviving ability and therefore may experience a negative selection [27]. In recent works most laboratories all over the world concentrated at finding driver mutations in target genes to defeat the growth and/or malignancy of tumors. The most common way of searching—comparing genomes of cancer samples and adjusting healthy tissues and identification of genes significantly mutated in cancer [11].

The approach that was proposed by Pyatnitskiy *et al.* revealed 91 gene [12]. It was suggested that these genes could be essential for melanoma surviving. Our knockdown experiments in melanoma cell lines could prove their theory.

According to Fantom5 data only less than a half of the genes (44 gene) were expressed in melanoma cell lines. Such discrepancy could be explained by a difference between patient’s samples on which the conclusions were made and cell lines presented in Fantom5: it is well known that immortal cell lines have high difference with patient samples. We also tried to exclude genes which are associated with any disease. To do it we used the most complete and numerous study of human genomes is the deCODE genetics research project.

In a list of 18 genes obtained after there were a part that confirm our hypothesis and a part that was not in a line with it. For instance, knockdown of UNC45A lead to decreased proliferation of cultured myogenic and ovarian cancer cells [28]; PTK2B inhibition by shRNA vectors led to reduction of multiple myeloma tumor growth in vivo as well as decreased cell proliferation, cell-cycle progression, and adhesion ability in vitro [29]. On the other hand, there are examples of driver mutation genes: MYCT1 was down-regulated in the majority of gastric carcinoma tissues and when overexpressed, could promote apoptosis of gastric
carcinoma cell lines [30]; TGM5 being overexpressed induces cell death, mutations in this gene are associated with acral peeling skin syndrome, knockdown effects are unknown [31].

For our work we selected 7 genes. For some of these genes it has been shown that knockdown can lead to decrease of viability of some cancer cell types but not for melanoma. Also, for some genes it has been shown the role in cell cycle but not in cancer cells viability. With these different genes that were interesting for our purpose we performed expression analysis in available melanoma cell lines: sk-mel-1, sk-mel-5, G361 and A375. In our experiments with knockdown we selected 4 genes (UNC45A, STK11IP, RHPN2 and ZNFX1), which have the highest expression in suitable cell line A375.

The results on knockdown experiments show that the selected genes are not essential for melanoma cells surviving, but they have influence at distinct processes in the cells. Particularly, knockdown of all of the tested genes increased the speed of cell migrating. Moreover, the viability of cells assessed by MTT test was not changed despite the Wound-healing results.

It indicates that although the genes play some role in melanoma cells life they, at least individually, are not as essential as we expected according to bioinformatical analysis. Perhaps the data shows that the contribution of the proteins of these genes to survivability is not crucial. They can make a significant contribution to the progression of turmeric processes when they are initiated by other mutations in the driver genes.

Anyway, this work of Pyatnitskiy et al. is the first try in such novel direction. For more accurate results we need to accumulate more data for every and each cancer type. For now, it seems to reveal a lot of false positive results. In this work, we used different methods to evaluate any sign of essentiality of the genes for melanoma cells surviving, but obtained controversial results that do not prove the theory. If the idea had had an extension, development and a good implementation, it could have led to appearance of novel approaches for cancer treatment.

**Conclusion**

Our study shows, that although some genes are hypomutated in distinct cell types, it does not mean they are essential for surviving of the cells. It is hard to evaluate information from negative data (absent of mutations) and this approach is associated with production of false positive results. Our work is one of ways to reveal it.

**DECLARATIONS**

**Declarations**

*Ethics approval and consent to participate*

Not applicable
Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

IK: acquisition of data, analysis and interpretation of data, drafting the article.

AF: conception and design, Acquisition of data, critical revision of the article.

SM: conception and design, drafting the article.

AB: analysis and interpretation of data, drafting the article.

MS: conception and design, critical revision of the article, final approval of the version to be published.

All authors have read and approved the submitted version.

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Figures
siRNAs show significant knockdown of target genes expression. Knockdown of target genes by siRNAs was assessed by RT-qPCR. Samples treated with control siRNA are referred as 1. Error bars are SD.
Knockdown of the chosen genes do not vary viability of melanoma cell lines. (A-D) Effect of siRNA-mediated silencing of the genes on cell viability evaluated by MTT assay. Five repeats of each sample were conducted. Error bars represent SD.
Figure 3

Knockdown of the chosen genes do not vary viability of melanoma cell lines. (A-D) Effect of siRNA-mediated silencing of the genes on cell viability evaluated by MTT assay. Five repeats of each sample were conducted. Error bars represent SD.
Figure 4

Knockdown of the chosen genes do not vary viability of melanoma cell lines. (A-D) Effect of siRNA-mediated silencing of the genes on cell viability evaluated by MTT assay. Five repeats of each sample were conducted. Error bars represent SD.
Figure 5

Knockdown of the chosen genes do not vary viability of melanoma cell lines. (A-D) Effect of siRNA-mediated silencing of the genes on cell viability evaluated by MTT assay. Five repeats of each sample were conducted. Error bars represent SD.
Figure 6

Knockdown of the chosen genes improves cell migration rate. Results of wound-healing assay shows significant improvement in cell motility. (A) − percent of remaining gap between cell carpet front. Area of gap at first time point is considered 1. (B) − speed of cell migrating calculated by the slope of A graphics. Error bars represent SD.
Figure 7

Knockdown of the chosen genes improves cell migration rate. Results of wound-healing assay shows significant improvement in cell motility. (A) – percent of remaining gap between cell carpet front. Area of gap at first time point is considered 1. (B) – speed of sell migrating calculated by the slope of A graphics. Error bars represent SD.